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^{1}H NMR-BASED METABOLOMICS: A POWERFUL TOOL TO UNRAVEL THE MECHANISMS OF TOXICITY OF MERCURY

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Summary

Environmental metabolomics is a high-throughput approach that has shown considerable potential in mechanistic research. Indeed, it is one of the most powerful "-omics" techniques that allow the simultaneous evaluation of a broad number of endogenous small metabolites, and offer the potential to unravel subtle alterations in biological pathways providing insights into the mechanisms that underlie various physiological conditions, identify new metabolite biomarkers as defensive or adaptive response, and thus provide an overview of the metabolic status of a biological system.

Mercury (Hg) is a hazardous pollutant because of its persistence, high toxicity and tendency to bioaccumulate and biomagnify throughout food chains, with implications to humans. Despite the efforts on evaluating the biological impact of Hg on aquatic organisms, the toxicity mechanism of Hg still need to be clarified, and its understanding requires new ways. In this regard, the use of environmental metabolomics may serve as a powerful tool focusing on metabolites involved in various metabolic pathways, which changes might reveal insights into the Hg toxicity mechanisms. Given the similarity of fish responses with higher vertebrates, fish can be used to screen potentially hazardous effects to humans. Considering that oxidative stress has been described as a key pathway to initiate Hg toxicity in fish, the combination of metabolomics and conventional oxidative stress biomarkers could be an innovative and promising approach to disclose Hg-induced toxicity.

Hence, this strategy was applied to the liver and gills of the golden grey mullet *Liza aurata* inhabiting an Hg contaminated system (Aveiro Lagoon, Portugal), with the aim to unmask the mechanisms of Hg toxicity. Furthermore, this thesis has also the merit to provide a detailed comparative analysis of the differential bioaccumulation of Hg and metabolic responses in

fish organs, which allowed to discern tissue-specific toxicological effects of Hg in fish liver and gills, attributable to their differential physiological functions.

In mullet liver, the major site for xenobiotic bioaccumulation and detoxification processes, metabolomics revealed that Hg accumulation has multiple levels of impact, interfering with membrane stabilization/degradation/repair processes, osmoregulation, energy metabolism, gene expression and antioxidant protection. Also, the oxidative stress biomarkers in liver revealed that Hg triggers adaptive responses of antioxidant system depicted in increased GST and CAT activities as well as GSHt content, compensating GPx activity depletion. Therefore, the combined use of metabolomics and oxidative stress endpoints allowed a better understanding of Hg hepatotoxicity, identifying GSH as a first line of defence against Hg and providing evidences of oxidative insults in cell membranes. Nonetheless, the induction of lipid peroxidation in mullet liver was efficiently prevented.

In mullet gills, metabolomics enabled an integrated description of numerous metabolites involved in various metabolic pathways. Interferences with the ion-osmoregulatory processes were revealed, with reduced taurine and glycerophosphocholine, along with increased creatine level. Impairments in energy metabolism were observed, as well as occurrence of protein catabolism supported by the increased levels in amino acids and alanine, the latter mainly involved in nitrogenous waste excretion. Vulnerability in the respiratory gas exchange activity was suggested by augmentation of isobutyrate, a known biomarker of anoxia. The combination of metabolomics and pro-oxidant status evaluation indicated the occurrence of massive GSH oxidation under Hg stress, and an inability to carry out its regeneration (GR activity was unaltered) or *de novo* synthesis (depletion in GSH constituent amino acids). The prevention of lipid peroxidative damage occurred, and it may be associated with the enhancement of membrane stabilization/repair processes, resulting from depletion in precursors of phosphatidylcholine. Though the exposure to Hg in gills led to a state of vulnerability due to limitations of antioxidant system

towards a physiological accommodation, the membranes were able to answer to oxidative insult through the enhancement of stabilization/repair processes.

Finally, from the detailed comparison of the differential bioaccumulation of Hg and metabolic responses in fish liver and gills, tissue-specific toxicological mechanisms of Hg, attributable to differential structural properties and physiological functions of the two organs under examination, were also detected. Specifically, Hg accumulation in fish tissues provoked severe disturbances in ion-osmoregulatory processes, mainly in gills, as highlighted by the depletion of osmolytes, namely taurine and glycerophosphocholine. However, the decrease in taurine level recorded in liver was associated to the metal-chelating property of taurine, thus resulting in the occurrence of hepatic detoxification processes. Differential disorders in energy-producing metabolic pathways were found between gills and liver, resulting in an enhancement of anaerobic metabolism as strategy to replenish insufficient energy supply in gills and promotion of gluconeogenesis in liver. Interferences with protein metabolism were also detected in both organs, depicting an ongoing protein catabolism in gills and, conversely, augmented protein synthesis in liver for repair of Hg-damaged proteins or activation of cytoprotective mechanisms to counteract Hg toxicity. Additionally, differential alteration in membrane stabilization/repair processes and perturbation of antioxidant defence system were also pointed out between mullet gills and liver.

The novel concurrent use of metabolomics and conventional oxidative stress endpoints, coupled with Hg tissue burdens characterization, demonstrated to be a sensitive and effective tool towards a mechanistically based assessment of Hg toxicity in fish, providing novel insights into the mechanisms underlying Hg-induced oxidative stress. Overall, the metabolomics approach demonstrated its effectiveness in the elucidation of the mechanisms of Hg toxicity in wild fish, and allowed to discern tissue-specific toxicological effects of Hg in fish gills and liver, attributable to their differential physiological functions, and potentially observable in humans.

Riassunto

La metabolomica ambientale rappresenta un approccio all'avanguardia che ha mostrato un potenziale considerevole nella ricerca meccanicistica. La metabolomica è la tecnica "-omica" che consente di effettuare una valutazione simultanea di un ampio numero di piccole molecole endogene, e offre il potenziale di mettere in luce minime alterazioni in pathways biologici fornendo quindi delucidazioni nei meccanismi che sottintendono diverse condizioni fisiologiche, di identificare nuovi biomarkers di stress metabolici come risposte di difesa o adattamento a diverse cause di stress, e di fornire quindi una panoramica dello stato metabolico di un sistema biologico.

Il mercurio (Hg) è un inquinante pericoloso a causa della sua persistenza, alta tossicità e tendenza a bioaccumulare e biomagnificare attraverso le reti trofiche, con conseguenze per l'uomo. Nonostante i numerosi studi condotti per valutare l'impatto biologico del Hg in organismi acquatici, i meccanismi di tossicità del Hg non sono ancora chiari, e la loro comprensione richiede nuovi strumenti di indagine. In questo contesto, l'uso della metabolomica ambientale può rappresentare uno valido strumento per lo studio dei metaboliti coinvolti in vari pathways metabolici, le cui variazioni possono rivelare indicazioni sui meccanismi di tossicità del Hg. Data la similarità delle risposte osservate in bassi vertebrati, come i pesci, con gli alti vertebrati, i pesci possono essere utilizzati per mettere in evidenza effetti potenzialmente pericolosi nell'uomo. Considerando che lo stress ossidativo è stato descritto come il pathway chiave che innesca la tossicità del Hg nei pesci, la combinazione della metabolomica e di convenzionali biomarkers di stress ossidativo potrebbe rappresentare un approccio innovativo e promettente per svelare la tossicità indotta da Hg.

Pertanto, questa strategia è stata applicata al fegato e alle branchie del cefalo dorato *Liza aurata* proveniente da un'area contaminata da Hg (Aveiro Lagoon, Portogallo), con lo scopo di comprendere i meccanismi di tossicità del Hg.

Inoltre, questa tesi ha anche il merito di fornire una dettagliata analisi comparativa del bioaccumulo differentiale del Hg e delle risposte metaboliche osservate nei campioni tissutali dei pesci, che hanno permesso di distinguere effetti tossicologici tessuto-specifici del Hg nel fegato e nelle branchie dei pesci, attribuibili alle loro diverse funzioni fisiologiche.

Nel fegato dei cefali, il sito principale per il bioaccumulo e i processi di detossificazione degli xenobiotici, la metabolomica ha rivelato che l'accumulo di Hg ha multipli livelli di impatto, interferendo con i processi di stabilizzazione/degradazione/riparo delle membrane, osmoregolazione, metabolismo energetico, espressione genica, e protezione antiossidante. Inoltre, i biomarkers di stress ossidativo hanno mostrato che il Hg innesca nel fegato risposte adattative del sistema antiossidante, risultanti nell'aumento delle attività del GST e CAT, e del contenuto del GSHt, che compensa la riduzione nell'attività del GPx. Pertanto, l'uso combinato della metabolomica e degli endpoints dello stress ossidativo hanno consentito una approfondita comprensione dell'epatotossicità del Hg, identificando il GSH come la prima linea di difesa contro il Hg e fornendo evidenze dell'insulto ossidativo nelle membrane cellulari. Tuttavia, l'induzione della perossidazione lipidica nel fegato dei cefali è stata efficacemente prevenuta.

Nelle branchie dei cefali, la metabolomica ha fornito una descrizione integrata di numerosi metaboliti coinvolti in vari pathways metabolici. In dettaglio, interferenze con i processsi di iono-osmoregolazione sono stati osservati mediante la riduzione della taurine e glicerofosfocolina, con incremento della creatina. Danni nei pathways energetici sono stati osservati, così come il catabolismo proteico, supportato dall'aumento di amminoacidi e di alanina, coinvolta principalmente nell'escrezione dei rifiuti azotati. Vulnerabilità nell'attività respiratoria è stata anche suggerita dall'incremento dell'isobutirato, un noto biomarker di anossia. L'uso combinato della metabolomica e della valutazione dello stato pro-ossidante hanno indicato una massiva ossidazione del GSH in risposta a Hg, e una incapacità della sua

rigenerazione (attività di GR era inalterata) o *de novo* sintesi (riduzione dei precursori del GSH, glicina e glutammato). Il danno perossidativo lipidico è stato prevenuto, probabilmente per un'attivazione dei processi di stabilizzazione/riparazione delle membrane, resultante dalla riduzione dei precursori della fosfatidilcolina. Sebbene l'esposizione al Hg nelle branchie ha portato a uno stato di vulnerabilità dovuto a limitazioni del sistema antiossidante verso un'accomodazione fisiologica, le membrane sono state in grado di rispondere all'insulto ossidativo mediante l'attivazione di processsi di stabilizzazione/riparazione.

Infine, dal confronto dettagliato del bioaccumulo differenziale del Hg e delle risposte metaboliche osservate nel fegato e nelle branchie dei pesci, meccanismi tossicologici tessuto-specifici del Hg, attribuibili alle diverse proprietà strutturali e funzioni fisiologiche dei due organi in eame, sono stati evidenziati. Specificamente, l'accumulo del Hg nei tesuti dei pesci ha provocato severi disturbi nei processi iono-osmoregolatori, principalmente nelle branchie, come evidenziato dalla riduzione in osmoliti, quali taurina e glicerofosfocolina. Comunque, la riduzione nel livello della taurina registrato nel fegato è stato associato alla proprietà di chelazione dei metalli della taurina, e risulta quindi nell'attuazione di processi epatici di detossificazione. Alterazioni differenziali nei pathways energetici sono stati osservati tra branchie e fegato, risultatnti nelle branchie nell'attivazione del metabolismo anaerobico come strategia per rifornire una riserva energetica insufficiente, e nel fegato nella promozione della gluconeogenesi. Interferenze nel metabolismo proteico sono state anche individuate in entrambi gli organi, indicando un catabolismo proteico in corso nelle branchie e, al contrario, un'attivazione della sintesi proteica nel fegato per la riparazione delle proteine danneggiate da Hg o per l'attivazione di meccanismi citoprotettivi al fine di contrastare la tossicità del Hg. In aggiunta, alterazioni diffeernziali nei processi di strabilizzazione/riparazione delle membrane e disturbi nel sistema di difesa antiossidante sono stati anche osservati fra branchie e fegato.

Pertanto, l'innovativo uso combinato della metabolomica e biomarker convezionali di stress ossidativo, in congiunzione con la caratterizzazione del Hg nei tessuti, si è dimostrato uno strumento sensibile ed efficace nella valutazione meccanicistica della tossicità del Hg nel fegato e nelle branchie dei pesci, fornendo nuove informazioni sui meccanismi che sottintendono il danno ossidativo indotto dal Hg. In generale, la metabolomica ha dimostrato la sua efficacia nella delucidazione dei meccanismi di tossicità del Hg nei pesci in natura, e ha permesso di distinguere effetti tossicologici tessuto-specifici del Hg fra le branchie e il fegato del cefalo dorato, attribuibili alla loro diverza funzione fisiologica, e potenzialmente osservabili nell'uomo.

List of abbreviation

1-D	One-dimensional
^{1}H NMR	Protonic Nuclear Magnetic Resonance
ADP	Adenosine Diphosphate
ANOVA	One-way Analysis of Variance
AAS	Atomic Aborption Spectrometry
ATP	Adenosine Triphosphate
BCCA	Branched Chain Amino Acids
CAT	Catalase
CH_3CH_2OH	Ethanol
CK	Creatine Kinase
CYP	Cytochrome P450
D_2O	Deuterated Water
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DHg	Dissolved Mercury
DMeHg	Dissolved Methylmercury
DSS	2,2-dimethyl-2-silapentane-5-sulfonate
DTNB	5,5´-dithiobis-2-nitrobenzoic Acid
DTPA	Diethylene Triamine Pentaacetic Acid
ERA	Ecological/environmental Risk Assessment
EU	European Union
GC	Gas Chromatography
GCS	γ-glutamylcysteine Synthetase
GPx	Glutathione Peroxidase

GR	Glutathione Reductase
GSH	Glutathione
GSHt	Total Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione S-transferase
Hg	Mercury
HMDB	Human Metabolome DataBase
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography-Ultraviolet
iHg	Inorganic Mercury
LAR	Largo do Laranjo
LPO	Lipid Peroxidation
MeHg	Methylmercury
MS	Mass Spectrometry
MS-222	Tricaine methanesulfonate
MTs	Metallothioneins
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
PAHs	Polycyclic Aromatic Hydrocarbons
PCA	Principal Components Analysis
PCBs	Polychlorinated Biphenyls
PMS	Post Mitochondrial Supernatant
RF	Radio Frequency
RLP	Rickettsiales-like Prokaryote
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RVD	Regulatory Volume Decrease
SJ	São Jacinto
SOD	Superoxide Dismutase
SVD	Singular Value Decomposition

TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic Acid
TMSP	Sodium 3-trimethylsilyl-2,2,3,3-d4-propionate
TNB	5-thio-2-nitrobenzoicacid
XOD	Xanthine Oxidase

List of figures

List of tables

Table of contents

Chapter IV: Metabolic and oxidative stress responses in liver of wild mullet *Liza aurata*

Chapter V: Metabolic and oxidative stress responses in gills of wild mullet *Liza aurata*

CHAPTER I

Introduction

1. Introduction

1.1. General introduction

The transitional ecosystems between land and sea, a narrow strip at the edge of both environments, contains some of the most productive and valuable habitats of the world (Valiela, 1995). Coastal lagoons are among these important ecosystems, since several organisms use lagoon habitats for nesting, feeding, reproduction or sheltering (Barnes, 1980). Despite a general decrease in the anthropogenic pressure on coastal ecosystems observed recently in developed countries, coastal lagoons are still undergoing major human impact (Lotze et al., 2006). The most important environmental concerns are associated with unplanned development (urbanization and industrialization), unregulated discharges (municipal sewage and industrial waste) and depletion of resources (over-fishing and bad use of agricultural land). Those activities could lead to the enhancement of contaminant availability and massive algal growth due to eutrophication, associated with an increase in the duration of intermittent periods of lower oxygenation (Rabouille et al., 2007). In fact, the deficient water renewal in coastal lagoons may slow down the dilution process and enhance sediment accumulation and/or retention of contaminants that are transferred to the biota (Mucha et al., 2004). Moreover, the fate of waterborne contaminants in shallow waters is regulated by resuspension and deposition, two physical processes that strongly depend on tidal currents and wind, which have low expression in some lagoon systems.

Therefore, there are concerns about risk to aquatic organisms inhabiting transitional ecosystems, because these organisms are exposed to high concentrations of environmental contaminants due to the intense anthropogenic activities. The major aim of environmental science is to make robust, practical and relatively low cost procedures for risk assessment, and to predict consequences of toxic compounds (Rice, 2003). Methods to effectively

monitor and quantify these effects are essential to provide an indication of ecosystem health status, an issue of both urgent and international concern.

Traditional approaches addressing this issue frequently use the concept of "biomarkers" to be applied in sentinel species, which may be both invertebrates and lower vertebrates, in order to provide an early warning system of exposure and toxic effects in the ecosystem. Sentinel species can be defined as biological indicators that accumulate a pollutant in their tissues (Beeby, 2001), offering a potentially simple solution to both the problem of measuring bioavailability and of summarizing complex patterns of contamination. Sentinel species should be widely distributed, easy to identify in the field, abundant and large enough to provide material for biomarker analysis (Beeby, 2001; Galloway et al., 2004; Phillips, 1977). The application of biomarkers in environmental research has been recently reinforced with the introduction of "-omics" technologies, which can offer greater insights into the effects of external insults on a biological system at a molecular level.

Among the "-omics" technologies, environmental metabolomics, which involves the study of low molecular weight metabolites, is in fact a cutting edge approach to assessing the health of organisms and discover novel metabolic biomarkers as organismal defensive or adaptive responses to various stress, thus providing an overview of the metabolic status of a biological system (Kell, 2004). In order to achieve a more detailed insight of the organismal health status, mainly in regard to mercury pollution, the aim of this thesis is to integrate environmental metabolomics to a combined approach of metal accumulation and conventional and well-established oxidative stress biomarkers, with the purpose to unravel the toxicity mechanisms of mercury.

1.2. Environmental monitoring

To a varying extent, human activities have adverse impacts on the health status of marine environments. As far as threats to the marine environment are concerned, pollution is by far the more significant. The internationally recognised definition of pollution for the marine sector was developed by the Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP, 1993) and reads: "Introduction of man, directly or indirectly, of substances or Energy into the marine environment (including estuaries) resulting in such deleterious effects as harm to living resources, hazard to human health, hindrance to marine activities including fishing, impairment of quality for use of seawater, and reduction of amenities."

Common class of pollutants arising from natural and anthropogenic sources include heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organic solvents and dioxins. Some of these compounds may have deleterious effects on both the target organism, and on many non-target species, including humans. Following exposure to such chemicals, these pollutants can be absorbed directly into the bodies of aquatic organisms such as fish and invertebrates via respiratory, as they remove oxygen from the water for respiration (De Zwaan and Eertman, 1996), dermal and oral routes, through the digestive tract following ingestion of contaminated food and water. These processes may also affect humans by their consumption of contaminated seafood (Martin et al., 1996). Therefore, there is an urgent need to understand the biological effects that these pollutants have on vulnerable organisms, such as those living in aquatic environments.

Connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects. Many of the hydrophobic organic compounds and their metabolites, which contaminate aquatic ecosystems, have yet to be identified and their impact on aquatic life has yet to be determined. Therefore, the exposure, fate and effects of chemical

contaminants or pollutants in the aquatic ecosystem have been extensively studied by environmental toxicologists. Indeed, in the early 20th century, researchers proposed the use of living organisms, in parallel with physico-chemical analysis, to evaluate the health state of the aquatic system (Amiard et al., 1998). In this context, biomonitoring was defined as the systematic use of biological responses to assess changes in the environment (Cairns and Van der Schalie, 1980).

Linking the adverse effects of environmental contaminants in individual animals to their ecosystem-level consequences is a key challenge in regulatory risk assessment (Moore et al., 2004). Ecological/environmental risk assessment (ERA) is defined as the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi, 1994; Van der Oost et al., 2003). The risk assessment process identifies and quantifies the risk resulting from a special use or occurrence of a chemical compound, and seeks a solution to the problem, whereas risk analysis determines the risk of a specific situation (Van der Oost et al., 2003). ERA has become increasingly important since environmental scientists as well as the general public have learned that chemicals which are not toxic to humans can have deleterious effects on natural resources which are generally valued (Bascietto et al., 1990). Although ERA is generally performed by predictive methods, the interest in the assessment of pollution that began in the past and may have ongoing consequences in the future is increasing. These so-called retrospective ERAs are primarily concerned with establishing the potential relationship between a pollutant source and an ecological effect caused by exposure of organisms to the pollutant (Suter, 1993).

The responses to pollutant stress within a biological system (Bayne et al., 1985) have triggered the research to establish early-warning signals, or biomarkers, reflecting the adverse biological responses towards anthropogenic

environmental toxins (Bucheli and Fent, 1995). A biomarker is defined as a change in a biological response starting at the subcellular level (e.g. interference with molecular pathways) and ultimately leading to adverse effects at higher levels of biological organization (De Coen and Janssen, 2003), which can be related to exposure to or toxic effects of environmental chemicals (Peakall, 1994). In principle, these early warning biomarkers should be capable of predicting reduced performance, impending pathology and damage to health (Moore et al., 2004). Hence, biomarkers should be able to identify those organisms that have been, or are being, exposed to certain chemicals or those organisms that are suffering, or will suffer, future impairments of ecological relevance (Forbes et al., 2006). In an environmental context, biomarkers thus offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed between tissues, and are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990).

Van Gastel and Van Brummelen (1996) redefined the terms 'biomarker', 'bioindicator' and 'ecological indicator', linking them to different levels of biological organization. They considered a biomarker as any biological response to an environmental chemical at the sub-individual level, measured inside an organism or in its products (urine, faeces, hair, feathers, etc.), indicating a deviation from the normal status that cannot be detected in the intact organism. A bioindicator is defined as an organism giving information on the environmental conditions of its habitat by its presence or absence or by its behavior, and an ecological indicator is an ecosystem parameter, describing the structure and functioning of ecosystems. Since many of the biomarkers are short-term indicators of long-term adverse effects, these data may permit intervention before irreversible detrimental effects become inevitabile (McCarthy and Shugart, 1990). Various biochemical parameters in aquatic organisms have been tested for their responses to toxic substances and their potential use as biomarkers of exposure or effect. Biomarkers, which have been investigated most extensively, are enzymes involved in the detoxication of

xenobiotics and their metabolites (biotransformation enzymes, antioxidant enzymes), as reported by Van der Oost et al. (2003).

Nevertheless, despite the common use of biomarkers in ecotoxicology, some authors have claimed that such approaches have failed to live up to their promise. Forbes et al. (2006) listed a number of different limitations that have blighted numerous biomarker studies, and made a series of demands on how such experiments could be improved. In order to achieve a more detailed insight of the organismal health status, the application of biomarkers has recently been reinforced with the introduction of "-omics" technologies, which can offer greater insights to the pollutant effects at a molecular level.

1.3. Environmental metal contamination

An increasing variety of industrial and agricultural chemicals are introduced in coastal ecosystems. Among the best studied contaminants, there are chemicals of both longstanding and more recent concern, such as polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (e.g. dichlorodiphenyltrichloroethane, DDT), industrial products (e.g. polychlorinated biphenyls, PCBs), dioxins, nitroaromatic compounds, organometallic compounds, pesticides, estrogenic compounds, and many metals including Cd, Cr, Cu, Fe, Hg and Zn (Livingston, 2001). Metals are of great environmental concern, since they tend to concentrate in aquatic organisms, are virtually non-degradable, and thus produce long lasting effects upon the environment even after their major sources have been removed.

Metals are introduced into coastal systems through fluvial inputs, direct effluent discharges and by the atmosphere. After discharged metals are mostly adsorbed on suspended particles and finally accumulate in the sediment, which can serve as sink or source of metals to the overlying water. Many aquatic

organisms spend a major portion of their life in or on sediment with the possibility to take up those metals. Moreover, numerous studies have shown that sediment-water interaction in aquatic systems play an important role on controlling metals transport processes (De Domenico et al., 2013, 2011; Gomez et al., 1999; Point et al., 2007; Thouzeau et al., 2007). Indeed, the mineralization of organic matter plays a fundamental role in the sediment-water exchanges of metals.

A number of metals are used by living organisms to stabilize protein structures, facilitate electron transfer reactions, and are essential cofactors for oxidative phosphorylation, in gene regulation and free-radical homeostasis. For example, Cu, Zn and Fe are essential as constituents of the catalytic sites of several enzymes (Siegel, 1973). Nevertheless, other metals like Pb, Hg and Cd may displace or substitute essential metals and interfere with the proper functioning of enzymes and associated cofactors. Trace elements may accumulate in aquatic organisms through different mechanisms: directly from water, via uptake from suspended particles and sediment, or by the consumption of lower trophic level organisms. The former is an essential point to consider in evaluating adverse effects on ecosystems (Van der Oost et al., 2003). In view of that, there are several works that use the accumulation of metals in organisms as mean to assess the environmental health status (Fernandes et al., 2007; Morrison et al., 2007; Pereira et al., 2009).

The absorption of metals by aquatic animals involves their transfer to the circulatory system by epithelial barrier of gills, digestive organs or integument. Dissolved metals are mainly taken up by exposed body surfaces such as the gills, whereas particulate metals are mostly ingested and then taken up after solubilization in the gut. Uptake of essential metals such as Ca, Cu, Fe and Zn, often involves specific pathways, such as calcium channels and specific membrane carriers for Fe and Cu (Sunda and Huntsman, 1998). For nonessential metals (e.g. Cd and Hg) specific uptake mechanisms are not known and, thus appear to follow existing pathways for essential metals

(Sunda and Huntsman, 1998).

Sequestration of metals in an immobilized form occurs throughout the various organs involved in pathways for metal uptake, transport, utilization and release. One of the best studied intracellular structures are the metallothioneins. These are low-molecular-weight cytosolic proteins rich in -SH groups, with high affinity for metal ions, known to be involved in metal homeostasis and over-expressed in organisms experiencing high metal contamination (Viarengo et al., 1998). Their expression in tissues is regarded as an indicator of metal contamination and widely used as a tool for biomonitoring programs (Fasulo et al., 2008; Viarengo et al., 1998).

Aquatic organisms utilize a variety of mechanisms to eliminate metals. The kinetic of metal release is complex and reflects the diverse compartments from which metals must be mobilized. Additionally, physical and chemical parameters, such as temperature and salinity, may affect the rate of release in aquatic animals, which can use several pathways to release metals (Mieiro et al., 2014, 2011; Pereira et al., 2014).

1.3.1. Toxicity of mercury

Among trace elements, mercury (Hg) is worldwide recognized as a hazardous pollutant mainly due to its persistence in water and sediments (Luoma and Rainbow, 2008), high toxicity to living organisms and tendency to bioaccumulate and biomagnify throughout food chains (Renzoni et al., 1998). The main sources of Hg to aquatic ecosystems derive both from natural processes (e.g. geological emissions) as well as anthropogenic activities, such as fossil fuel combustions (Pacyna et al., 2001), mining and smelting operations, and chlor-alkali industries (Driscoll et al., 2013; UNEP, 2011).

Despite the great efforts on evaluating the biological impact of Hg on aquatic organisms (De Domenico et al., 2013, 2011; Mieiro et al., 2014, 2011), the toxicity mechanisms of Hg still need to be clarified. In recent field and laboratory studies, oxidative stress has been described as a key pathway to initiate Hg toxicity in fish (Elia et al., 2003; Larose et al., 2008; Mieiro et al., 2014, 2011; Monteiro et al., 2010). Hence, both the modulation of antioxidant enzymes and changes in glutathione (GSH) content have been frequently employed as useful biomarkers for monitoring Hg contamination on aquatic organisms (Brandão et al., 2015; Guilherme et al., 2008; Mieiro et al., 2011).

However, these currently used biochemical assays are often inconclusive on elucidation of the mechanisms underlying the Hg-induced oxidative stress in fish. Hence, understanding the Hg toxicity requires new ways. In this regard, the use of environmental metabolomics may serve as a powerful tool focusing on a number of key metabolites, which changes might reveal insights into the mechanisms of oxidative damage due to Hg. Although metabolomics has been extensively used for mechanistic research, no studies have been developed to specifically investigate this scientific question. Therefore, the present thesis aims to addresses this specific issue and represents a very recent example of the potential of metabolomics to clarify the toxicity mechanisms of Hg, providing insights on the metabolic and oxidative stress responses in wild fish.

1.4. Metabolomics

The "-omics" sciences are readily increasing disciplines aimed at the study of biological systems (Berry et al., 2011). They include, among the others, genomics, transcriptomics, proteomics, and metabolomics. Whereas genomics, transcriptomics, and proteomics are based on the analysis of the genome, gene expression and proteins, respectively, metabolomics is deemed as the end point

of the "-omics cascade" (Dettmer and Hammock, 2004).

Indeed, one of the most recent additions to the "-omics" family is metabolomics, which in 2002 was defined by Fiehn as "the qualitative and quantitative study of the metabolome in a biological system" (Fiehn, 2002). Metabolomics is focused on the study of endogenous low molecular weight metabolites (<1000 Da), whose production and levels vary with the physiological, developmental, or pathological state of cells, tissues, organs or whole organisms (Lin et al., 2006; Viant, 2007). The metabolome describes the composition of low molecular weight metabolites at the time of sampling, and includes compounds such as lipids, sugars, and amino acids that can provide important clues about the health of individuals and a functional measure of cellular status at that moment in time (Lin et al., 2006; Schmidt, 2004).

There are several advantages for the application of this technique. One of the greatest advantages of metabolomics is that the metabolome is often the first to respond to anthropogenic stressors, where in some cases no changes in the transcriptome and proteome occur (Viant, 2007). Hence, it has been suggested that metabolomics may provide the most functional information of the "-omics" technologies (Sumner et al., 2003), as transcript and protein changes do not necessarily lead to a biochemical change in the study organism (Fiehn et al., 2002, 2000). Furthermore, the metabolome, a term coined by Oliver et al. (1998) to describe the set of metabolites synthesised by an organism in a fashion analogous to that of the genome and proteome, represents the final "-omic" level in a biological system, and metabolites represent functional entities, unlike messenger RNA molecules, which are further upstream of biological processes (Raamsdonk et al., 2001). Metabolites thus have a clear function in the life of the biological system and are also contextual allied with the further advantage that there are far fewer metabolites than genes or gene products to be studied (Raamsdonk et al., 2001). However, it should be duly noted that all "-omics" technologies provide valuable information, and integration of these techniques promises to provide the most

complete understanding of biological systems.

Basically, metabolomic studies can be divided in *targeted* and *untargeted* analyses (Fernández-Peralbo and Luque de Castro, 2012). In untargeted approaches, significant metabolites are, by definition, unknown prior to analysis, while in targeted analysis, the physico-chemical characteristics of the metabolites are known and an exhaustive separation of them from the matrix is usually required for quantification. Therefore, metabolomics investigations can be designed as targeted studies looking for specific metabolite changes, although this requires some prior knowledge on the metabolic action of whatever toxicant is being tested. Otherwise, an alternative metabolomic approach is where the global metabolome is analysed, although constrained by the efficiency and sensitivity of the techniques used to extract and detect the metabolites. This method is a relatively non-targeted approach where there is little, if any, prior selection of which metabolic components to measure. Thus, a similar study design can be used in both a screening mode and for mechanistic exploration (Keun, 2006).

A further differentiation of metabolomic analyses can be done based on the scientific application, namely *metabolic profiling*, *metabolic fingerprinting*, *metabolic footprinting*, and *metabolomics* (Oldiges et al., 2007). *Metabolic profiling* is the quantitative analysis of a group of pre- defined metabolites, like members of a particular pathway. *Metabolic fingerprinting* and *metabolic footprinting* are metabolomic studies focused on the classification of samples by analysing their intracellular metabolites (endometabolome) and extracellular metabolites (exometabolome), respectively. Lastly, *metabolomics* can be defined as the complete analysis of the entire cellular metabolome, in which all the metabolites are quantified and identified. While *target analysis*, *metabolic profiling*, and *metabolomics* are all quantitative approaches that require unique identification of all metabolites, *metabolic fingerprinting* and *metabolic footprinting* are semi-quantitative approaches and even unknown metabolites can be used to get deeper insights into samples metabolic profiles (untargeted).

1.4.1. Metabolomics workflow

Independently of the field of application, metabolomic studies have to pass through numerous steps in order to achieve profitable and reliable results. Indeed, a typical workflow of a metabolomic strudy includes hypothesis, experimental design, sampling, analytical platforms, data collection, multivariate data analysis, and interpretation of the results, which represent the basic steps in metabolomics. These aspects are summarized in Figure 1.1.

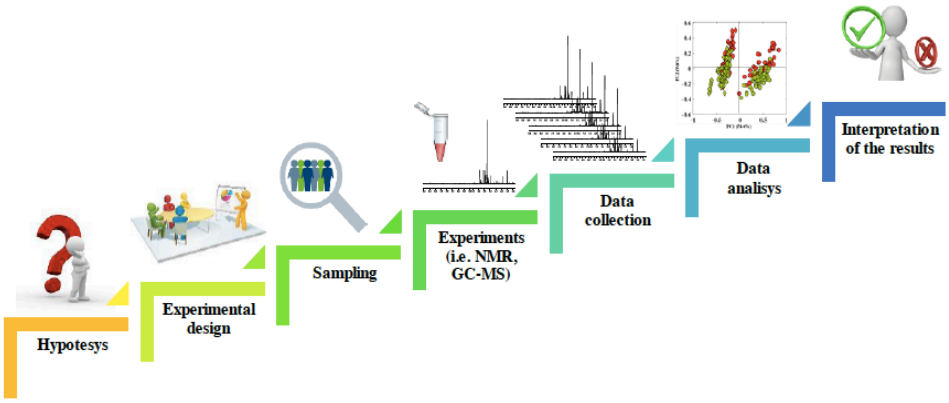


Figure 1.1. Typical workflow of a metabolomic strudy: hypothesis, experimental design, sampling, analytical platforms, data collection, multivariate data analysis, and interpretation of the results, representing the basic steps in metabolomics.

Among these aspects, sample selection and preparation is a crucial point in metabolomic experiments. A correct sampling provides a real snapshot of the metabolome at a certain point in time, hence the necessity to adopt procedures fostering an unbiased sampling. Strategies of sampling and sample preparation vary according to the experimental setup, thus different strategies for the metabolites sampling can be performed. Extracellular metabolites present in human or animal biofluids are sampled using either non-invasive (urine) or invasive (serum, plasma) methods. As a matter of fact, the process of sampling

can change the metabolome composition (Dunn and Ellis, 2005).

Samples storage is another crucial point in metabolomic analysis as the continued freezing/defrosting of samples could damage their molecular stability. The inhibition of the enzymatic activity, aimed to preserve sample biochemical composition, is normally achieved through the freeze clamping or freezing in liquid nitrogen followed by storage at -80 °C (Dunn and Ellis, 2005).

In regard to metabolite extraction, the adopted procedures dictate the nature and levels of the extracted metabolites. For non-targeted approaches, the objective is to extract the maximum number of metabolites from many chemical classes in a quantitative and non-biased manner with minimal losses of metabolites. For metabolic profiling, extraction is generally performed by the disruption of cell walls and subsequent distribution of metabolites into polar (methanol, water) and non-polar (chloroform, hexane, ethyl acetate) solvents followed by the removal of the cellular residue.

The preparation of samples for the analysis is also dependent on the metabolomics strategy employed. Targeted analyses require separation of the metabolome into chemical classes, whereas for metabolic profiling and fingerprinting analyses, samples are mainly analysed directly without further separation of metabolites into subclasses (Emwas et al., 2013).

However, in spite of the great variability related to the sampling procedure, it is generally recognized that the impact of unpredictable biological variability is much higher than that related to the analytical one.

1.4.2. NMR-based metabolomics

Metabolites separation and identification is made possible thanks to several advanced analytical techniques. Nuclear Magnetic Resonance (NMR)

spectroscopy, Mass Spectrometry (MS), and chromatographic methods, such as Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC), are the most commonly used platforms (Dunn and Ellis, 2005). All techniques have advantages and drawbacks and there is not an analytical technique completely suitable for metabolomic studies. In particular, NMR and MS have been demonstrated to be complementary and powerful analytical approaches for the complete characterization of the metabolome (Pan and Raftery, 2007).

By virtue of its numerous advantages, NMR spectroscopy is largely used in metabolomic studies for the analysis of bulk metabolites. High-resolution NMR spectroscopy is a quantitative technique that can report on hundreds of compounds in a single measurement. NMR can provide information on metabolites that comprise nuclei such as ^{1}H, ^{13}C, ^{31}P. These nuclei can exist at different energy states in a strong magnetic field because they possess nuclear spin, allowing the generation of valuable structural information. NMR spectroscopy (and most spectroscopic techniques) is based on the principle that lines that can be seen in the spectra are due to transitions between these energy states or levels. Such a transition can be caused by a photon of light whose frequency, υ, is related to the energy gap, ΔE, between the two levels according to:

$$\Delta E = h\upsilon$$

where h is the universal constant, namely Plank's constant.

The appearance of multiplets and other peaks in an NMR spectrum are predicted by using different rules to this approach but are still related to energy levels. The splitting of energy levels in a magnetic field is dependent on the nuclear spin of the atom. For atoms that possess spin (such as ^{1}H, ^{13}C, ^{31}P), the net spin of the nucleus can be determined from the number of protons plus neutrons. When the number of protons and number of neutrons are even, there

is no spin. If the number of protons plus neutrons is odd then the nucleus has half integer spin (i.e. 1/2, 3/2, 5/2) and if the number of protons and neutrons are both odd then the nucleus has an integer spin (i.e. 1, 2, 3). The overall spin, I, is important and a nucleus of spin I will have $2I + 1$ possible orientations. When a magnetic field is applied, the energy levels split with each level given a magnetic quantum number, m. In a magnetic field a nucleus with spin 1/2 will have two orientations: $m = +1/2$, a low energy state aligned parallel to the magnetic field; and $m = -1/2$, a high energy state aligned anti-parallel to the magnetic field. The initial populations of the energy levels are determined by thermodynamics, as described by the Boltzmann distribution and means the lower energy level will contain slightly more nuclei than the higher level. However, these nuclei can be excited into the higher level by electromagnetic radiation with the frequency of radiation needed determined by the difference between the energy levels.

In an NMR experiment, each nucleus has a magnetic moment and their relative contributions add up to create a net magnetic field along the direction of the applied field (B_0). This is called bulk magnetisation and can be represented by a vector pointing along the direction of the applied field (z). If this magnetisation vector is tipped away from the z axis, which can be brought about by the application of a radiofrequency pulse, it rotates about the direction of the magnetic field sweeping out a constant angle. The vector is said to precess about the field in a motion known as Larmor precession at the Larmor frequency (Keeler, 2005). The precession of the magnetisation vector is what is detected in an NMR experiment, known as the free induction signal. If these signals were to be plotted they would represent simple oscillations at the Larmor frequency, and Fourier transformation of these signals can produce an NMR spectrum.

The magnetisation can be rotated away from its equilibrium position along the z axis using the idea of resonance by applying a small magnetic field along the x axis that is resonant with the Larmor frequency. Different types of pulses

exist and when the transmitter frequency is exactly the same as the Larmor frequency, the pulse is said to be exactly on resonance (Keeler, 2005). Pulses can be modified by changing the time at which they are applied. This alters the angle (known as "flip angle of the pulse") through which the magnetisation has been rotated. Commonly used flip angles are 90º where the process is called a 90º pulse, and 180º where the magnetisation is taken all the way from $+z$ to $-z$. In practical NMR spectroscopy multiple resonances are usually present in the spectrum with different Larmor frequencies. A sufficiently strong radio frequency (RF) pulse is therefore needed to overcome the induced field to move all the signals at different Larmor frequencies away from equilibrium and this is called a hard pulse. When the pulse is weaker it is possible to excite a single signal within the spectrum and can be done by choosing a radio frequency identical to a selected signal reducing the influence of the pulse on other signals. These pulses are known as selective pulses or soft pulses and can be used to help suppress resonances that are not of interest in the sample (for example solvent-based resonances).

1.4.3. Chemical shifts in NMR spectra

Depending on the local chemical environment, different protons in a molecule resonate at slightly different frequencies, and frequencies at which NMR absorptions (lines) occur scale linearly with the magnetic field strength. As this frequency shift and the fundamental resonant frequency are directly proportional to the strength of the magnectc field, the shift can be converted into a dimensionless value known as the chemical shift. The chemical shift is then reported relative to a reference resonance frequency (a commonly used compound is sodium 3-trimethylsilyl-2,2,3,3-d4-propionate (TMSP), or 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), which is used in this thesis). The

difference between the frequency of the signal and the frequency of the reference is divided by the frequency of the reference signal to give the chemical shift and is expressed in parts per million (ppm) (Keleer, 2005).

The chemical shift can be used to obtain structural information. For the ^{1}H-NMR spectrum of ethanol (CH_3CH_2OH), three specific signals are expected at three specific chemical shifts: one for the CH_3 group, one for the CH_2 group and one for the OH group. The three methyl groups average out during the course of the NMR experiment and the protons become degenerate forming a peak at the same chemical shift. The size and shape of peaks also give further information and the CH_3 peak would be three times as large as the OH, and the CH_2 peak only twice the size of the OH peak but 2/3 the size of the CH_3 peak. The integrated areas under the peaks are also important, and in a complex mixture they can be used to predict the concentration of a particular compound.

1.4.4. Metabolomics in environmental monitoring

Nuclear magnetic resonance (NMR) spectroscopy-based metabolomics, when linked with pattern recognition techniques and data mining tools, can detect differences in the profile of metabolites (metabolite biomarkers) in response to environmental stressors, diseases or exposure to toxicants (Cappello et al., 2016a, 2016b, 2015, 2013b; Fiehn, 2002; Hines et al., 2007; Liu et al., 2015; Tuffnail et al., 2009; Viant et al., 2003; Xu et al., 2015), thus providing an overview of the metabolic status of a biological system. Among the pattern recognition techniques, Principal Component Analysis (PCA) is a multivariate statistical technique which can be used to identify correlations amongst a set of variables and to transform the original set of variables to a new set of uncorrelated variables called principal components (PCs). PCs are linear combinations of the original variables and are derived in decreasing

order of importance. Therefore, the first PC accounts for the maximum variation among the samples, and subsequent PCs are chosen to account for progressively decreasing variance (Chatfield and Collins, 1980; Jolliffe, 1986; Martens and Naes, 1989). PCA is a variable-directed technique and therefore does not use any a priori knowledge of the groupings within samples in the data set. It is an unsupervised method, and thus plots of PCs are thought to display the natural relationships between the samples on a score plot.

Metabolite profiling, originally developed for human biomedical applications (Nicholson et al., 1988) has now been increasingly employed in several research areas, including plant science (Kim et al., 2010), food quality (Tarachiwin et al., 2008), microbial metabolomics (Boroujerdi et al., 2009) and environmental metabolomics (Viant, 2009). Because metabolomics can provide valuable information on how xenobiotics influence physiological functions, this technique has also been applied to experimental studies of selective exposure on various aquatic organisms, both invertebrates (Cappello et al., 2013b; Wu and Wang, 2010) and fish (Cappello et al., 2016a, 2016b; Iacono et al., 2010; Santos et al., 2010).

In ecotoxicology, metabolomics is of particular value for the risk assessment of chemicals in the environment (Lin et al., 2006). Recently, there have been numerous studies applying metabolomic techniques to this area of research. Various studies have been published in aquatic species on the effects of pesticides and other xenobiotics using NMR-based metabolomics. Experiments with Japanese medaka (*Oryzias latipes*) have shown that metabolic changes in embryos exposed to dinoseb correlated with traditional toxic endpoints such as reduced growth and heart rates, abnormal development and post-exposure mortality (Viant et al., 2006a). Results from ^{1}H NMR metabolomics were also in agreement with findings from a previous study using *in vivo* ^{31}P NMR and HPLC-UV, in which phosphocreatine utilisation for compensation of ATP loss could be used as an indicator of medaka embryotoxicity (Pincetich et al., 2005).

The advantages of NMR-based metabolomics has also been proven in studies involving salmon (*Oncorhynchus tshawytscha*) alevins exposed to dinoseb, diazinon and esfenvalerate where identifiable metabolic changes were detected with greater sensitivity using NMR compared to HPLC-UV (Viant et al., 2006b). Furthermore, the metabolic fingerprints of eyed egg and alevin extracts revealed both dose-dependent and mechanism-specific responses induced by pesticides.

Metabolomics applications to disease monitoring in wild species have been shown by studies in Californian red abalone (*Haliotis rufescens*) (Rosemblun et al., 2006; Viant et al., 2003). NMR metabolomics was successfully able to distinguish between healthy, stunted and diseased groups afflicted with a disease known as withering syndrome associated with a Rickettsiales-like prokaryote (RLP) infection. The ratio between the metabolites homarine and glucose in foot muscle was able to effectively predict the disease status of RLP infected animals. Other environmental stressors such as increased water temperature were also assessed and judged to be a contributing factor in disease development since RLP infection alone did not cause animals to develop withering syndrome.

A further parasitic infection investigated using NMR-based metabolomics involved Atlantic salmon (*Salmo salar*) exposed to the bacterium *Aeromonas salmonicida*. The metabolic response consisted primarily of changes in lipoprotein and choline levels for infected salmon highlighting the potential of metabolomics for studying the disease status in this and other wild species (Solanky et al., 2005).

CHAPTER II

Aims of the thesis

2. Aims of the thesis

Overall, the primary objective of this thesis is to evaluate the potential of NMR-based metabolomics in the mechanistic research, in conjunction with a combined approach of conventional oxidative stress biomarkers, in order to achieve a comprehensive assessment of the mechanisms of toxicity of mercury (Hg), and discover novel biomarkers that may function as a diagnostic tool for detecting and monitoring the environmental impact of Hg on fish and, given the similarity of fish responses with higher vertebrates, potentially on humans.

Specifically, the three main aims of this thesis are:

- To determine Hg bioaccumulation, identify potential metablite shifts as early warming indicators of Hg toxicity, and elucidate the modulation of the antioxidant system, and subsequent emergence of oxidative damage, in liver of the golden grey mullet *Liza aurata* inhabiting an Hg contaminated system. Liver is the major site for xenobiotic bioaccumulation and detoxification processes.

- To determine Hg bioaccumulation, identify potential metablite shifts as early warming indicators of Hg toxicity, and elucidate the modulation of the antioxidant system, and subsequent emergence of oxidative damage, in gills of the golden grey mullet *Liza aurata* inhabiting an Hg contaminated system. Gills are the main route for the uptake of pollutants because of their anatomic location and large surface constantly exposed to seawater and its substances, harmful or not.

- To provide a detailed comparative analysis of the differential bioaccumulation of Hg in liver and gills of *L. aurata*, together with their metabolic profiles, in order to achieve a more comprehensive assessment of the mechanisms of Hg toxicity.

CHAPTER III

Environmental availability of mercury in the Aveiro Lagoon, Portugal

3. Environmental availability of mercury in the Aveiro lagoon, Portugal

3.1. General introduction

Aquatic ecosystems are often impacted by a number of human activities that, as a consequence of the release into the environment of toxic chemicals, may adversely affect the biota and human health. Aquatic ecotoxicology is the research field that focuses on the elucidation of the adverse effects of environmental pollutants at various organizational levels of biological systems to unravel the modes of action of toxicants altering normal biological performances (Connon et al., 2012). In ecotoxicological studies, fish have been widely used as worthy sentinel organisms for the assessment of the environment health status (Brunelli et al., 2011; Fasulo et al., 2012b, 2010; Ferrando et al., 2005; Guilherme et al., 2008; Mauceri et al., 2005; Mieiro et al., 2014, 2009; Xu et al., 2015). This is mainly because of their ability to efficiently metabolize, accumulate, and concentrate environmental contaminants in their tissues, and elicit measurable responses to toxic insults.

Among metals, mercury (Hg) is worldwide recognized as one of the most harmful pollutants mainly due to its persistence in water and sediments (Luoma and Rainbow, 2008), high toxicity to living organisms and tendency to bioaccumulate and biomagnify throughout food chains (Renzoni et al., 1998). The recognition of Hg as a priority hazardous contaminant in the aquatic environment has triggered numerous studies over the past decades, in an attempt to appraise the implications for the aquatic organisms and human health. Indeed, Hg tends to bioaccumulate and biomagnify through the food chains, posing a risk for humans through fish consumption, as evidenced by recent epidemiological studies addressing human health effects of methylmercury (Murata et al., 2011). In this context, fish have deserved particular attention due to their major ecological role in aquatic food webs as a

carrier of energy from lower to higher trophic levels. Despite the efforts devoted by aquatic ecotoxicologists to evaluating the biological impact of Hg on aquatic lower vertebrates (De Domenico et al., 2013, 2011; Mieiro et al., 2014, 2011; Pereira et al., 2014), the toxicity mechanisms of Hg still need to be clarified.

In recent field and laboratory studies, oxidative stress has been described as a key pathway to initiate Hg toxicity in fish (Elia et al., 2003; Larose et al., 2008; Mieiro et al., 2014, 2011; Monteiro et al., 2010). Oxidative damage occurs when the animal's defence mechanisms, responsible for removal of reactive oxidative species (ROS), are inadequate or insufficient, disturbing the prooxidant–antioxidant balance and promoting oxidative stress (Lushchak, 2011). To prevent damage caused by ROS, fish usually react by enhancing the activity of protective antioxidants enzymes, such as catalase, superoxide dismutases, and peroxidases, as well as the levels of non-enzymatic free radical scavengers such as reduced glutathione (GSH). Hence, both the modulation of antioxidant enzymes and changes in GSH content have been frequently employed as useful biomarkers for monitoring Hg contamination on aquatic organisms (Cappello et al., 2013a; Guilherme et al., 2008; Mieiro et al., 2011; Natalotto et al., 2015). However, these currently used biochemical assays are often inconclusive on elucidation of the mechanisms underlying the Hg-induced oxidative stress in fish. For instance, it is still not clear why, in some cases, lipid peroxidation does not occur with the depletion of antioxidants (Mieiro et al., 2010). Hence, understanding the Hg toxicity requires new ways.

Indeed, due to their unidimensional nature, the conventional ecotoxicological methods for stress evaluation in sentinel organisms cannot provide the "full picture" of biological effects on organisms upon exposure to stressful conditions. Therefore, in order to overcome such limitations, a current approach is to employ "-omics" techniques that allow for the simultaneous evaluation of a broad number of biomolecules. Metabolomics, defined as the comprehensive analysis of all endogenous small metabolites (molecular weight

<1000 Da) in cells, tissues, biofluids, or whole organisms (Lin et al., 2006), has demonstrated notable potential in the field of ecotoxicology. In particular, metabolomics based on protonic nuclear magnetic resonance (^{1}H NMR), coupled with chemometric approaches, offer the potential to elucidate organism-environment interactions and identify new metabolite biomarkers of stress in organisms in response to changes in abiotic factors, diseases, or environmental pollutants (Brandão et al., 2015; Cappello et al., 2015, 2013b, 2016a, 2016b; Fasulo et al., 2012a; Iacono et al., 2010; Liu et al., 2015; Xu et al., 2015). Moreover, it is widely recognised the value of metabolomics in disclosing the toxicity mechanisms of environmental contaminants, including herbicides (Xu et al., 2015), endocrine disrupting chemicals (Katsiadaki et al., 2010), polyaromatic hydrocarbons (Cappello et al., 2015, 2013b; Fasulo et al., 2012a), and metals (Santos et al., 2010).

Therefore, the use of environmental metabolomics may serve as a powerful tool to reveal insights into the mechanisms of oxidative damage due to Hg. Although metabolomics has been extensively used for mechanistic research, no studies have been developed to specifically investigate this scientific question. Thus, the research conducted within this doctoral thesis, in part already published (Brandão et al., 2015; Cappello et al., 2016a, 2016b), appears as a very recent example of the potential of metabolomics to clarify the mechanisms of toxicity of Hg in fish.

Hence, considering that ROS-associated processes have been suggested as a precocious expression of Hg-induced toxicity in fish and that molecular processes are the deepest level of the problem, the combination of metabolomics and conventional oxidative stress biomarkers was applied to the golden grey mullet (*Liza aurata*) inhabiting an Hg contaminated system, choosing liver and gills as target organs. *Liza aurata* is an abundant European mugilide, euryhaline and widely distributed in both Atlantic and Mediterranean coastal waters. Its feeding behaviour is characterized by a regular contact with the sediment, often extended to the whole water column. Therefore, it is a

valuable bio-indicator species for monitoring water contaminants within a large lipophilicity range. Moreover, it has been demonstrated to be sensitive to Hg exposure (Brandão et al., 2015; Guilherme et al., 2008; Mieiro et al., 2011).

Overall, ^{1}H NMR metabolomics and biochemical assays were applied in conjunction on liver and gills of *L. aurata* with the aims to (i) identify potential metabolite shifts as early warning indicators of Hg toxicity, (ii) elucidate the modulation of the antioxidant system and subsequent emergence of oxidative damage, and (iii) clarify crosslinking processes between the two previous levels of response and Hg accumulation, as a crucial step towards the understanding of Hg toxicity. Moreover, a detailed comparative analysis of the differential bioaccumulation of Hg in liver and gills of *L. aurata*, together with their metabolic profiles, was also included herein in order to highlight potential tissue-specific responses to mercury, and therefore provide a more comprehensive assessment of the mechanisms of Hg toxicity.

3.2. Materials and Methods

This study was conducted in accordance with the EU Directive 2010/63/EU of 22nd September on the protection of animals used for scientific purposes, under the supervision of a team member (Mário Pacheco) authorized by the competent authorities.

3.2.1. Study area characterization

Ria de Aveiro is a coastal lagoon located along the northwestern coast of Portugal, which has an inner basin, Largo do Laranjo (LAR), located near Estarreja (Fig. 3.1) that is a confined area severely contaminated by Hg. This area continuously received chlor-alkali plant discharges for almost five decades

from 1950. About 20 years ago, the improvement in the production process by the industry resulted in a considerable reduction of Hg release. However, the high Hg amount still stored in the sediments (Coelho et al., 2005) and its progressive resuspension, represent a source of contamination for aquatic organisms inhabiting this area (Mieiro et al., 2014, 2011; Pereira et al., 2014). Due to the absence of other important sources of contaminants, Laranjo basin is considered to be a ‘‘field laboratory”, offering a unique opportunity to assess Hg toxicity under realistic conditions (Guilherme et al., 2008; Mieiro et al., 2009). Conversely, the area of São Jacinto (SJ), located in proximity of the lagoon entrance (Fig. 3.1), was considered as a reference site known to be unaffected by Hg contamination (Guilherme et al., 2008). Additionally, according to the Portuguese legislation for sediment quality (Portaria nº1450/2007), SJ sediments belong to class 1 in what concerns trace metals, meaning non-contaminated dredge material.

At both sampling sites, sub-surface water (at 0.2 m depth) was sampled in triplicate with polypropylene acid decontaminated bottles for the determination of total dissolved Hg (DHg) and methylmercury (DMeHg). At the same depth, temperature, salinity and dissolved oxygen were measured *in situ* in triplicate with an YSI 650 meter (Yellow Springs, USA).

Additionally, surface sediments (about the first 2 cm) were collected at the two sites for the determination of total mercury (tHg) and MeHg.

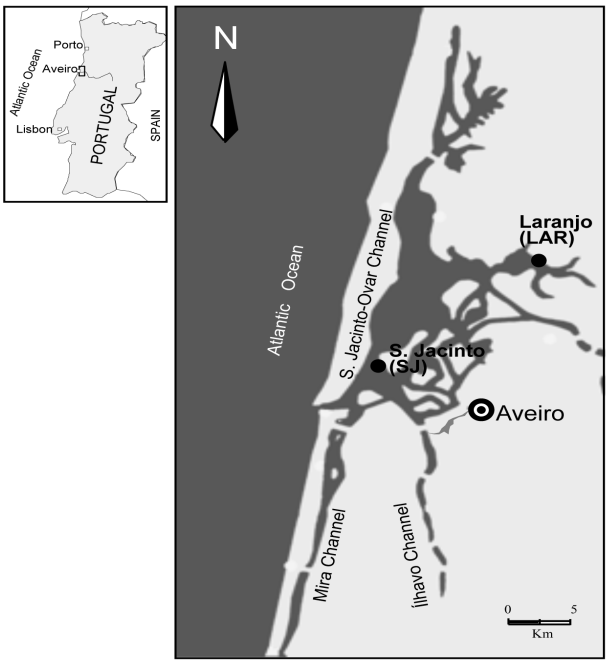


Figure 3.1. Location of the sampling sites at Aveiro lagoon (Portugal): São Jacinto (SJ) (40°41′00″ N, 8°42′44″ W); Laranjo (LAR) (40º43'28.98'' N, 8º37'35.80'' W).

3.2.2. Sampling

Juveniles of golden grey mullets (*L. aurata*) were collected in summer (June 2013) at S. Jacinto (SJ) (16.5 ± 2.1 cm mean total length) and Laranjo (LAR) (13.6 ± 2.1 cm mean total length) during low-tide using a traditional beach-seine net named ''chincha". The sites were selected taking into account previous ecotoxicological studies (Guilherme et al., 2008; Mieiro et al., 2014, 2011; Pereira et al., 2014). Juvenile specimens were used to minimize the interference of variables such as gender. In compliance with the ethical guidelines of the European Union Council (Directive 2010/63/EU), fish were anesthetized with tricaine methanesulfonate (MS-222) immediately after catching, sacrificed and properly bled. Liver and gill samples were rapidly excised and flash-frozen in liquid nitrogen in three sets of samples: one for the

quantification of Hg, one for metabolic profile evaluation, and another one for oxidative stress quantifications. Samples were then transferred to the laboratory and stored at -80 ºC until further processing for the quantification of Hg, metabolite assessment and oxidative stress assays.

Mercury bioaccumulation, metabolic and oxidative stress responses of wild mullet livers and gills are reported in Chapter IV and V, respectively. Furthermore, a detailed comparative analysis of the differential bioaccumulation of Hg in liver and gills of *L. aurata*, together with their metabolic responses to Hg, is also included in Chapter VI.

3.2.3. Mercury in the water column

Total dissolved mercury (DHg) was determined following U.S. EPA method 1630 (U.S.EPA., 2002). Briefly, water samples were preserved by the addition of 0.5% BrCl until analyses (less than one week after collection). Samples were then analyzed by cold-vapour atomic fluorescence spectrometry (CV-AFS) with a PSA model Merlin 10.023 equipped with a detector PSA model 10.003 using $SnCl_2$ reduction. BCR-579 certified reference material was used to control the accuracy of the procedure (Table 3.1).

Dissolved methylmercury (DMeHg) in water samples was determined following U.S. EPA method 1631 (U.S.EPA, 2001) by distillation of 50 mL sub-samples, after addition of 1% $C_5H_9NS_2.NH_3$ as a complexing agent. Mercury was ethylated with $NaB(C_2H_5)_4$, purged with argon, collected on Tenax^{TM} traps, separated with a gas chromatography (GC), thermally desorbed to Hg(0) for detection of DMeHg with a Brooks Rand Model III CV-AFS. All sample batches analyzed for DMeHg included at least one method replicate, and at least three analytical standards prepared by dilution of MeHgCl (Sigma-Aldrich RTC) in ethanol and after diluted with Milli-Q water. Recoveries were determined by spiking samples with MeHg and varied between 92-101%.

Certified Reference Material		Total Hg (ng kg^{-1})	Total Hg (µg g^{-1})	MeHg (ng g^{-1})
BCR-579	Obtained	2.2±0.3	-	-
	Certified	1.9±0.5	-	-
MESS-2	Obtained	-	0.095±0.004	-
	Certified	-	0.092±0.009	-
IAEA-405	Obtained	-	0.85±0.02	5.51±0.08
	Certified	-	0.81±0.04	5.49±0.53
BCR-580	Obtained	-	-	71±3
	Certified	-	-	75±4
DORM-4	Obtained	-	0.403±0.012	0.358±0.020
	Certified	-	0.410±0.055	0.354±0.031

Table 3.1. Total mercury and methylmercury levels in coastal seawater (BCR-579), three estuarine sediments (MESS-2, IAEA-405 and BCR-580) and fish protein (DORM-4), as well the respective certified values. Mean levels and the associated uncertainty are presented.

3.2.4. Mercury in sediments

Sediment samples were analysed for tHg by atomic absorption spectrometry (AAS) by thermal decomposition following by gold amalgamation in an Hg analyser (AMA) LECO 254 (Costley et al., 2000). Methylmercury was determined in dry sediments by alkaline digestion (KOH/MeOH), organic extraction with dichloromethane (DCM) pre-concentration in aqueous sulphide solution, back-extraction into DCM and quantification by GC-AFS in a Agilent Chomatograph coupled with a pyroliser unit and a PSA florescence detector (Canário et al., 2004). Recoveries and the possible MeHg artifact formation were evaluated by spiking several samples with Hg(II) and MeHg standard solutions with different concentrations. Recoveries varied between 97 and

103%, and no artefact MeHg formation was observed during the procedure. Precision of Hg analysis, expressed as relative standard deviation of 4 replicate samples, was less than 4% ($p<0.05$). Certified reference materials (MESS-2, IAEA-405 and BCR-580) were used to ensure the accuracy of the procedure. Levels of tHg and MeHg obtained in the reference materials were consistent within the ranges of the certified values (Table 3.1).

3.3. Results

3.3.1. Water and sediment characteristics

Water characteristics are described in Table 3.2. Water temperature was similar between the two sites (SJ and LAR). Salinity and dissolved oxygen were lower at LAR than SJ. Dissolved oxygen was around 100% at SJ but undersaturation was recorded at LAR. This site presented higher levels of tHg and MeHg, and the percentage of MeHg with respect to tHg in water, when compared to SJ.

Levels of tHg and MeHg in sediments, as well as the percentage of MeHg, were higher at LAR than SJ (Table 3.3).

Site	T (ºC)	Salinity	DO (%)	DHg (ng L^{-1})	DMeHg (ng L^{-1})	% MeHg
SJ	18±0.12	33±0.11	102±1.9	1.0±0.02	0.016±0.007	1.4±0.79
LAR	18±0.05	21±0.07	65±0.50	1.5±0.77	0.040±0.008	3.0±0.83

Table 3.2. Water temperature (T), salinity, dissolved oxygen (DO), total dissolved Hg (DHg), dissolved methylmercury (DMeHg), and the percentage of MeHg with respect to total mercury. Data measured at low-tide are presented for São Jacinto (SJ) and Laranjo (LAR) at Aveiro lagoon. Means and associated standard deviations are presented.

Site	Total Hg (ng g^{-1})	MeHg (ng g^{-1})	% MeHg
SJ	25±5	0.1±0.02	0.44±0.04
LAR	440±250	8±3	1.9±0.42

Table 3.3. Total Hg, methylmercury (MeHg) and the percentage of MeHg with respect to total Hg in surface sediment. Data are presented for São Jacinto (SJ) and Laranjo (LAR) at Aveiro lagoon. Means and associated standard deviations are presented.

3.4. Discussion

Water physicochemical parameters (e.g. temperature, dissolved oxygen, salinity) can determine the fate of pollutants in the environment, influencing also fish metabolic/oxidative stress profiles both directly and indirectly through the modulation of Hg bioavailability. In the present study, the water physico-chemical parameters were evaluated and it was found that salinity and dissolved oxygen diverge considerably among the sampling sites, both being lower in LAR. These differences have been taken into account on the interpretation of Hg effects. Although it has been recognized that salinity can affect metals bioavailability (Monserrat et al., 2007), under the present conditions its influence should be undervalued, since the Hg levels found in the water column were low.

CHAPTER IV

Metabolic and oxidative stress responses in liver of wild mullet *Liza aurata*

4. Metabolic and oxidative stress responses in liver of wild mullet *Liza aurata*

4.1. General introduction

The liver of lower vertebrates has been extensively investigated in environmental monitoring studies as a target organ to evaluate the fish health status (Berntssen et al., 2003; Fasulo et al., 2010a; Guilherme et al., 2010; Mieiro et al., 2014). The liver is the major site for several metabolic activities and detoxification processes, and it has been demonstrated to play a main role in accumulation (Mieiro et al., 2014), biotransformation (Van der Oost et al., 2003) and cycling of Hg (Ung et al., 2010). Therefore, the liver is an important organ to understand toxic effects and malfunctions that may elicit metabolic disorders induced by toxicants.

The mechanism of detoxification of organic pollutants consists of two steps essential to modify the toxicity of lipophilic xenobiotics, converting them to water-soluble and easily excretable metabolites (Bucheli and Fent, 1995; Kleinow et al., 1987; Livingstone, 1993). Phase I is a non-synthetic alteration (oxidation, reduction or hydrolysis) of the original foreign molecule, and represent the introduction of functional groups. The phase I metabolites can then act as substrates for phase II enzyme reactions, also known as conjugation pathways (Buhler and Williams, 1988; Daly, 1995). Hence, induction and/or inhibition of certain enzymes may indicate the presence in the environment of biologically significant levels of xenobiotics, and thus measurement of the activities of biotransformation enzymes is strongly recommended in environmental monitoring programs (Bebianno et al., 2007; Bucheli and Fent, 1995; Cappello et al., 2013a; Fasulo et al., 2010a; Iacono et al., 2010; Pereira et al., 2010; Sureda et al., 2011; Van der Oost et al., 2003).

The phase II of metabolism involves a conjugation of the parent compound or its metabolites (produced in phase I) with an endogenous ligand. Some

xenobiotics contain the functional groups for direct metabolism by conjugative phase II enzyme systems, while others are metabolized by integrated steps involving prior action of the phase I enzymes (Sijm and Opperhuizen, 1989). The major pathway for electrophilic compounds and metabolites is the conjugation with glutathione (GSH), which is catalysed by the glutathione S-transferases (GSTs). GST is thus involved in the detoxification of many environmental chemicals, rendering them less reactive and more water-soluble (Cheung et al., 2001; Pan et al., 2009). Apart from their essential functions in intracellular transport and biosynthesis, GSTs have a critical role in defence against oxidative damage and peroxidative products of DNA and lipids (George, 1994).

Another important mechanism of toxicity associated with xenobiotic products is oxidative damage (Sanchez et al., 2005), which is due to the imbalance between generation and neutralization of reactive oxygen species (ROS) by antioxidant mechanisms within an organism (Davies, 1995). Biological systems have developed during their evolution adequate enzymatic and non-enzymatic antioxidant mechanisms to protect their cellular components from oxidative damage. Indeed, These include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and GST, as well as some molecules with antioxidant action such as GSH. The activity of these enzymes can be used as a biomarker of oxidative stress, although it does not respond specifically to a group of pollutants but can be induced by a wide range of contaminants, including organic xenobiotics, heavy metals and PAHs (Altenburger et al., 2003; Fernández et al., 2012; 2010; Livingstone, 2001; Roméo et al., 2003; Sureda et al., 2011; Winston and Di Giulio, 1991).

Therefore, the aim of this study was to assess the metabolic and oxidative stress responses to mercury elicited in liver of wild golden grey mullet *Liza aurata* inhabitating an Hg contaminated system, in order to elucidate the hepatotoxicity mechanisms of mercury in fish, and potentially in humans.

4.2. Materials and Methods

This study was conducted in accordance with the EU Directive 2010/63/EU of 22nd September on the protection of animals used for scientific purposes, under the supervision of a team member (Mário Pacheco) authorized by the competent authorities.

4.2.1. Chemicals

Deuterated water (D_2O) was acquired from Armar AG (Switzerland), and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and the other chemicals for metabolomics analysis were purchased from Sigma-Aldrich (Italy). Bovine serum albumin was purchased from E. Merck-Darmstadt (Germany) and all the other chemicals used for oxidative stress conventional parameters were obtained from the Sigma–Aldrich Chemical Company (Spain). Other routine chemicals (analytical grade) were purchased from local suppliers.

4.2.2. Mercury in fish liver

Liver samples (n=10) were firstly lyophilised and macerated. Samples were then analysed for tHg as previously described for sediment. For MeHg analysis, a modified methodology was used (Armstrong et al., 1999; Westöö, 1967). Briefly, approximately 2 mL of Milli-Q water and 3 mL of KOH (6 M) solution were added to 200 mg of dried sample. The mixture was shaken for 2 hours and then 3 mL of HCl (6 M) and 4 mL of a $KBr/CuSO_4$ (3:1) solution was added. After 10 min of shaking, 5 mL of DCM was then added, the mixture centrifuged and finally the organic phase separated. A slight sulphide solution (≈ 0.06 mM) was used to extract MeHg from the organic phase and then MeHg was back extracted to DCM. Methylmercury in DCM was

quantified by GC-AFS using the chromatographic equipment described above. Again, the possible MeHg artifact formation were also evaluated by spiking several samples with Hg(II) and MeHg standard solutions of different concentrations. Recoveries varied between 92 and 103% and no artefact MeHg formation was observed. For all the analysis, precision expressed as the relative standard deviation of 3 replicate samples, was less than 2% ($p<0.05$). Certified reference material (DORM-4) was used to ensure the accuracy of the procedures (Table 3.1).

A crude estimation of the inorganic mercury (iHg) concentrations in the liver was done by subtracting tHg levels by the corresponding MeHg concentrations. For this estimation it was assumed that MeHg is the only organic mercury compound that is bioaccumulated in fish (Zhang and Adeloju, 2012).

4.2.3. Metabolomics analysis

4.2.3.1. Tissue metabolite extraction

Polar metabolites were extracted from liver of eight fish from each sampling site using a "two-step" methanol/chloroform/water procedure (Wu et al., 2008). Briefly, a 100 mg subsample of each frozen liver was homogenized in 4 mL/g of cold methanol and 0.85 mL/g of cold water by a TissueLyser LT bead mill (Qiagen) with 3.2 mm stainless steel beads, for 10 min at 50 vibrations/s. The homogenates were transferred to glass vials, and 4 mL/g chloroform and 2 mL/g water were added. Samples were vortexed for 60 s, left on ice for 10 min for phase separation, and then centrifuged for 5 min at 2000 g at 4 °C. Six hundred µL of the upper methanol layer with polar metabolites were transferred to glass vials, dried using a centrifugal vacuum concentrator (Eppendorf 5301) and stored at -80 °C. Immediately prior to NMR analysis,

the dried polar extracts were resuspended in 600 μL 0.1 M sodium phosphate buffer (pH 7.0, 10% D_2O) containing 1 mM DSS, vortexed, and then transferred to a 5 mm diameter NMR tube. DSS acts as an internal standard and provides a chemical shift reference (δ=0.0 ppm) for the NMR spectra, while D_2O provides a deuterium lock for the NMR spectrometer.

4.2.3.2. ^{1}H NMR metabolomics and spectral pre-processing

Extracts of liver tissue were analyzed on a Varian-500 NMR spectrometer operating at a spectral frequency of 499.74 MHz at 298 K. One-dimensional (1-D) ^{1}H NMR spectra were obtained using a PRESAT pulse sequence to suppress the residual water resonance and 6,983 Hz spectral width with a 2.0 s relaxation delay. A total of 128 transients were collected into 16,384 data points requiring a 10 min acquisition time. All data sets were zero filled to 32,768 data points and exponential line-broadenings of 0.5 Hz were applied before Fourier transformation. All ^{1}H NMR spectra were manually phased, baseline-corrected, and calibrated (DSS at 0.0 ppm) using Chenomx Processor, a module of Chenomx NMR Suite (version 5.1; Chenomx Inc., Edmonton, Canada) software. Peaks within the ^{1}H NMR spectra were assigned with reference to known chemical shifts and peak multiplicities, using public databases such as the HMDB, Human Metabolome DataBase (Wishart et al., 2009), and the Chenomx 500-MHz library database. The latter was also used for metabolite quantification as it uses the concentration of a known DSS signal to determine the concentrations of individual metabolites.

All 1-D ^{1}H NMR spectra were converted to a data matrix using Chenomx Profiler, another module included in Chenomx NMR Suite software. Each spectrum was segmented into 0.005 ppm chemical shift bins between 0.5 and 9.0 ppm, with bins from 0.60 to 0.64 and 2.87 to 2.93 ppm (DSS), and 4.66 to

5.19 ppm (water) excluded from all the NMR spectra to prevent interference in subsequent multivariable analyses. To conduct comparison between spectra, the integrated spectral area of the remaining bins was normalized to the total integrated area of the spectra.

4.2.4. Oxidative stress endpoints

Tissue samples (n=10) were homogenized in a 1:10 ratio (liver weight:buffer volume) of ice-cold phosphate buffer (0.1 mM and pH 7.4) using a Potter–Elvehjem glass–Teflon homogenizer. An aliquot of homogenate (50 µL) was removed to measure thiobarbituric acid reactive substances (TBARS). The remaining homogenate was centrifuged at 13,400 g for 25 min, and post mitochondrial supernatant (PMS) was divided into aliquots to be used for the different determinations. Homogenate and PMS were stored at -80 ºC until analyses.

Catalase (CAT) activity was measured following the decomposition of H_2O_2, according with the Claiborne (1985) protocol as described by Giri and coworkers (1996), adapted to 96-well microplate. The assay mixture consisted of 5 µL of PMS (previously diluted 1:10) and 195 µL of hydrogen peroxide (10 mM, prepared in phosphate buffer 0.05 M, pH 7.0) in a final volume of 200 µL. Changes in absorbance were spectrophotometrically monitored at 240 nm during 1 min, and activity was expressed as micromoles of hydrogen peroxide consumed/min/mg of protein (ε= 43.5 $M^{-1}cm^{-1}$).

Glutathione peroxidase (GPx) activity was determined by spectrophotometry, according to Flohé and Günzler (1984), adapted to 96-well microplate. In this assay, the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) was monitored at 340 nm during 5 min, when oxidized glutathione (GSSG) is converted to GSH by glutathione reductase (GR). The

assay mixture consisted of 90 µL phosphate buffer (10 mM; pH7.0), 30 µL PMS (previously diluted 1:2 with phosphate buffer), 30 µL GR (2.4 U/mL), 30 µL GSH (10 mM), 30 µL sodium azide (10 mM), 30 µL EDTA (10 mM), 30 µL NADPH (1.5 mM) and 30 µL hydrogen peroxide (2.5 mM) in a total volume of 300 µL. Enzymatic activity was expressed as nanomoles of NADPH oxidized/min/mg of protein (ε= 6.22 $mM^{-1}cm^{-1}$).

GR activity was determined by spectrophotometry, according to the procedure of Cribb et al. (1989), adapted to 96-well microplate. Briefly, the reaction medium consisted in 0.05 M phosphate buffer (pH7.0), diethylene triamine pentaacetic acid (DTPA) 0.549 mM, NADPH 0.206 mM and GSSG 1.068 mM. In microtiter plate, 250 µL of reaction medium was added to 50 µL of PMS (previously diluted 1:3). GR-mediated oxidation of NADPH was monitored at 340 nm and enzymatic activity was expressed as micromoles of NADPH oxidized/min/mg of protein (ε= 6.22 $mM^{-1}cm^{-1}$).

Glutathione-S-transferase (GST) activity was determined according to Habig and coworkers (1974), after adjustments to 96-well microplate. The assay was carried out using 100 µL of PMS (previously diluted 1:65) and 175 µL of GSH (1.765 mM; prepared in phosphate buffer 0.2 M, pH 7.9). The reaction was initiated by addition of 30 µL of 1-chloro-2,4-dinitrobenzene (CDNB; 10 mM), and the increase in absorbance was recorded spectrophotometrically at 340 nm, during 5 min. GST activity was expressed as nanomoles of thioether produced/min/mg of protein (ε=9.6 $mM^{-1}cm^{-1}$).

Superoxide dismutase (SOD) activity was measured using a commercial kit (RX Monza - RANSOD - SD 125) according to the manufacturer's instructions, and adapted to microplate. The assay was carried out in a 96-well microtiter plate with 10 µL of standard or PMS (adequately diluted) and 210 µL of mixed substrate. The reaction was initiated by the addition of 30 µL of xanthine oxidase (80 U/L), and the absorbance was recorded during 3 min. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-

phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is defined by a 50% inhibition of the rate of reduction of INT under the conditions of the assay. Results were expressed as SOD units/mg protein.

Total glutathione (GSHt) content was measured following the method of Baker et al. (1990), adapted to 96-well microplate by Vandeputte and coworkers (1994). Protein content in the PMS was precipitated with trichloroacetic acid (TCA; 12%) for 1 h and then centrifuged at 12,000 g for 5 min at 4 ºC. GSHt was determined (in deproteinated PMS) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with DTNB (5,5´-dithiobis-2-nitrobenzoic acid, Ellman's reagent) producing a yellow colored 5-thio-2-nitrobenzoicacid (TNB). Reaction mixture containing 1 mM DTNB, 0.34 mM NADPH dissolved in a stock sodium phosphate buffer (143 mM with 6.3 mM EDTA, pH 7.4) was added to wells containing 40 µL of deproteinated PMS (previously diluted 1:3) and the reaction was started by adding 40 µL of 8.5 U/mL GR. Formation of TNB was monitored by spectrophotometry at 415 nm, for 7 min. The results were expressed as nmol TNB conjugated/min/mg of protein (ε=14.1mM^{-1} cm^{-1}).

The determination of lipid peroxidation (LPO) was performed in the tissue homogenate, according to the procedure of Ohkawa et al. (1979) and Bird and Draper (1984), as adapted by Filho and coworkers (2001a, 2001b). To 50 µL of homogenate, 5 µL of 1:1 butylated hydroxytoluene (4% in methanol) and 45 µL of ice-cold phosphate buffer (0.1 mM and pH 7.4) were added and well mixed. To this aliquot, 1000 µL of 12% TCA in aqueous solution, 900 µL Tris-HCl (60 mM, pH 7.4, and 0.1 mM DTPA) and 1000 µL 0.73% thiobarbituric acid (TBA) were added and well mixed. The mixture was heated for 1 h in a water bath set at boiling temperature and then cooled to room temperature, decanted into 2-mL microtubes and centrifuged at 15,800 g for 5 min. Absorbance was measured at 535 nm, and LPO was expressed as nanomoles of TBARS formed per milligram of protein (ε=1.56×10^{5} M^{-1} cm^{-1}).

Protein concentrations were determined (at 550 nm) according to the Biuret method (Gornal et al., 1949), adapted to microplate, using bovine serum albumin as standard, in order to express enzymatic activities, GSHt and TBARS levels as a function of the protein content.

All the spectrophotometric determinations were carried out in triplicate (at 25 ºC) using a SpectraMax 190 microplate reader.

4.2.5. Data analysis

Statistical software (Statistica 8.0) was used for statistical analyses of Hg levels and oxidative stress endpoints. These data were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) to meet statistical demands. In turn, metabolomic data were mean-centred before Principal Components Analysis (PCA) using the Unscrambler X package (version 10.0.1; Camo Software AS, Oslo, NO) and the singular value decomposition (SVD) algorithm was applied to perform a PCA with cross validation. PCA, an unsupervised pattern recognition technique, allowed the differences and similarities between NMR metabolic fingerprints to be visualized in a score plot, where samples that are metabolically similar cluster together. The metabolites responsible for the clustering within the pattern recognition models were identified by examining the corresponding loading plot. Metabolite changes were calculated via the ratio between the averages of the LAR and SJ peak areas. Both for Hg levels and oxidative stress data with normal distribution (using Statistica 8.0) as metabolomic data (using Graph Pad software, Instat, La Jolla, CA, US) the Student's t-test was applied for comparison between group means. Non-parametric test (Mann-Whitney test) for Hg levels and oxidative stress data was applied when data distribution significantly deviated from normality. The probability level of 0.05 was used as the criterion of significance.

4.3. Results

4.3.1. Mercury levels in the liver

The liver of fish from LAR showed significantly higher accumulation of tHg, MeHg and iHg than those from SJ. No statistical differences were found between sites for the percentage of MeHg with respect to tHg (Fig. 4.1).

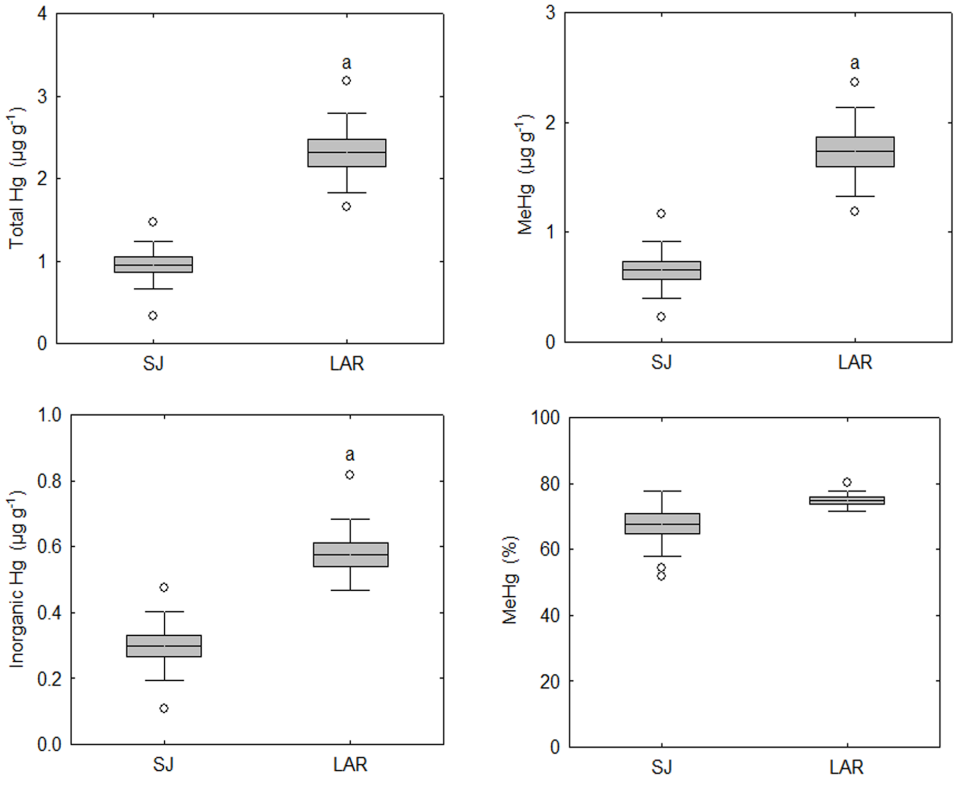


Figure 4.1. Total Hg (tHg), MeHg, inorganic Hg (µg g^{-1}, dry weight) and % of MeHg (in relation with tHg) in the liver of *L. aurata* captured in Laranjo (LAR) and São Jacinto (SJ) at Aveiro lagoon. Mean, standard deviation, standard error, outliers (o) are presented. Significant differences are indicated by 'a' *vs.* SJ.

4.3.2. Metabolomics analysis

4.3.2.1. ^{1}H NMR spectroscopy of liver tissue extracts

A representative 1-D ^{1}H NMR spectrum of liver extract from golden grey mullet sampled from SJ is shown in Figure 4.2. Although several metabolites were identified, all spectra were found to be dominated by two organic osmolytes, taurine and glycerophosphocholine, which were *ca.* 45 and 15 times higher in intensity than other metabolites, respectively. Other major classes of compounds included amino acids (e.g. alanine, glutamate, glycine), carbohydrates (e.g. glucose), glycolytic products (e.g. lactate), tricarboxylic acid cycle intermediates (e.g. succinate), and nucleotides (e.g. uracil).

4.3.2.2. Pattern recognition analysis of ^{1}H NMR spectra

The PCA scores plot of the ^{1}H NMR metabolic fingerprints of *L. aurata* liver (Fig. 4.3) shows a clear separation between the two fish groups collected from SJ and LAR along the PC2 axis (explaining 24.62 percent of variance). The corresponding PC2 loading plot, depicted in Figure 4.4, was used to determine the metabolites responsible for the observed separation and the direction of their changes. Specifically, peaks with positive loadings correspond to metabolites that have higher concentrations in specimens from LAR than in those from SJ, whereas negative loadings correspond to metabolites whose concentration is decreased in LAR group relative to SJ. From the PC2 loading plot, the metabolic profiles of liver extracts from LAR individuals were characterized by significantly elevated levels of alanine, phosphocholine, glucose, and glutathione, together with the significantly decreased concentration of tyrosine, phenylalanine, taurine, and hypoxanthine, as reported in Table 4.1.

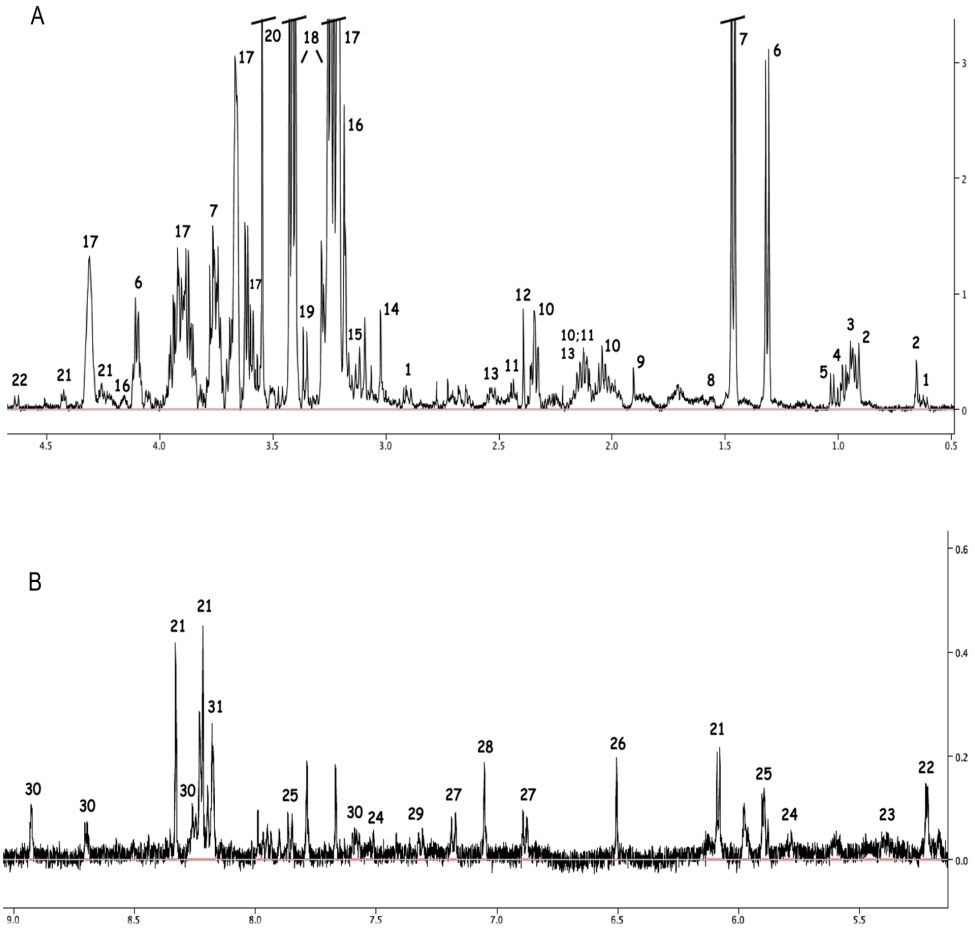


Figure 4.2. A representative 1-D 500 MHz ^{1}H NMR spectrum of liver tissue extracts from *L. aurata* collected from SJ, with (A) representing the aliphatic region and (B) a vertical expansion of the aromatic region. Keys: (1) DSS, (2) taurocholic acid, (3) leucine, (4) isoleucine, (5) valine, (6) lactate, (7) alanine, (8) unknown #1, (9) acetate, (10) glutamate, (11) glutamine, (12) succinate, (13) glutathione, (14) creatine, (15) malonate, (16) phosphocholine, (17) glycerophosphocholine, (18) taurine, (19) unknown #2, (20) glycine, (21) inosine, (22) glucose, (23) glycogen, (24) uracil, (25) uridine, (26) fumarate, (27) tyrosine, (28) unknown #3, (29) phenylalanine, (30) niacinamide, and (31) hypoxanthine.

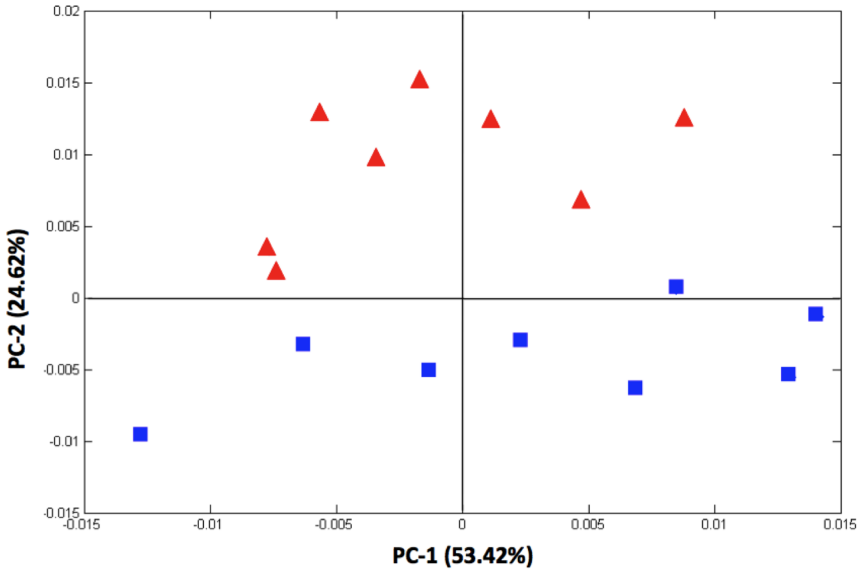


Figure 4.3. Principal components analysis (PCA) of ^{1}H NMR spectra of liver extracts showing separation (PC1 vs. PC2) between *L. aurata* collected from SJ (blue square) and those from LAR (red triangle).

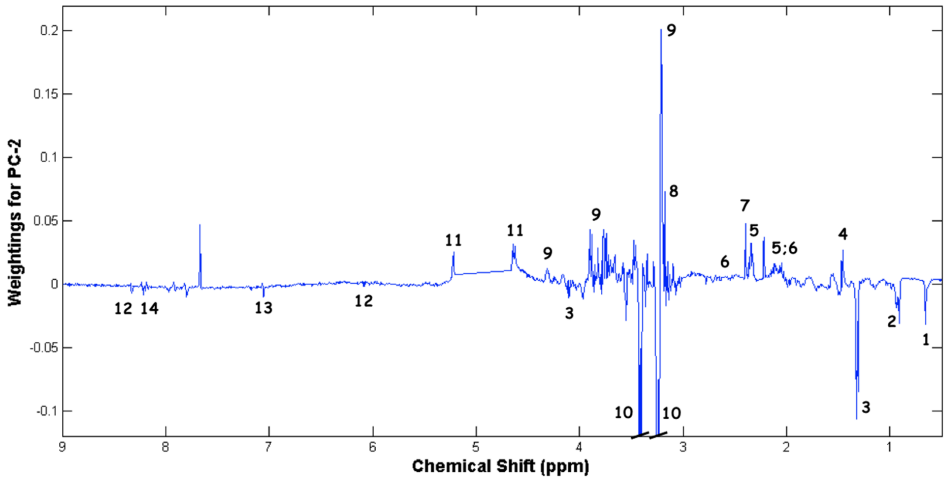


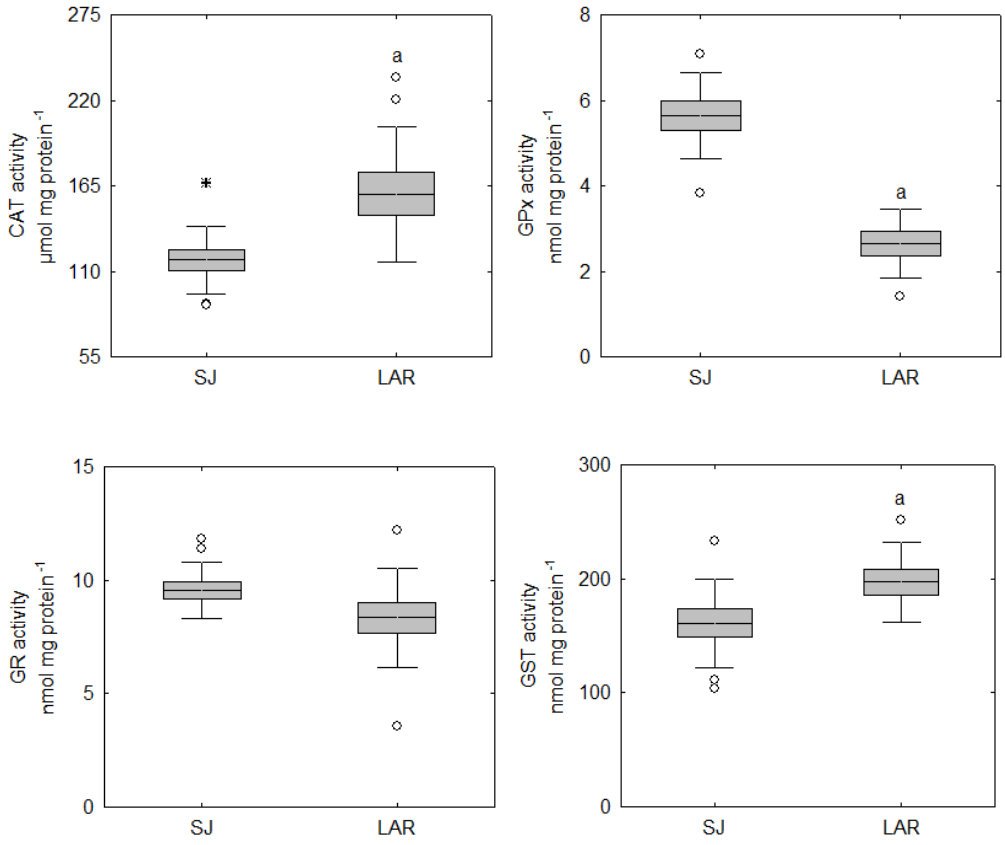
Figure 4.4. Corresponding PC2 loading plot showing the metabolic differences between individuals collected from SJ and LAR. Keys: (1) taurocholic acid, (2) branched chain amino acids: isoleucine, leucine, valine, (3) lactate, (4) alanine, (5) glutamate, (6) glutathione, (7) succinate, (8) phosphocholine, (9) glycerophosphocholine, (10) taurine, (11) glucose, (12) inosine, (13) tyrosine, and (14) hypoxanthine.

Metabolites	Chemical shift and peak shape, ppm	
Amino acids		
Isoleucine	0.92 (t), 1.00 (d), 1.26 (m), 1.44 (m), 1.96 (m), 3.66 (d)	15% ↓
Leucine	0.94 (d), 0.96 (d), 1.66 (m), 3.71 (t)	21% ↓
Valine	0.98 (d), 1.03 (d), 2.25 (m), 3.59 (d)	17% ↓
Alanine	1.46 (d), 3.76 (m)	37% ↑[a]
Glutamate	2.08 (m), 2.34 (m), 3.74 (t)	24% ↑
Tyrosine	6.89 (d), 7.19 (d)	28% ↓[b]
Phenylalanine	3.13 (m), 3.28 (m), 3.98 (m), 7.31 (d), 7.36 (t), 7.41 (m)	25% ↓[b]
Energy metabolites		
Lactate	1.33 (d), 4.12 (q)	44% ↓
Succinate	2.41 (s)	12% ↑
Glucose	3.23 (m), 3.40 (m), 3.45 (m), 3.52 (dd), 3.73 (m), 3.82 (m), 3.88 (dd), 4.63 (d), 5.22 (d)	52% ↑[c]
Glycogen	3.40 (m), 3.60 (m), 3.80 (m), 3.96 (s), 5.40 (s)	no change
Osmolytes		
Taurine	3.25 (s), 3.41 (t)	38% ↓[b]
Glycerophosphocholine	3.21 (s), 3.60 (dd), 3.67 (m), 3.90 (m), 4.31 (m)	23% ↑
Nucleosides and derivatives		
Inosine	3.83 (dd), 3.90 (dd), 4.27 (dd), 4.25 (t), 4.76 (t), 6.08 (d), 8.21 (s), 8.33 (s)	16% ↓
Uracil	5.81 (d), 7.54 (d)	32% ↓
Hypoxanthine	8.17 (s), 8.20 (s)	45% ↓[c]
Bile acids		
Taurocholic acid	0.65 (s), 0.91 (s), 0.98 (m), 1.36 (m), 1.42 (m), 1.61 (m), 1.96 (m), 2.13 (d), 2.20 (m), 2.53 (t), 3.18 (m), 3.28 (dd), 3.61 (s), 3.78 (s)	24% ↓
Miscellaneous metabolites		
Glutathione	2.13 (m), 2.54 (m), 2.97 (dd), 3.75 (m), 4.53 (m)\	56% ↑[b]
Phosphocholine	3.21 (s), 3.57 (t), 4.16 (m)	93% ↑[a]

Table 4.1. Relative changes in metabolite concentrations between LAR and SJ golden grey mullets ($p < 0.05^{a}$; $p < 0.01^{b}$; $p < 0.005^{c}$; Student's t test). (s: singlet; d: doublet; t: triplet; dd: doublet of doublets; q: quartet; m: multiplet)

4.3.3. Oxidative stress endpoints

A significant increase of mean value of CAT (1.4-fold) and GST (1.2-fold) activities in liver, as well as GSHt content (2.0-fold) was recorded at fish from LAR (Fig. 4.5). Contrarily, a significant decrease of hepatic GPx activity (2.1-fold) was found at LAR relatively to SJ. Moreover, no significant differences were found between sites for GR and SOD activities, as well as for LPO.



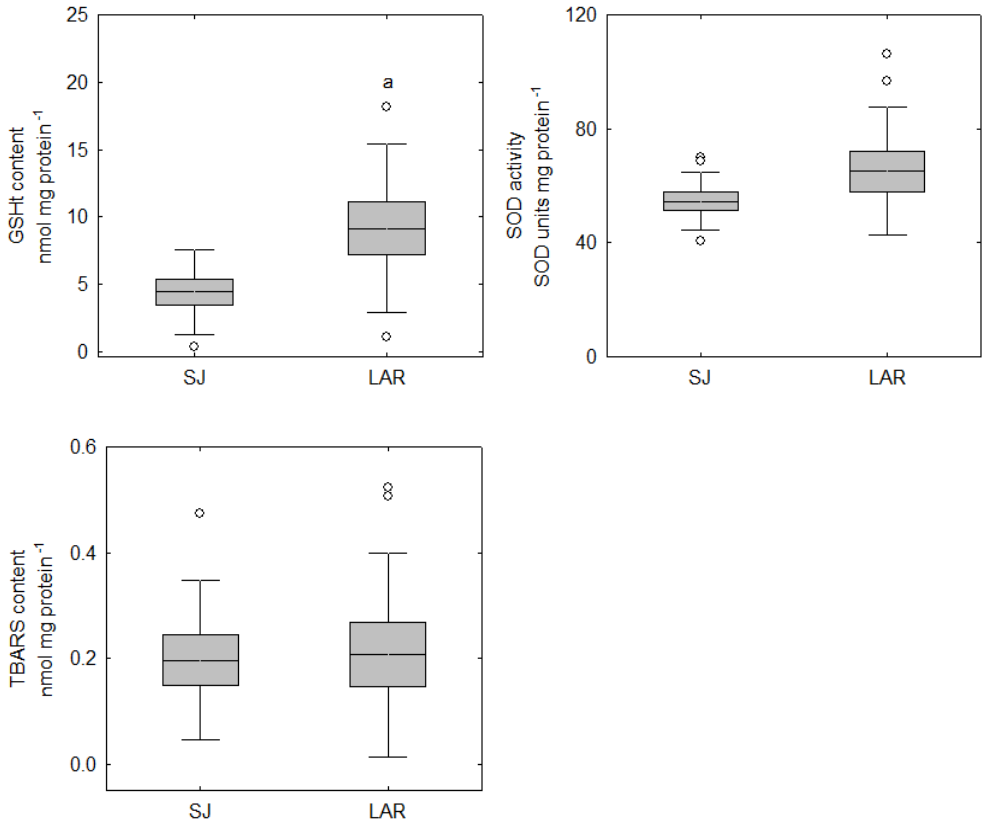


Figure 4.5. Oxidative stress responses (CAT, GPx, GR, GST and SOD activities and GSHt and TBARS content) in the liver of *L. aurata* captured in Laranjo (LAR) and São Jacinto (SJ) at Aveiro lagoon. Mean, standard deviation, standard error, outliers (o) and extreme values (※) are presented. Significant differences are indicated by 'a' *vs.* SJ.

4.4. Discussion

4.4.1. Discussion on metabolic responses to mercury

The liver of fish from LAR (contaminated area) accumulated higher levels of MeHg and iHg (and consequently tHg) than those from SJ, clearly evidencing an Hg exposure. These results are in line with previous findings of Mieiro et al. (2011, 2009), who demonstrated that *L. aurata* liver was able to

reflect environmental variations of Hg in a field study performed at Aveiro lagoon. Nevertheless, the current study reports for the first time MeHg accumulation in liver of fish from this aquatic system. Data herein reported demonstrated that MeHg, the most toxic Hg form, is substantially higher in LAR than in SJ, and it corresponds to the most accumulated Hg form in liver, both in SJ and LAR. These results are consistent with Mieiro and coworkers (2014), who observed percentages of organic Hg varying between 54.1 and 83.3% in liver of *Dicentrarchus labrax* collected from the same area.

The NMR metabolic data provided an integrated description of the Hg-induced hepatotoxic metabolic responses in the golden grey mullets, allowing the identification of a considerable number of metabolites from several different classes, including amino acids, organic acids and bases, carbohydrates, and nucleotides. PCA analysis indicated that individuals from SJ clustered separately from those collected at LAR, suggesting a clear differentiation in their metabolic profile.

The largest change occurred in the concentrations of phosphocholine, which was significantly increased by 93% in fish from LAR in respect to those from SJ. The synthesis of phosphocholine is catalysed by a choline kinase that phosphorylates choline as the first step in the Kennedy pathway towards synthesis of phosphatidylcholine, the major cell membrane phospholipid. Therefore, the observed increase in phosphocholine may be related to a breakdown of phosphatidylcholine and/or membrane turnover, as suggested by Karakach et al. (2009) after investigation of the metabolic response of juvenile Atlantic salmon to long-term handling stress. Noteworthy, phosphocholine has been suggested as a biomarker of cancer, as its accumulation was shown in human tumor tissues and cells in comparison with normal human epithelial cells (Eliyahu et al., 2007), although phosphocholine functional role in this context remains unknown. Phosphocholine is one of the two major forms of choline storage along with glycerophosphocholine, which was found at higher levels (although not statistically significant) in fish from LAR relatively to SJ.

Glycerophosphocholine mainly serves as an organic osmolyte, and thus its accumulation may indicate an attempt to compensate a hypothetical perturbation in osmoregulation, occurring due to the difference in salinity observed between the two sites (salinity is lower in LAR). However, it can be also attributed to Hg accumulation, as demonstrated for rats (Wang et al., 2006).

Further evidences of interference with the osmoregulatory processes are also provided by the significant change in taurine, also associated with the maintenance of homeostasis (El-Sayed, 2014), which decreased in liver of mullets capture at LAR. Previous *in vitro* studies with fish cells found a taurine efflux in response to hyposmotic stress (Avella et al., 2009; Guizouarn et al., 2000; Michel et al., 1994). In turn, Liu et al. (2011) observed a decrease of taurine in gills from Manila clam *Ruditapes philippinarum* exposed to Hg. This way, the significant decrease of taurine observed in *L. aurata* livers could be associated with an osmotic adjustment related both with the difference of salinity between the two sites and an Hg-induced osmotic imbalance. Here, it should be noted that the taurine efflux above described in response to hypotonicity occurred in cells directly challenged by the external medium, which cannot be cogitated for the liver (an internal organ) in the present study. Hence, the possibility that the taurine decrease observed in LAR fish is determined by the lower water salinity loses plausibility. The main functions of taurine include membrane stabilization and bile acid conjugation (Kokushi et al., 2012), and thus, its depletion may be responsible for a dramatic change in membrane structure and function, as well as for the decrease currently observed (not significant) in taurocholic acid level, the product of conjugation of cholic acid with taurine. Furthermore, taurine is also known to have a potent antioxidant capacity against various reactive radicals (radical scavenger properties) (El-Sayed, 2014), and thus, the depletion of taurine can be also indicative of a vulnerability in the protection of liver against oxidative stress. Studies with rats demonstrated that taurine supplementation reduces oxidative

stress caused by Hg, and thus it could protect the liver (Jagadeesan and Pillai, 2007).

Important metabolic changes associated with energetic pathways were also observed in fish of LAR. Generally, the fish response to physical and chemical stressors involves an elevation of lactate, specifically the exposure to hypoxic conditions (Omlin and Weber, 2010; Richards, 2009) or contaminants (Fasulo et al., 2012a). However, in this work a depletion (although not statistically significant) of lactate in the liver of mullets sampled at LAR was observed. The lactate-lowering tendency may be indicative of a decrease in lactate production or an increase in lactate removal. Since the former explanation seems improbable, it gains plausibility the utilization of lactate as substrate for gluconeogenesis, which has been previously demonstrated in fish liver (Pereira et al., 1995). This explanation fits with the observed increase in hepatic glucose concomitantly with unaltered glycogen levels. Previous studies referred that gluconeogenesis is stimulated during hypoxia (Omlin and Weber, 2010), and following exposure to Hg (Ung et al., 2010). Thus, the significant increase of glucose is likely a combined effect of higher Hg accumulation and lower dissolved oxygen observed in LAR relatively to SJ, via a Cori cycle increment.

Amino acids can also be oxidized in the liver and used as glucose precursors and indirect energy sources (Pereira et al., 1995). Indeed, the significant decrease in tyrosine and phenylalanine detected in fish from LAR with respect to those from SJ (together with non-significant decreases in leucine, valine and isoleucine) reinforces that assertion. It is well known that amino acid catabolism releases ammonia, which may induce serious metabolic problems due to its toxicity. Taking into account that alanine is the main carrier of nitrogen to the liver, its increase may result in an attempt to minimize the amount of free ammonia, and thus intoxication.

Overall, the alterations observed in the pool of amino acids may also indicate changes in expression of protein synthetic genes, as an attempt to induce the activation of cytoprotective mechanisms in response to Hg

hepatotoxicity. In support of this hypothesis, a non-significant depletion in uracil level was found in the liver of fish from LAR, suggesting an enhancement of ongoing transcriptional activities. Furthermore, the significant decrease in hypoxanthine in combination with the reduction in inosine, which are a purine derivative and nucleoside, respectively, formed during ATP degradation, may result in an increase in ATP, in order to cope with the metabolically expensive processes occurring in fish exposed to Hg.

Interestingly, in the liver of fish from LAR a significant increase of 56% in glutathione level was observed, as well as elevation in glutamate. It is well known that glutamate is a constituent of some oligopeptides such as glutathione, which plays a central role in protective processes against oxidative insult (Storey, 1996). Evidence of Hg-induced hepatotoxicity triggered by oxidative stress were documented in zebrafish by Ung and coworkers (2010), using transcriptome analysis, phenotypic anchoring and validation of targeted gene expression. Therefore, it may be supposed the activation of antioxidant defence mechanisms in liver of golden grey mullets naturally exposed to Hg.

4.4.2. Discussion on oxidative stress responses to mercury

The significant increase of glutathione observed in the metabolomic profile is in agreement with the significant increase in the GSHt content found in the antioxidant parameters, demonstrating that this increase is associated with an elevation of GSH levels in liver. Mercury is highly reactive with protein sulphydryl groups, forming covalent bonds with GSH and protein cysteine residues. GSH is the primary antioxidant and conjugating agent, being the first line of defence against Hg (Farina et al., 2013). Data herein reported demonstrated an increase of GSH content in fish liver from LAR, corresponding to an adaptive response to Hg accumulation, in line with several

studies (Guilherme et al., 2008; Mieiro et al., 2014; Monteiro et al., 2010). As a protective response, the organisms may increase GSH levels through biosynthesis or incrementing its regeneration by glutathione reductase (GR), which was not confirmed by the current GR data. Consequently, the increase of GSH amount must be associated to *de novo* synthesis. In accordance, results from studies in rats indicated that short- and long-term exposure to MeHg in drinking water resulted in a two- to three-fold up-regulation of mRNA encoding for γ-glutamylcysteine synthetase (GCS) (Woods and Ellis, 1995). Indeed, liver is the most important site of GSH synthesis, which is subsequently exported to other tissues such as the kidney, brain and muscle (Monteiro et al., 2010).

Besides the high affinity of Hg to thiol groups (Farina et al., 2013; Sweet and Zelikoff, 2001), selenohydryl groups have also been reported as critical and primary targets in mediating Hg-induced toxicity (Branco et al., 2012; Franco et al., 2009; García-Sevillano et al., 2014). This way, selenoproteins such as GPx, the most abundant selenoenzyme (playing an important role in preventing the production of ROS by reducing hydrogen peroxide and free fatty acid hydroperoxides), correspond to MeHg preferred molecular target (Farina et al., 2013). In accordance, a GPx activity depletion was detected in *L. aurata* liver from LAR. Such effect was in line with the higher accumulation of Hg, mainly as MeHg, in this fish group. This enzymatic impairment will probably lead to the enhancement of cellular levels of hydrogen peroxide and lipid hydroperoxides, mainly due to Hg, a well-known pro-oxidant that exerts oxidative stress via hydrogen peroxide production (Stohs and Bagchi, 1995). Data reported in this thesis about livers of *L. aurata* from LAR are in agreement with Larose et al. (2008), who also observed an inhibitory effect of MeHg in hepatic GPx activity of *Perca flavesnescens*.

It is well known that the action of an antioxidant enzyme can be replaced by the activity of other antioxidants (Bagnyukova et al., 2005). Thus, the GPx inhibition could be compensated by an increment of GST and CAT activities.

GSTs are a major group of xenobiotic detoxifying enzymes that catalyse the transformation of a wide variety of electrophilic compounds to less toxic substances by conjugating them with GSH (Van der Oost et al., 2003). In addition, some GST isozymes display peroxidase activity with respect to lipid hydroperoxides. Thus, the increment of GST activity perceived in liver of fish captured at LAR illustrates a protective and adaptive response to Hg accumulation in hepatic cells, and may be associated with the increase of lipid hydroperoxides due to the depletion of GPx. Previous studies also showed that Hg exposure induces GST activity (Larose et al., 2008; Monteiro et al., 2010). In turn, the increment of CAT in liver of *L. aurata* from LAR could be also associated to a replacement of GPx activity since both enzymes catalyse protective reactions against hydrogen peroxide. Although no differences were observed in SOD activity (responsible for the formation of hydrogen peroxide), the inhibition of GPx activity may lead to enhancement of cellular levels of hydrogen peroxide. This way, higher CAT activity reflects more ability to breakdown hydrogen peroxide, the major cellular precursor of the hydroxyl radical, the most toxic ROS. In agreement, CAT increments with Hg exposure have also been reported in previous fish studies (Berntssen et al., 2003; Huang et al., 2010; Mieiro et al., 2011).

The alterations reported on oxidative stress biomarkers may also be associated with non-contaminant related abiotic factors such as lower salinity and dissolved oxygen verified at LAR, since previous studies verified that these two variables influence both the toxicity of metals and oxidative stress responses (Rodrigues et al., 2014, 2012). The ability of liver to induce CAT and GST activities, as well as to raise GSHt content, in response to Hg exposure/accumulation or abiotic variations evidenced an elevated antioxidant protection, characteristic of this organ.

Lipid peroxidation is a chemical mechanism capable of disrupting the structure and function of the biological membranes occurring as a result of free radical attack on lipids. Since the prevention of LPO greatly depends of the

action of antioxidants, the *L. aurata* liver demonstrated some degree of vulnerability due to the depletion of GPx activity and taurine, which are essential antioxidants in the protection of the membrane. However, no LPO increase was found, demonstrating a hepatic protection efficiency against Hg-induced oxidative stress. These results are in accordance with previous studies on *L. aurata* liver collected in the same area (Guilherme et al., 2008; Mieiro et al., 2011). Moreover, as mentioned earlier, the observed increase in phosphocholine may be related to a breakdown of phosphatidylcholine and/or membrane turnover. The membrane turnover is a process involved in membrane repair following oxidative stress that is influenced both by pro-oxidants as antioxidants. Thus, the depletion of GPx activity and taurine, that are essential antioxidants in the protection of the membrane against lipid peroxidation, together with the increase of phosphatidylcholine degradation products (phosphocholine and glycerophosphocholine) observed by metabolomcs, suggest that the membrane possibly suffered from an oxidative insult from other nature, but substantial lipid peroxidation was prevented. Thus, liver cells were able to recover from this insult through activation of membrane repair processes and elevated antioxidant protection.

4.5. Conclusions

Data reported in this study revealed that environmental exposure to Hg induces marked changes both in metabolic profile and antioxidant responses in the liver of *L. aurata*, pointing out a compromised health status of mullets.

The metabolomics analysis displayed a clear differentiation between the two sites (SJ and LAR), revealing that accumulation of Hg has multiple levels of impact, interfering with membrane stabilization/degradation/repair processes,

osmoregulation, energy metabolism, gene expression and antioxidant protection.

The research on oxidative stress biomarkers revealed that Hg triggers adaptive responses of antioxidant system depicted in the increase of GST and CAT activities as well as GSHt content, compensating GPx activity depletion. The GPx inhibition revealed some vulnerability and provides evidences of the role of selenohydryl groups as a primary target of Hg.

The combined approach of metabolomics and oxidative stress endpoints allowed a better understanding of Hg toxicity, namely the identification of GSH as a first line of defence against Hg and evidences of oxidative insults in cell membranes recognized through the increase of phosphatidylcholine degradation products, namely phosphocholine and glycerophosphocholine. Nonetheless, the induction of lipid peroxidation was efficiently prevented.

Overall, the combined use of the novel NMR-based environmental metabolomics and oxidative stress biomarkers, coupled with the Hg tissue burdens characterization, demonstrated to be a sensitive and effective approach for a mechanistically based assessment of Hg hepatotoxicity.

CHAPTER V

Metabolic and oxidative stress responses in gills of wild mullet *Liza aurata*

5. Metabolic and oxidative stress responses in gills of wild mullet *Liza aurata*

5.1. General introduction

Fish gills serve a multitude of physiological functions. Besides the respiratory gas exchange activity, the gills are a crucial organ for osmoregulation (Fugelli and Thoroed, 1990), active ion transport (Brunelli et al., 2010), acid-base balance, and nitrogenous wastes excretion (Evans, 1987). Fish gills are the main target organs for toxicants, being the main route for uptake of environmental pollutants. This is related to their anatomic location, being constantly immersed in the surrounding water, wide surface, and thin epithelium that facilitates the interactions with toxicants (Evans, 1987).

Furthermore, high cell regeneration occurs in fish gills to counteract exfoliation of the branchial epithelium, which may reflect a recent exposure to contaminants (De Domenico et al., 2013; Pereira et al., 2010). Owing to their morpho-physiological significance, gills are target organs that suffer instantaneously from ambient toxicants, and thus they are suitable to study the alterations due to variations in water chemical composition (Bernet et al., 1999; Brunelli et al. 2011; Gagnon and Holdway, 1999; Prasad, 1988). Pollutants not only enter into the organism through the gills, but also exert their primary toxic effects on the branchial epithelium, which in turn may influence the general gill functions (Fernandes et al., 2007). Hence, to date fish gills have been successfully employed in environmental biomonitoring studies (Brunelli et al., 2011; De Domenico et al., 2013, 2011; Fasulo et al., 2012b; Guilherme et al., 2008; Liu et al., 2015; Maisano et al., 2016a, 2016c; Pereira et al., 2010).

Therefore, the aim of this study was to assess the metabolic and oxidative stress responses to mercury elicited in gills of wild golden grey mullet *Liza aurata* inhabitating an Hg contaminated system, in order to elucidate the toxicity mechanisms of mercury in fish, and potentially in humans.

5.2. Materials and Methods

This study was conducted in accordance with the EU Directive 2010/63/EU of 22nd September on the protection of animals used for scientific purposes, under the supervision of a team member (Mário Pacheco) authorized by the competent authorities.

5.2.1. Chemicals

Deuterated water (D_2O) was acquired from Armar AG (Switzerland), and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and the other chemicals for metabolomics analysis were purchased from Sigma-Aldrich (Italy). Bovine serum albumin was purchased from E. Merck-Darmstadt (Germany) and all the other chemicals used for oxidative stress conventional parameters were obtained from the Sigma–Aldrich Chemical Company (Spain). Other routine chemicals (analytical grade) were purchased from local suppliers.

5.2.2. Mercury in fish gills

Gills samples (n=10) were firstly freeze-dried (Labconco), grounded and homogenized. Samples were then analysed for tHg as previously described for sediment. A modified methodology was used to determine MeHg (Armstrong et al., 1999; Westöö, 1967). Briefly, approximately 2 mL of Milli-Q water and 3 mL of KOH (6 M) solution were added to 200 mg of dried sample. The mixture was shaken for 2 hours and then 3 mL of HCl (6 M) and 4 mL of a $KBr/CuSO_4$ (3:1) solution was added. After 10 min of shaking, 5 mL of DCM was then added, the mixture centrifuged and finally the organic phase separated. A slight sulphide solution (≈ 0.06 mM) was used to extract MeHg from the organic phase and then MeHg was back extracted to DCM.

Methylmercury in DCM was quantified by GC-AFS using the chromatographic equipment described above. Again, the possible MeHg artifact formation were also evaluated by spiking several samples with Hg(II) and MeHg standard solutions of different concentrations. Recoveries varied between 92 and 103% and no artefact MeHg formation was observed. For all the analysis, precision expressed as the relative standard deviation of 3 replicate samples, was less than 2% ($p<0.05$). Certified reference material (DORM-4) was used to ensure the accuracy of the procedures (Table 3.1).

A crude estimation of the inorganic mercury (iHg) content in the gills was done by subtracting tHg levels by the corresponding MeHg concentrations. For this estimation it was assumed that MeHg is the only organic Hg compound that is bioaccumulated in fish (Zhang and Adeloju, 2012).

5.2.3. Metabolomics analysis

5.2.3.1. Tissue metabolite extraction

Extraction of polar metabolites from fish gill tissues (n=8 per sampling site) was performed according to a "two-step" methanol/chloroform/water protocol, as reported in detail in previous works (Cappello et al., 2013b, 2015; Maisano et al., 2016a; Wu et al., 2008). Briefly, 150 mg sub-sample of gill tissues were homogenized in 4 mL/g of cold methanol and 0.85 mL/g of cold by a TissueLyser LT bead mill (Qiagen) with 3.2 mm stainless steel beads, for 10 min at 50 vibrations/s. The homogenates were transferred into glass vials, and 4 mL/g chloroform and 2 mL/g water were added. Samples were vortexed, left on ice for 10 min for phase separation, and centrifuged for 5 min at 2000 g at 4 °C. A 600 µL volume of the upper methanol layer containing the polar metabolites were transferred into glass vials, dried in a centrifugal vacuum concentrator (Eppendorf 5301), and stored at -80 °C. Prior to NMR analysis,

the dried polar extracts were resolvated in 600 μL of a 0.1 M sodium phosphate buffer (pH 7.0, 10% D_2O) containing 1 mM DSS as internal reference. The mixture was vortexed and transferred to a 5 mm diameter NMR tube. The DSS acted as an internal standard and provided a chemical shift reference (δ=0.0 ppm) for the NMR spectra, whereas the D_2O provided a deuterium lock for the NMR spectrometer.

5.2.3.2. ^{1}H NMR metabolomics and spectral pre-processing

Extracts of gill tissues were analyzed by a Varian-500 NMR spectrometer operating at a spectral frequency of 499.74 MHz at 298 K. One-dimensional (1-D) ^{1}H NMR spectra were obtained using a PRESAT pulse sequence to suppress the residual water resonance and 6,983 Hz spectral width with a 2.0 s relaxation delay. A total of 128 transients were collected into 16,384 data points requiring a 10 min acquisition time. All data sets were zero filled to 32,768 data points and exponential line-broadenings of 0.5 Hz were applied before Fourier transformation. All ^{1}H NMR spectra were phased, baseline-corrected, and calibrated (DSS at 0.0 ppm) under manual correction using Chenomx Processor, a module of Chenomx NMR Suite (version 5.1; Chenomx Inc., Edmonton, Canada) software. Peaks within the ^{1}H NMR spectra were assigned with reference to known chemical shifts and peak multiplicities, using public databases such as the Human Metabolome DataBase (HMDB; Wishart et al., 2009), and the Chenomx 500-MHz library database. The latter was also used for metabolite quantification as it uses the concentration of a known DSS signal to determine the concentrations of individual metabolites (Cappello et al., 2015, 2016a; Maisano et al., 2016a, 2016b).

All 1-D ^{1}H NMR spectra were converted to a data matrix using Chenomx Profiler, another module included in Chenomx NMR Suite software. Each

spectrum was segmented into 0.005 ppm chemical shift bins within a range of 0.7 and 9.5 ppm, from which the residual water resonances (4.65 - 5.15 ppm) were cut from all the NMR spectra to prevent interference in subsequent multivariable analyses. To conduct comparison between spectra, the integrated spectral area of the remaining bins was normalized to the total integrated area of the spectra. Data were mean-centered before carry out multivariate statistical analysis.

5.2.4. Oxidative stress endpoints

Tissue samples (n=10) were homogenized in a 1:10 ratio (gills weight:buffer volume) of ice-cold phosphate buffer (0.1 mM and pH 7.4) using a Potter–Elvehjem glass–Teflon homogenizer. An aliquot of homogenate (50 µL) was removed to measure thiobarbituric acid reactive substances (TBARS), to which 5 µL of 1:1 butylated hydroxytoluene (4% in methanol) and 45 µL of ice-cold phosphate buffer (0.1 mM and pH 7.4) were added and well mixed. The remaining homogenate was centrifuged at 13,400 g, 4 ºC for 25 min, and post mitochondrial supernatant (PMS) was divided into aliquots to be used for the different determinations. Homogenate and PMS were stored at -80 ºC until analyses.

Catalase (CAT) activity was measured following the decomposition of H_2O_2, according with the Claiborne (1985) protocol as described by Giri et al. (1996), adapted to 96-well microplate. The assay mixture consisted of 5 µL of PMS and 195 µL of hydrogen peroxide (10 mM, prepared in phosphate buffer 0.05 M, pH 7.0) in a final volume of 200 µL. Changes in absorbance were spectrophotometrically monitored at 240 nm during 1 min, and activity was expressed as micromoles of hydrogen peroxide consumed/min/mg of protein (ε= 43.5 $M^{-1}cm^{-1}$).

Glutathione peroxidase (GPx) activity was determined by spectrophotometry, according to Flohé and Günzler (1984), adapted to 96-well microplate. In this assay, the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) was monitored at 340 nm during 5 min, when oxidized glutathione (GSSG) is converted to GSH by glutathione reductase (GR). The assay mixture consisted of 90 µL phosphate buffer (10 mM; pH7.0), 30 µL PMS (previously diluted 1:2 with phosphate buffer), 30 µL GR (2.4 U/mL), 30 µL GSH (10 mM), 30 µL sodium azide (10 mM), 30 µL EDTA (10 mM), 30 µL NADPH (1.5 mM) and 30 µL hydrogen peroxide (2.5 mM) in a total volume of 300 µL. Enzymatic activity was expressed as nanomoles of NADPH oxidized/min/mg of protein (ε= 6.22 $mM^{-1}cm^{-1}$).

GR activity was determined by spectrophotometry, according to the procedure of Cribb et al. (1989) adapted to 96-well microplate. Briefly, the reaction medium consisted in 0.05 M phosphate buffer (pH7.0), diethylene triamine pentaacetic acid (DTPA) 0.549 mM, NADPH 0.206 mM and GSSG 1.068 mM. In microtiter plate, 250 µL of reaction medium was added to 50 µL of PMS (previously diluted 1:3). GR-mediated oxidation of NADPH was monitored at 340 nm and enzymatic activity was expressed as micromoles of NADPH oxidized/min/mg of protein (ε= 6.22 $mM^{-1}cm^{-1}$).

Glutathione-S-transferase (GST) activity was determined according to Habig et al. (1974), after adjustments to 96-well microplate. The assay was carried out using 100 µL of PMS (previously diluted 1:20) and 175 µL of GSH (1.765 mM; prepared in phosphate buffer 0.2 M, pH 7.9). The reaction was initiated by addition of 30 µL of 1-chloro-2,4-dinitrobenzene (CDNB; 10 mM), and the increase in absorbance was recorded spectrophotometrically at 340 nm, during 5 min. GST activity was expressed as nanomoles of thioether produced/min/mg of protein (ε=9.6 $mM^{-1}cm^{-1}$).

Superoxide dismutase (SOD) activity was measured using a commercial kit (RX Monza - RANSOD - SD 125) according to the manufacturer's instructions, and adapted to microplate. The assay was carried out in a 96-well

microtiter plate with 10 µL of standard or PMS (adequately diluted) and 210 µL of mixed substrate. The reaction was initiated by the addition of 30 µL of xanthine oxidase (80 U/L), and the absorbance was recorded during 3 min. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is defined by a 50% inhibition of the rate of reduction of INT under the conditions of the assay. Results were expressed as SOD units/mg protein.

Total glutathione (GSHt) content was measured following the method of Baker et al. (1990) adapted to 96-well microplate by Vandeputte et al. (1994). Protein content in the PMS was precipitated with trichloroacetic acid (TCA; 12%) for 1 h and then centrifuged at 12,000 g for 5 min at 4 ºC. GSHt was determined (in deproteinated PMS) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with DTNB (5,5´-dithiobis-2-nitrobenzoic acid, Ellman's reagent) producing a yellow colored 5-thio-2-nitrobenzoicacid (TNB). Reaction mixture containing 1 mM DTNB, 0.34 mM NADPH dissolved in a stock sodium phosphate buffer (143 mM with 6.3 mM EDTA, pH 7.4) was added to wells containing 40 µL of deproteinated PMS (previously diluted 1:3) and the reaction was started by adding 40 µL of 8.5 U/mL GR. Formation of TNB was monitored by spectrophotometry at 415 nm, for 7 min. The results were expressed as nmol TNB conjugated/min/mg of protein (ε=14.1mM^{-1} cm^{-1}).

The determination of lipid peroxidation (LPO) was performed in the tissue homogenate, according to the procedure of Ohkawa et al. (1979) and Bird and Draper (1984), as adapted by Filho et al. (2001a, 2001b). To the aliquot stored for determination of LPO, 1000 µL of 12% TCA in aqueous solution, 900 µL Tris-HCl (60 mM, pH 7.4, and 0.1 mM DTPA) and 1000 µL 0.73% thiobarbituric acid (TBA) were added and well mixed. The mixture was heated for 1 h in a water bath set at boiling temperature and then cooled to room

temperature, decanted into 2-mL microtubes and centrifuged at 15,800 g for 5 min. Absorbance was measured at 535 nm, and LPO was expressed as nanomoles of TBARS formed per milligram of protein ($\varepsilon=1.56\times10^5\ M^{-1}\ cm^{-1}$). According to the literature, this is a sensitive and widely used assay, although non-specific since TBA reacts with a variety of components in biological samples (Devasagayam et al., 2003; Meagher and FitzGerald, 2000). Nevertheless, previous studies with *L. aurata* from Aveiro lagoon used TBARS assay to evaluate LPO, demonstrating the adequacy of this methodology, as well as the responsiveness of the corresponding endpoint to Hg exposure (Guilherme et al., 2008; Mieiro et al., 2014). The same approach was adopted in the current work in order to allow comparisons between current and previous data.

Protein concentrations were determined (at 550 nm) according to the Biuret method (Gornall et al., 1949), adapted to microplate, using bovine serum albumin as standard, in order to express enzymatic activities, GSHt and TBARS levels as a function of the protein content.

All the spectrophotometric determinations were carried out in triplicate (at 25 ºC) using a SpectraMax 190 microplate reader.

5.2.5. Data analysis

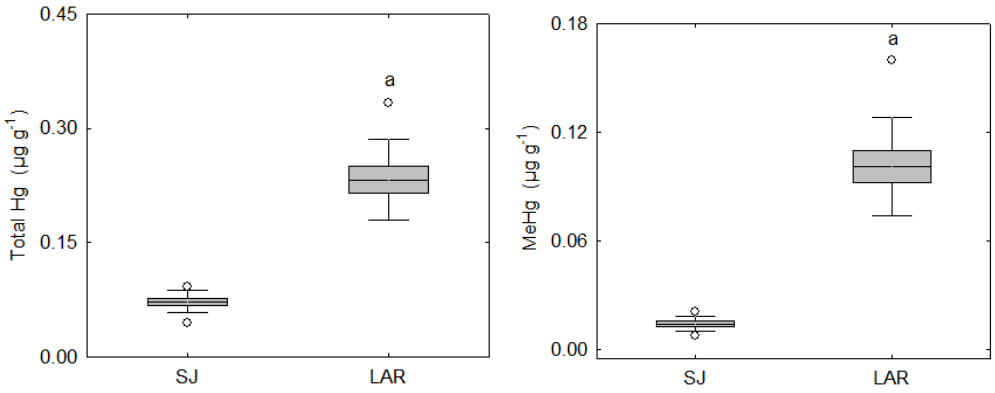
Data of Hg levels and oxidative stress endpoints were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) to meet statistical demands by using the Statistica 8.0 software. In turn, an unsupervised chemometric technique, the Principal Component Analysis (PCA), was carried out by MATLAB to reduce the dimensionality of metabolomic data and distinguish the two groups of fish. Indeed, each sample is visualized in a score plot in accordance with its metabolic fingerprint, and

thus samples that are metabolically similar are grouped. The corresponding loading plot was then examined to identify the metabolites contributing to the group separation. Metabolite changes were calculated via the ratio between the averages of the LAR and SJ peak areas. For comparison between group means, the parametric Student's *t*-test was applied on both Hg levels and oxidative stress data with normal distribution using Statistica 8.0, and metabolomic data using Graph Pad software. Non-parametric Mann-Whitney test for Hg levels and oxidative stress data was used when data distribution significantly deviated from normality. The threshold for significance was $p < 0.05$ for all tests, recognized as the criterion of statistical significance.

5.3. Results

5.3.1. Mercury levels in the gills

Levels of mercury measured in the gills of *L. aurata* from SJ and LAR are reported in Fig. 5.1. Fish gills from LAR displayed significantly higher accumulated levels of tHg, MeHg and iHg in respect to those from SJ. Accordingly, also the percentage of MeHg relative to tHg was significantly higher in fish gills from LAR in comparison with fish gills from SJ.



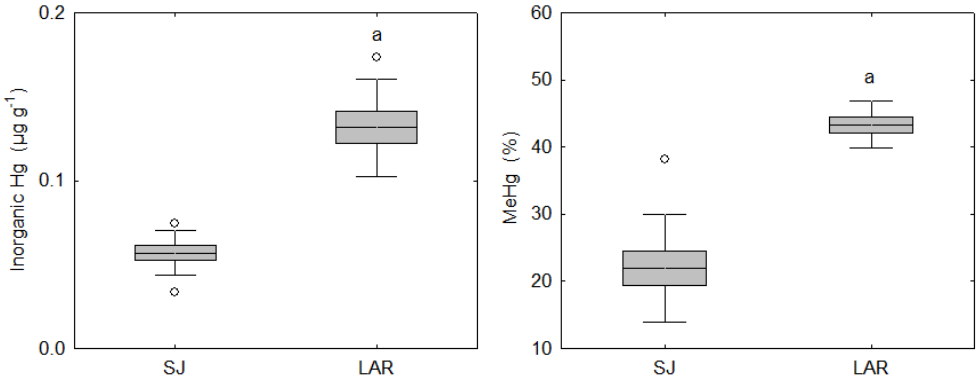


Figure 5.1. Total Hg (tHg), MeHg, inorganic Hg (µg g^{-1}, dry weight) and % of MeHg (in relation with tHg) in the gills of *L. aurata* captured in Laranjo (LAR) and São Jacinto (SJ) at Aveiro lagoon. Mean, standard deviation, standard error, outliers (o) are presented. Significant differences are indicated by 'a' *vs.* SJ.

5.3.2. Metabolomics analysis

5.3.2.1. ^{1}H NMR spectroscopy of gill tissue extracts

A typical 1-D ^{1}H NMR spectrum of gill tissue extracts from golden grey mullet collected at SJ is shown in Fig. 5.2. Numerous metabolites were identified, however all spectra were dominated by taurine (3.25 and 3.41 ppm) and lactate (1.33 and 4.12 ppm), which were *ca.* 45 and 20 times more concentrated than other metabolites, respectively. Among the other prominent categories, amino acids (e.g. glutamate, alanine), energy storage compounds (e.g. glucose), Kreb's cycle intermediates (e.g. malonate, fumarate), and nucleotides (e.g. uracil) were observed.

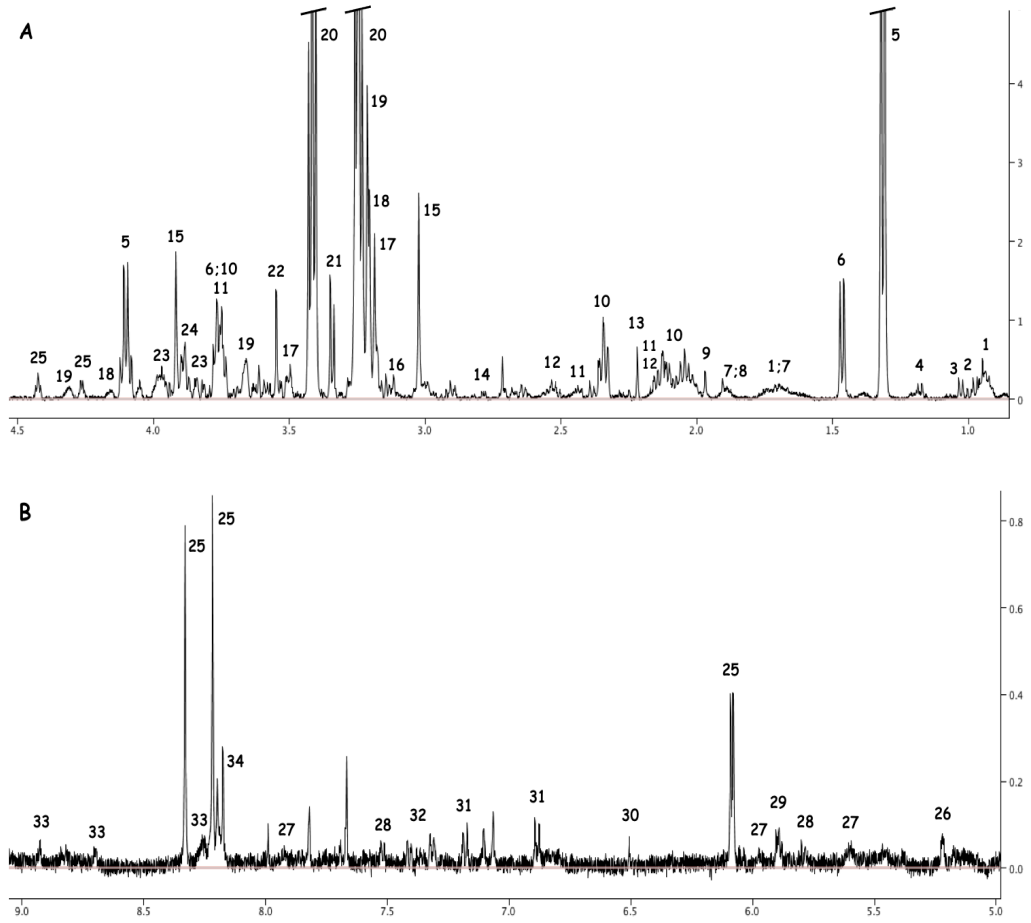


Figure 5.2. A representative 1-D 500 MHz ^{1}H NMR spectrum of gill tissue extracts from *L. aurata* collected from SJ, with (A) representing the aliphatic region and (B) a vertical expansion of the aromatic region. Keys: (1) leucine, (2) isoleucine, (3) valine, (4) isobutyrate, (5) lactate, (6) alanine, (7) lysine, (8) arginine, (9) N6-acetyl lysine, (10) glutamate, (11) glutamine, (12) glutathione, (13) acetone, (14) aspartate, (15) creatine, (16) malonate, (17) choline, (18) phosphocholine, (19) glycerophosphocholine, (20) taurine, (21) unknown #1, (22) glycine, (23) serine, (24) betaine, (25) inosine, (26) glucose, (27) UDP-glucose, (28) uracil, (29) uridine, (30) fumarate, (31) tyrosine, (32) phenylalanine, (33) niacinamide, and (34) hypoxanthine.

5.3.2.2. Pattern recognition analysis of ^{1}H NMR spectra

PCA scores plot of the ^{1}H NMR metabolic profiles of *L. aurata* gills (Fig. 5.3) displays a clear clustering of the fish groups from the two sampling sites along the PC1 axis (accounting for 47.82 percent of variance). To identify the metabolites responsible for grouping samples and the direction of their changes, the corresponding PC1 loading plot, depicted in Figure 5.4, was analyzed. In detail, peaks with negative loadings correspond to metabolites whose concentrations were higher in fish from LAR relative to those from SJ, whilst positive loadings correspond to metabolites whose level was lower in LAR group in respect to SJ. From the analysis of PC1 loading plot it was revealed that the metabolic profiles of fish gills from LAR presented significantly elevated concentrations of alanine, creatine, lactate, uracil, and choline, together with significantly reduced levels of taurine, glycerophosphocholine, and glutathione, as reported in Table 5.1.

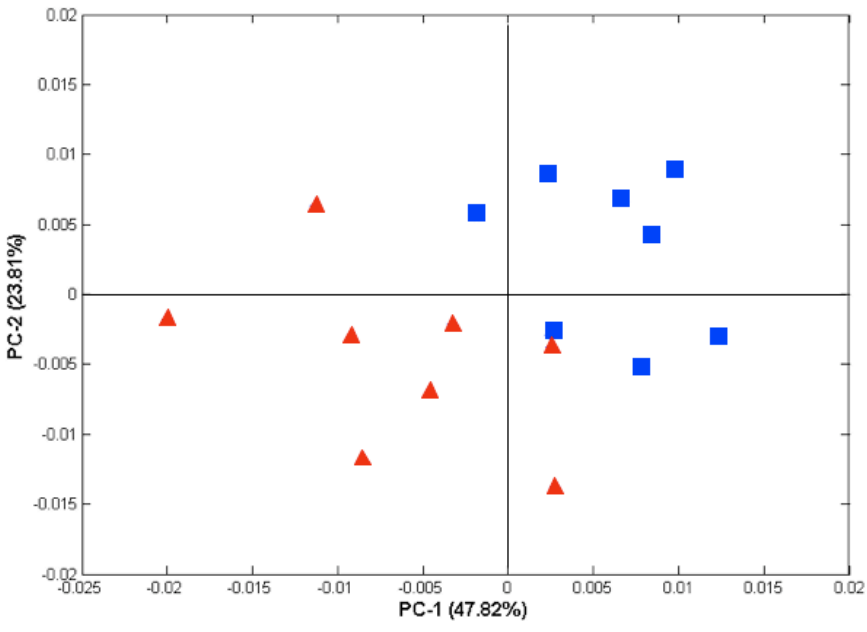


Figure 5.3. Principal components analysis (PCA) of ^{1}H NMR spectra of gill extracts showing separation (PC1 vs. PC2) between *L. aurata* collected from SJ (blue square) and those from LAR (red triangle).

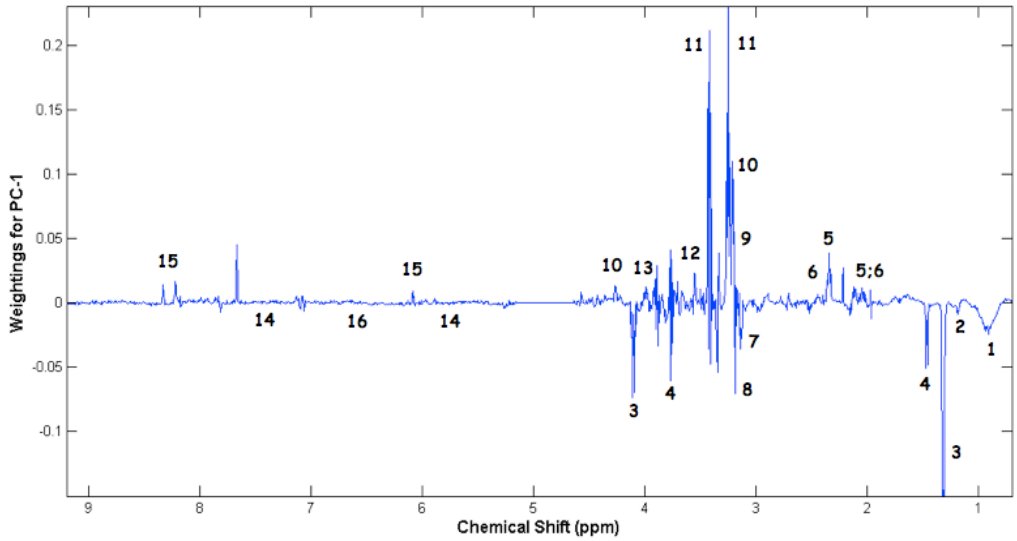


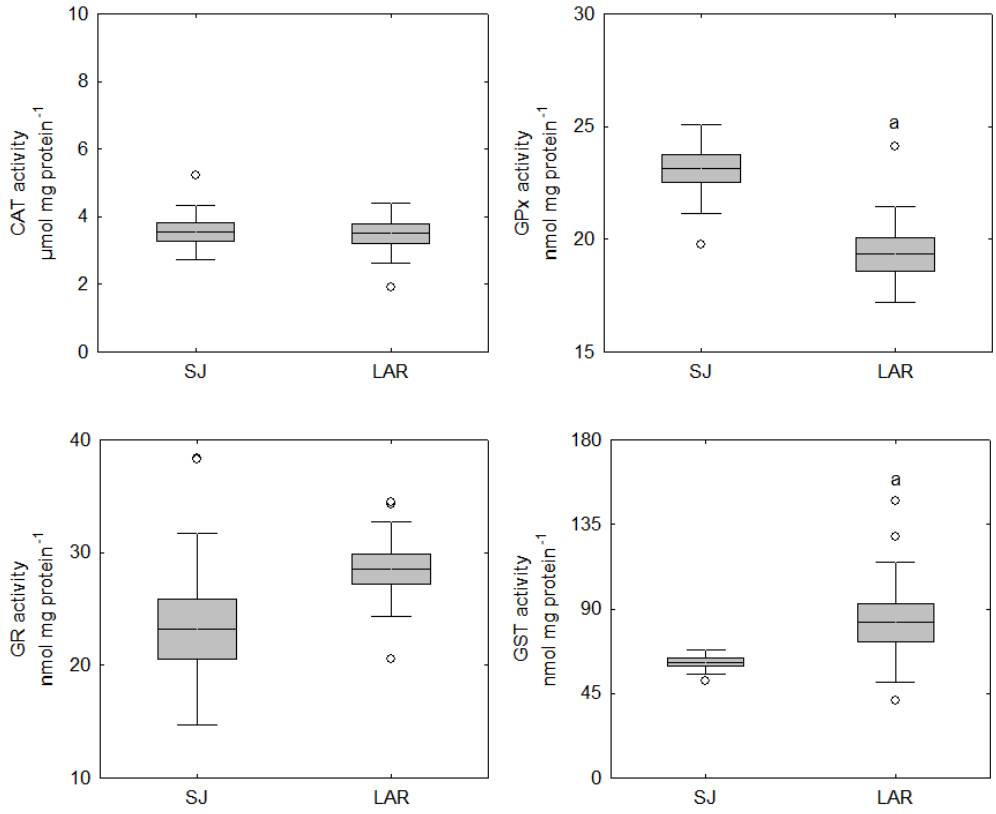
Figure 5.4. Corresponding PC1 loading plot showing the metabolic differences between gills of *L. aurata* collected from SJ and LAR. Keys: (1) branched chain amino acids: isoleucine, leucine, valine, (2) isobutyrate, (3) lactate, (4) alanine, (5) glutamate, (6) glutathione, (7) creatine, (8) choline, (9) phosphocholine, (10) glycerophosphocholine, (11) taurine, (12) glycine, (13) serine, (14) uracil, (15) inosine, and (16) fumarate.

Metabolites	Chemical shift and peak shape, ppm	
Amino acids		
Isoleucine	0.92 (t), 1.00 (d), 1.26 (m), 1.44 (m), 1.96 (m), 3.66 (d)	34% ↑
Leucine	0.94 (d), 0.96 (d), 1.66 (m), 3.71 (t)	27% ↑
Valine	0.98 (d), 1.03 (d), 2.25 (m), 3.59 (d)	21% ↑
Isobutyrate	1.19 (d), 2.59 (m)	42% ↑
Alanine	1.46 (d), 3.76 (m)	24% ↑[a]
Glutamate	2.08 (m), 2.34 (m), 3.74 (t)	22% ↓
Creatine	3.02 (s), 3.91(s)	43% ↑[a]
Glycine	3.54 (s)	26% ↓
Serine	3.84 (dd), 3.95 (m)	18% ↓
Energy metabolites		
Lactate	1.33 (d), 4.12 (q)	38% ↑[a]
Fumarate	6.50 (s)	35% ↑
Osmolytes		
Taurine	3.25 (s), 3.41 (t)	52% ↓[a]
Glycerophosphocholine	3.21 (s), 3.60 (dd), 3.67 (m), 3.90 (m), 4.31 (m)	32% ↓[a]
Nucleosides and derivatives		
Inosine	3.83 (dd), 3.90 (dd), 4.27 (dd), 4.25 (t), 4.76 (t), 6.08 (d), 8.21 (s), 8.33 (s)	18% ↓
Uracil	5.81 (d), 7.54 (d)	25% ↑[a]
Miscellaneous metabolites		
Glutathione	2.13 (m), 2.54 (m), 2.97 (dd), 3.75 (m), 4.53 (m)	38% ↓[a]
Choline	3.21 (s), 3.52 (s), 4.07 (m)	48% ↑[a]
Phosphocholine	3.21 (s), 3.57 (t), 4.16 (m)	19% ↓

Table 5.1. Relative changes in metabolite concentrations in gills between LAR and SJ golden grey mullets ($p < 0.05$[a]; Student's *t* test). (s: singlet; d: doublet; t: triplet; dd: doublet of doublets; q: quartet; m: multiplet)

5.3.3. Oxidative stress endpoints

A significant increase of GSHt content and GST activity was recorded in gills of fish from LAR in comparison to SJ, together with a significant decrease of gills GPx activity. Moreover, no significant differences were found between sites for GR activity (Fig. 5.5). Also, a significant decrease of gills SOD activity, as well as LPO levels, was found at LAR relatively to SJ, while no significant differences were found between sites for CAT activity.



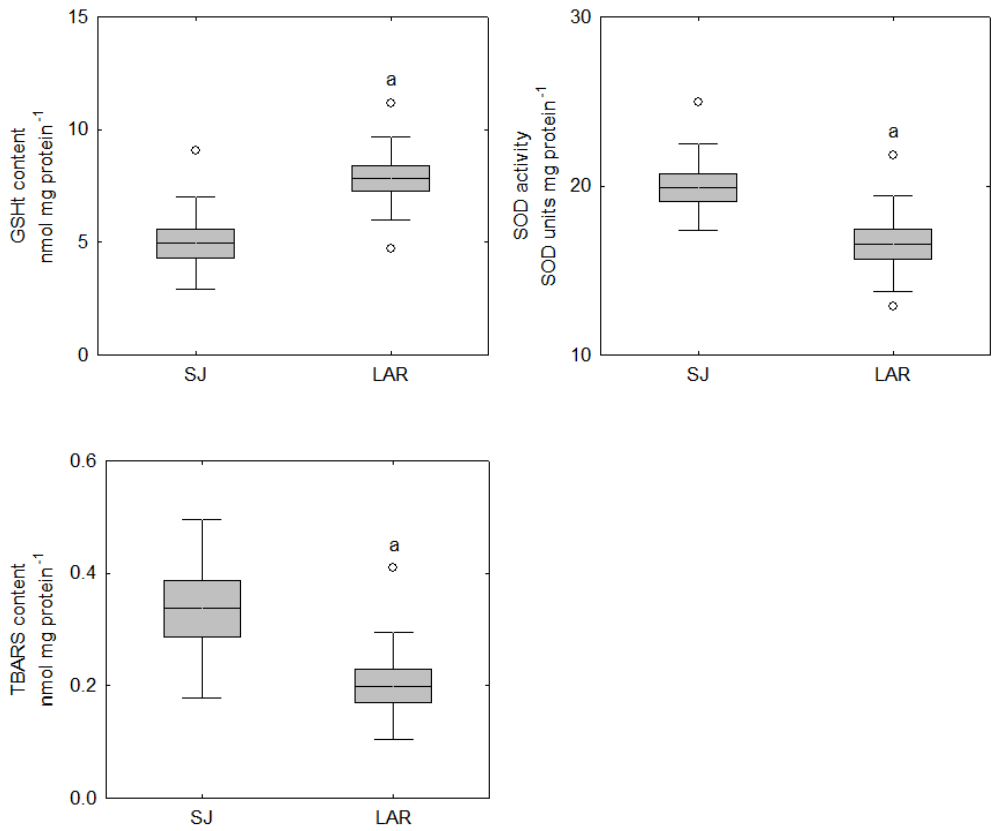


Figure 5.5. Oxidative stress responses (CAT, GPx, GR, GST and SOD activities and GSHt and TBARS content) in the gills of *L. aurata* captured in Laranjo (LAR) and São Jacinto (SJ) at Aveiro lagoon. Mean, standard deviation, standard error, outliers (o) and extreme values (※) are presented. Significant differences are indicated by 'a' *vs.* SJ.

5.4. Discussion

5.4.1. Discussion on metabolic responses to mercury

The gills of *L. aurata* from LAR (contaminated area) accumulated higher levels of MeHg and iHg, and consequently tHg, than those from SJ (uncontaminated area), clearly evidencing an exposure to environmental Hg. These results are in line with previous findings from Mieiro et al. (2011, 2009),

who demonstrated that *L. aurata* gills were able to reflect environmental variations of total Hg in a field study performed at the Aveiro lagoon. In addition, the present study reports for the first time MeHg accumulation in fish gills from this aquatic system, which was significantly higher at LAR than SJ. The percentage of MeHg was significantly higher in LAR with respect to tHg (43% of tHg) than that in SJ (only 22% of tHg). Although Hg in water is mainly in inorganic form, different accumulation patterns of Hg in gills were found for both sites. At LAR, the accumulation of MeHg and iHg was almost similar (0.10 μg/g of MeHg and 0.13 μg/g of iHg), while iHg was mainly accumulated at SJ.

The fate of contaminants in the environment depends on many factors, including water physico-chemical parameters (i.e. temperature, dissolved oxygen and salinity), which may interfere, both directly and indirectly, with the bioavailability of metals (Luoma and Rainbow, 2005), thereby influencing fish metabolic/oxidative stress profiles. As reported in Chapter III of this thesis, in both sites the Hg in water is mainly in inorganic form, and even though the environmental availability of both iHg and MeHg is higher at LAR, this fact is not sufficient *per se* to explain the difference observed in accumulation patterns of Hg in gills between the two sites. Thus, the different accumulation patterns in the two sampling sites may be associated with the divergence in salinity and dissolved oxygen, being both lower at LAR. The uptake of Hg is greatly dependent on its speciation in aquatic environments, which can be directly influenced by the water physico-chemical parameters (Fitzgerald et al., 2007). It has been shown that a decrease in salinity is usually associated with an increase in bioavailability and uptake rates of contaminants (Monserrat et al., 2007). Specifically, Wang and Wang (2010) demonstrated in their study with tilapia (*Oreochromis niloticus*) that the aqueous uptake rates of Hg(II) were significantly higher at lower salinity. In turn, Wang et al. (2011) observed that a decrease in dissolved oxygen level lead to an increase in the MeHg availability and water uptake rates in the Nile tilapia (*Oreochromis niloticus*),

suggesting the coupling of water flux and MeHg uptake. Thus, both iHg and MeHg uptake by fish gills from LAR may be potentiated by the lower salinity and dissolved oxygen. Moreover, an increase in aqueous uptake of dissolved Hg (iHg and MeHg) could directly affect the aqueous uptake at all trophic levels and even further affect the dietary uptake in higher consumers via food chain transfer. Because the dietary uptake is usually considered the major accumulation pathway of MeHg and the gills are between the venous and arterial circulation, receiving nearly all of the cardiac output, it is possible that this food chain transfer may be associated with an increase in the accumulation of MeHg in gills of the fish from LAR.

The NMR metabolic data provided an integrated description of the Hg-induced metabolic responses in the gills of golden grey mullets, allowing the identification of a considerable number of metabolites from several different classes, including amino acids, osmolytes, carbohydrates and nucleotides. PCA analysis indicated that individuals from SJ clustered separately from those collected at LAR, suggesting a clear differentiation in their metabolic profile.

The largest change in the gill metabolic profile of mullets collected from the two sites occurred in the concentration of taurine, which decreased significantly by 52% in fish from LAR in respect to fish from SJ. Taurine is a sulfur-containing amino acid mainly involved in the maintenance of cellular homeostasis. It is known that cell swelling caused by hypo-osmotic stress is followed by a regulatory volume decrease (RVD) that occurs via cellular extrusion of ions, such as K^+ and Cl^-, and organic osmolytes, mainly taurine (Fugelli and Thoroed, 1990). Euryhaline fish, such as *L. aurata*, naturally deal with fluctuations of water salinity and the fish gill epithelium, which is constantly in contact with the surrounding water, is particularly sensitive to environmental osmolarity, as demonstrated by *in vitro* studies conducted by Avella et al. (2009) on the swelling-activated efflux of taurine in cultured branchial cells of the European sea bass *D. labrax*. However, even the exposure to metals, especially Hg, can affect water permeability of fish gills

and influence ion-osmoregulation (De Domenico et al., 2011). Therefore, the reduction in taurine observed in the present study can be associated to interferences in osmotic balance both due to differences in salinity recorded in the two areas investigated (salinity decreased in LAR) and due to Hg accumulation. In accordance, the concomitant depletion at LAR of glycerophosphocholine, which plays a major role as organic osmolyte (Burg and Ferraris, 2008), provides further evidence of osmoregulatory disturbances in gills of *L. aurata*.

Moreover, the statistically significant increase in creatine content reported in gills of *L. aurata* from LAR is supportive of alteration in ion-osmoregulatory processes. In a number of osmoregulatory epithelia, including fish gills, the enzyme Na^+-K^+-ATPase plays a crucial role in the mechanisms of active ion transport and water balance (Brunelli et al., 2010; McCormick, 1995). Creatine kinase (CK) serves for the transfer of energy between phosphagens in a reaction reversible between phosphocreatine and ADP, in which creatine is the end product of the reaction along with ATP, necessary for functioning of Na^+-K^+-ATPase. Previous studies conducted by Weng et al. (2002) have shown the strict correlation in the activity between gill Na^+-K^+-ATPase and CK in the euryhaline fish tilapia, suggesting that CK provides energy for Na^+-K^+-ATPase to carry out osmoregulation during acute salinity challenges. In addition, a time-dependent increased activity of Na^+-K^+-ATPase was also documented in gills of *D. labrax* after exposure to Hg-enriched sediments from a petrochemical area (De Domenico et al., 2011).

Impairments in energy-producing metabolic pathways were also found in gills of fish collected from the Hg-contaminated site. Specifically, the statistically significant rise in the level of lactate, accompanied by the concomitant increase (not significant) of fumarate, may indicate an enhancement in anaerobic metabolism as strategy to replenish insufficient energy supply, attributable to Hg contamination as well as lower oxygen availability recorded at LAR. Similar findings were reported by Xu et al.

(2015) in gill tissues of butachlor intoxicated goldfish *C. auratus*, which showed accumulation of lactate and some intermediates of Krebs cycle, i.e. citrate and fumarate, reflecting a hampered tricarboxylic acid (TCA) cycle that could be due to damages in mitochondria and consequent short supply of ATP. Also, the gills of mussels *M. galloprovincialis* caged in a petrochemical area, mainly impacted by Hg and PAHs, exhibited elevated level of Krebs cycle intermediates together with the occurrence of glycogenolysis, as a compensatory mechanism in marine mussels to counteract with the impairment of branchial ciliary activities (Cappello et al., 2013b).

Changes in the pool of amino acids were also observed in mullet gills presumably in response to Hg contamination. Indeed, the concentrations of three important branched-chain amino acids (BCAAs), namely isoleucine, leucine, and valine, were found increased, though not significantly. It is known that BCAAs are essential proteinogenic amino acids, but also play a crucial role in the regulation of protein turnover process. The increased levels of BCAAs in fish gills might thus suggest an enhancement of protein catabolism, attributable to the Hg-induced formation of reactive oxygen species that could attack proteins provoking their damage and consequent fragmentation (Liu et al., 2015).

Among the changes in amino acids in response to Hg-exposure, it must be pointed out the significant increase in alanine in gills of mullets. Nitrogen is mainly carried to the liver by alanine. Therefore, its arise might be an adaptive response to deal with an excess of free ammonia, thus preventing intoxication resulting from the protein catabolism occurring in gills, which also play a major role in nitrogenous waste excretion (Evans, 1987).

Finally, from the gill metabolome of mullets from the Hg-contaminated area, it is worth to be noted the increased level of isobutyrate by 42%, although not significant. Isobutyrate is a marker of anoxia, thus its elevation in gills is attributable to the onset of a hypoxic condition (Giannetto et al., 2015), that may be a direct consequence of the lower dissolved oxygen content measured

at LAR relatively to SJ, or be indirectly related to Hg toxicity. Perturbations in the oxygen intake were in fact reported in the gills of seabass *D. labrax* experimentally exposed to Hg-enriched sediments (De Domenico et al., 2011). Furthermore, Kokushi et al. (2012) observed increase of isobutyrate in blood plasma of the freshwater carp *C. carpio* treated with heavy oil, while the dissolved oxygen in test waters were kept at normal levels.

In gills of *L. aurata* from LAR, the significant depletion of glycerophosphocholine, besides being attributable to its involvement in the mechanisms of osmoregulation as above discussed, may be associated to occurring membrane stabilization/repair processes. In fact, the concomitant reduction in level of glycerophosphocholine and phosphocholine, though the latter not statistically significant, may indicate an ongoing biosynthesis of phosphatidylcholine, the major structural phospholipid of cell membranes, thus reflecting the occurrence of membrane repair processes in gills. This explanation gains plausibility from the observed significant decrease in lipid peroxidation (LPO) level, discussed more in detail below. This finding is suggestive of the occurrence of adaptive cell-protecting antioxidant mechanisms elicited by mullets to prevent peroxidation of membrane lipids in gills. Furthermore, from the analysis of the gill metabolome, a significant 48% increase in choline was found in fish from LAR relatively to those from SJ. This data is in compliance with the decreased levels of glycerophosphocholine and phosphocholine, which are the main storage forms for choline within the cytosol (Rohlfs et al., 1993).

The NMR-based metabolomics approach revealed also a significant decrease in reduced glutathione (GSH) and its constituent amino acids (i.e. glycine and glutamate) in the gills of mullets from the Hg-contaminated area. It is known that Hg is highly reactive with protein sulphydryl groups and cysteine residues, including GSH. Furthermore, it is recognized that GSH is the primary antioxidant and conjugating agent, being the first line of defence against Hg (Farina et al., 2013; Mieiro et al., 2014; Ung et al., 2010). Hence, this results in

the formation of Hg-GSH complexes and, accordingly to what found in the gill metabolome of mullets from LAR compared to SJ, in a consequent reduction of the intracellular pool of GSH molecules available (Sears, 2013). Overall, the depletion of GSH and its constituent amino acids indicate some vulnerability in mullet gills to Hg toxicity, as it enhances the risk of oxidative stress due to the accumulation of reactive oxidative species (ROS), that are generally neutralized by GSH.

Besides its role of glutathione precursor, glutamate has long been known to be a major excitatory neurotransmitter in vertebrates (von Bohlen und Halbach and Dermietzel, 2002). At elevated concentrations, glutamate acts as a neurotoxin capable of inducing severe neuronal damage – a phenomenon known as excitotoxicity (Dingledine and Mcbain, 1994). However, it was reported that glutamic uptake could be affected by compounds that react with -SH groups on cysteine residues. As a matter of fact, MeHg potentially and specifically inhibits glutamate uptake in astrocytes (Trotti et al., 1997), which is consistent with the reduction (not significant) in glutamate level observed in the current study in the gills of *L. aurata* from LAR.

5.4.2. Discussion on oxidative stress responses to mercury

Several studies indicated that the binding of Hg with GSH (formation of Hg-GSH complexes) reduces the intracellular pool of available GSH molecules (Elia et al., 2003; Lushchak, 2011; Sevcikova et al., 2011). Accordingly, in the current work the metabolomic data obtained by NMR spectroscopy revealed a depletion of GSH in the gills of fish from LAR when compared to SJ, as discussed above. However, an increase of total glutathione content (reduced glutathione + oxidized glutathione) was observed in fish gills at LAR that, in parallel with the GSH depletion above mentioned, indicates the occurrence of

massive GSH oxidation under Hg stress. These results, together with the unaltered GR activity and the depletion of GSH constituent amino acids (i.e. glycine and glutamate), revealed an inability of the gills to carry out efficiently the regeneration or *de novo* synthesis of GSH. Previous studies observed that Hg exposure increased GR activity (Mieiro et al., 2014; Monteiro et al., 2010). However, as above mentioned, no differences were observed in the GR activity, which is in agreement with other studies conducted on *L. aurata* collected from the same area, namely Aveiro Lagoon in Portugal (Brandão et al., 2015; Mieiro et al., 2011). Previous studies performed in the same area reported an increase of GSHt content in the gills of *Dicentrarchus labrax* (Ahmad et al., 2008; Mieiro et al., 2014, 2011). However, it was not possible to confirm if this increase correspond to an enrichment of GSSG or GSH. Contrarily to data presented herein, Monteiro et al. (2010) observed an increase in the GSH levels in the gills of the fish *Brycon amazonicus* exposed to $HgCl_2$. The apparent dissimilarities observed between the different studies both for GSH content and GR activity may be associated with the type (field or laboratory), duration (short or chronic) and Hg form (iHg and MeHg) of exposure (Mieiro et al., 2014). However, a new mechanistic insight related with Hg toxicity was found, revealing that it does not only led to GSH depletion through its oxidation but also interfered with the processes of its regeneration and *de novo* synthesis.

The selenohydryl groups have also been reported as critical and primary targets in mediating Hg-induced toxicity (Branco et al., 2012; García-Sevillano et al., 2014). Thus, a preferential target of MeHg is GPx, a selenoenzyme that prevents the production of ROS by reducing hydrogen peroxide and free fatty acid hydroperoxides (Farina et al., 2013). In accordance, a drop in the GPx activity was recorded in *L. aurata* gills from LAR. Such effect was in line with the higher tHg accumulation (as well iHg and MeHg) in this fish group. The impairment of GPx activity will probably lead to an enhancement in the cellular levels of hydrogen peroxide and lipid hydroperoxides. Contradicting

the results presented in this thesis, Mieiro et al. (2014) observed an increase of GPx activity in *Dicentrarchus labrax* gills collected in the same area. Taking into account that Hg is mostly accumulated in the sediment, different feeding behaviours cannot be overlooked, since *L. aurata,* as a mud feeder, is potentially more vulnerable to Hg effects comparing to *D. labrax*. However, a similar pattern of GPx inhibition was verified for liver of *L. aurata* from LAR, as described in Chapter IV of this thesis.

It is known that the action of an antioxidant enzyme can be replaced by the activity of other antioxidants (Bagnyukova et al., 2005). Thus, the GPx inhibition could be partially compensated by an enhancement in the GST activity. This increase of GST activity was observed in the present study, suggesting a protective and adaptive response to Hg accumulation, which may be associated with the increase of lipid hydroperoxides. GST enzymes are involved in the transformation of a wide variety of electrophilic compounds to less toxic substances by conjugating them with GSH (Van der Oost et al., 2003) and some of them display peroxidase activity with respect to lipid hydroperoxides (Singhal et al., 1992). These two roles of GSTs are associated with concomitant oxidation of GSH to GSSG (Regoli and Giuliani, 2014), and thus, the observed depletion of GSH and increase of GSSG referred above may be associated with this process. Previous studies also showed that Hg exposure induces GST activity in gills of fish (Mieiro et al., 2014; Monteiro et al., 2010).

SOD is the enzyme responsible for the degradation of the superoxide radical ($O_2^{\bullet-}$) (it is dismutated to H_2O and H_2O_2), which is among the most unstable and reactive forms of oxygen. In turn, CAT is a heme-containing enzyme that decomposes the H_2O_2, produced by the dismutation of $O_2^{\bullet-}$, into water and molecular oxygen. In the present study, the exposure to Hg was responsible for SOD inhibition, suggesting some vulnerability of the defence system in the presence of Hg. The SOD–CAT system provides the first line of enzymatic defence against oxygen toxicity, displaying suppressive effects on hydroxyl radical formation (Pandey et al., 2003). Thus, the inhibition of SOD

and the absence of response of CAT indicates some vulnerability of the antioxidant system of *L. aurata* gills that may contribute to oxidative stress (Hussain et al., 1997). In agreement with the present lack of CAT response, Mieiro et al. (2011) also observed limitations on CAT response in the gills of *L. aurata* caught in the same area. In turn, Berntssen et al. (2003) observed a decrease of SOD activity in the brain of the *Salmo salar* after chronic dietary MeHg exposure, which is in line with that perceived in the current study. However, in the same work, both the liver and kidney of fish exposed to MeHg, and the three tissues (i.e. brain, liver and kidney) of fish exposed to iHg revealed a contrary pattern. Monteiro et al. (2010) also observed the induction of the SOD–CAT system in the liver, gills, white muscle and heart of *Brycon amazonicus* exposed for 96 h to iHg, indicating a fast adaptive response of the redox-defence system. Overall, the responses of SOD and CAT in fish may vary with the exposure pathway, duration of exposure, Hg form and accumulation levels. Additionally, there is an organ-specific response for both endpoints in fish.

Furthermore, lipid peroxidation was also evaluated in gills of *Liza aurata* collected from an Hg-contaminated system. Lipid peroxidation is a chemical mechanism capable of disrupting the structure and function of the biological membranes occurring as a result of free radical attack on lipids. Since the prevention of LPO greatly depends on the action of antioxidants, the *L. aurata* gills demonstrated some degree of vulnerability due to the depletion of SOD and GPx activity and GSH content, which are essential antioxidants in the protection of the membrane. However, no LPO increase was found and the only significant effect was exerted in terms of decreased levels of TBARS, which is not demonstrative of any noteworthy physiological outcome. The absence of oxidative response observed here could be related to an enhancement of membrane stabilization/repair processes, as suggested by the depletion of phosphocholine and glycerophosphocholine revealed by the metabolomic analysis conducted in fish gills, and discussed above. However,

other metabolites may have markedly varying roles in membrane metabolism. For instance taurine, besides functioning as an effective osmolyte, plays a key role in the maintenance of cell membrane, regulating the ethanolamine:choline ratio of some membranes (Huxtable, 1992). Therefore, the depletion in taurine, detected in the gill metabolome profile of fish from LAR, may have a dramatic influence on the structure of biological membranes, which in turn alters both membrane fluidity and the activity of membrane enzymes and transporters (Schaffer et al., 1995). Nevertheless, findings from this work suggest that membrane stabilization/repair processes are ongoing, as discussed above.

Moreover, the absence of oxidative response may also be associated to an over expression of metallothioneins (MTs), which are proteins directly involved with intracellular storage and detoxification of metals. By scavenging metallic species, and consequently reducing the fraction of biologically active metal, MTs prevent the establishment of redox deleterious cycles that could otherwise lead to oxidative damage, acting as indirect antioxidant compounds (Nunes et al., 2014). Several studies have shown that induction of MTs is a suitable biomarker of metal exposure, as reported in invertebrates (Ciacci et al., 2012; Fasulo et al., 2008) and lower vertebrates (De Domenico et al., 2011; Mauceri et al., 2005; Sevcikova et al., 2011). More specifically, it has been demonstrated that MTs play a protective role in response to Hg exposure (Navarro et al., 2009; Oliveira et al., 2009). Oliveira et al. (2009) showed significantly higher levels of MTs in gills of *L. aurata* from LAR. MTs responsiveness in gills is controversial and some authors claimed that it does not constitute a good organ for MT quantification in fish (Hamza-Chaffai et al., 1997; Olsvik et al., 2001). However, gills of the *L. aurata* seemed to have the capacity to increase MTs levels as a response to metal exposure, as aforementioned. Although MTs were not measured in the current study, its over expression is a plausible hypothesis to the prevention of oxidative damage (namely LPO).

5.5. Conclusions

From the findings herein reported, it emerges that Hg contamination induced multiple toxicological effects in the gills of wild *L. aurata*, suggesting a compromised health status of mullets. However, the influences of salinity and oxygen with the observed effects were not disregarded.

The ^{1}H NMR-based metabolomics approach enabled an integrated description of a number of metabolites involved in different metabolic pathways, including ion-osmoregulation, energy and protein metabolism, membrane stabilization/repair processes, and antioxidant defence system. In regard to the latter, Hg accumulation interfered with the antioxidant protection of gills, leading to depletion of important antioxidants (GSH, GPx and SOD). Nonetheless, the induction of lipid peroxidation was efficiently prevented.

In detail, in gills of fish from the Hg-contaminated area interferences in the ion-osmoregulatory processes were revealed, seen as decreases in taurine and glycerophosphocholine, along with increased creatine level. Impairments in energy-producing metabolic pathways were also observed, as well as the occurrence of protein catabolism supported by the increased levels in amino acids and alanine, the latter mainly involved in nitrogenous waste excretion. Moreover, some vulnerability in the respiratory gas exchange activity of mullets from the Hg-polluted area was suggested by augmentation of isobutyrate, a recognized biomarker of anoxia.

The combination of the metabolomics and pro-oxidant status evaluation indicated the occurrence of massive GSH oxidation under Hg stress, and an inability to carry out its regeneration (GR activity was unaltered) or *de novo* synthesis (depletion in GSH constituent amino acids, glycine and glutamate). Moreover, the prevention of lipid peroxidative damage may be associated with the enhancement of membrane stabilization/repair processes, resulting from depletion in precursors of phosphatidylcholine (phosphocholine and glycerophosphocholine). Though the exposure to Hg led to a state of

vulnerability due to limitations of antioxidant system towards a physiological accommodation, the membranes were able to answer to oxidative insult through the enhancement of stabilization/repair processes.

Therefore, the novel concurrent use of metabolomics and conventional oxidative stress endpoints demonstrated to be effective towards a mechanistically based assessment of Hg toxicity in gills of fish, providing novel insights into the mechanisms underlying the oxidative damage.

CHAPTER VI

Tissue-specific responses to mercury in wild mullet *Liza aurata*

6. Tissue-specific responses to mercury in wild mullet *Liza aurata*

6.1. General introduction

As reported previously in this thesis, fish are widely used as sentinel organisms for evaluation of the health of aquatic ecosystems and environmental risk assessment (Fasulo et al., 2010a, 2010b; Lushchak et al., 2011; Mieiro et al., 2014, 2011; Sweet and Zelikoff, 2001). Indeed, fish are generally regarded as good indicators of environmental conditions, since are able to provide evidence of alterations in the environments induced by anthropogenic activities, namely, the occurrence of a range of toxic pollutants. Although contaminants might lead to a wide range of responses in fish, the induction of measurable biochemical responses has received increasing relevance in the fields of ecotoxicology and environmental risk assessment. Fish are ubiquitous in the aquatic environment, playing a relevant ecological role in the aquatic food webs due to their ability to transfer energy from lower to higher trophic levels (Beyer et al., 1996). Moreover, fish are permanently in direct contact with the contaminants present in the water bodies (Kane et al., 2005) and, given the similarity of their responses with higher vertebrates, they can be used to screen potentially hazardous effects to humans (El-Shehawi et al., 2007).

It has been extensively reported in the literature that the variety of responses elicited by fish in response to a toxicant insult are dependent on the species (Mieiro et al., 2011, 2010), as well as on the organ (Miero et al., 2014; Oliveira et al., 2008). Indeed, organ-specific sensitivity reflected a different threshold limit within which antioxidants are able to respond (Pereira et al. 2010; Mieiro et al. 2011), and it is thus very likely that tissue-specific responses may be appreciated also at metabolite level. Therefore, there is an urgent need to understand the responses elicited by organisms to a given toxicant compound

using a multi-organ approach, in order to understand which feature might be the most relevant amog physiological and anatomical properties, bioaccumulation capacity or the exposure pathway.

Taking into account that the risk associated to mercury is widely acknowledged, considering the importance of fish in aquatic food chains and given the similarity of their responses with higher vertebrates, and thus with humans, it is crucial to understand the influence of tissue physiological functions in the Hg accumulation and biological responses in different key organs (i.e. liver and gills) of wild fish. In this way, by a detailed comparative analysis of the differential bioaccumulation of Hg and metabolic responses elicited in different fish tissues, it will be possible to improve the understanding of biological markers in the context of environmental health research, and thus to improve risk assessment approaches.

Therefore, the aim of this study was to assess both mercury accumulation and organs' specific metabolic responses to mercury elicited in liver and gills of wild golden grey mullet *Liza aurata* inhabitating an Hg contaminated system, in order to provide a more comprehensive assessment of the mechanisms of Hg toxicity in fish, and potentially in humans.

6.2. Comparative analysis of mullet tissues responses

As described in detail in the previous Chapter IV and Chapter V of this thesis, different forms of mercury, namely total Hg (tHg), MeHg, inorganic Hg, and percentage of MeHg in relation to tHg, were measured respectively in liver and gills of golden grey mullet *L. aurata* collected from the Aveiro Lagoon (Portugal), a site highly contaminated by mercury. Results obtained in mullet gills and livers are reported in Table 6.1.

	Gills				Liver			
Site	**tHg** (μg g^{-1})	**MeHg** (μg g^{-1})	**iHg** (μg g^{-1})	**% MeHg**	**tHg** (μg g^{-1})	**MeHg** (μg g^{-1})	**iHg** (μg g^{-1})	**% MeHg**
SJ	0.7 (± 0.04)	0.018 (± 0.001)	0.06 (± 0.002)	22 (± 0.3)	1 (± 0.13)	0.75 (± 0.16)	0.3 (± 0.08)	67 (± 0.44)
LAR	0.25 (± 0.02)	0.11 (± 0.14)	0.14 (± 0.02)	43 (± 0.15)	2.38 (± 0.25)	1.82 (± 0.18)	0.58 (± 0.04)	78 (± 0.02)

Table 6.1. Total Hg (tHg), MeHg, inorganic Hg (μg g^{-1}, dry weight) and % of MeHg (in relation with tHg) in the gills and liver of *L. aurata* captured in Laranjo (LAR) and São Jacinto (SJ) at Aveiro lagoon. Data are presented as mean (± standard deviation).

Moreover, a NMR-based metabolomics approach was applied in gills and liver of the same organisms in order to unravel the mechanisms of toxicity of mercury at metabolite level, as reported in Chapter IV and Chapter V, respectively.

From the comparison of ^{1}H NMR spectra of liver and gills from *L. aurata*, it was observed that numerous metabolites, belonging to a variety of biomolecule categories, are present in both fish organ profiles. In particular, liver spectra were dominated by two organic osmolytes, namely taurine and glycerophosphocholine, which were *ca.* 45 and 15 times respectively higher in intensity than the other metabolites, while the gill metabolome showed elevated level of taurine and lactate, which were *ca.* 45 and 20 times more concentrated than other metabolites, respectively. Other major classes of compounds included amino acids (i.e. alanine, glutamate), carbohydrates (i.e. glucose), Krebs cycle intermediates (i.e. fumarate), and nucleotides (i.e. uracil). Taurocholic acid was detected solely in liver, whereas isobutyrate, choline and serine were observed exclusively in gill profiles.

In both organs, PCA score plots described the majority of variance between spectra (i.e. 78% in liver and 72% in gills), and displayed a clear clustering of the fish groups from the two sampling sites along PC2 and PC1 for liver and gills, respectively. To identify the metabolites responsible for grouping samples of liver and gills, as well as the direction of their changes, the corresponding PC loading plots were examined.

In detail, the metabolic profiles of liver extracts of fish from LAR were characterized by significantly ($p < 0.05$) elevated levels of alanine, phosphocholine, glucose, and glutathione, and decreased concentration of tyrosine, phenylalanine, taurine, and hypoxanthine. Conversely, the gills of fish from LAR showed significantly increased concentrations of alanine, creatine, lactate, uracil, and choline, together with significantly reduced levels of taurine, glycerophosphocholine, and glutathione. The relative changes in liver and gill metabolite concentrations between LAR and SJ golden grey mullets are reported in Table 6.2, together with their significance.

Metabolites		
Amino acids	**GILLS**	**LIVER**
Isoleucine	34% ↑	15% ↓
Leucine	27% ↑	21% ↓
Valine	21% ↑	17% ↓
Isobutyrate	42% ↑	Not found
Alanine	24% ↑[a]	37% ↑[a]
Glutamate	22% ↓	24% ↑
Creatine	43% ↑[a]	-
Glycine	26% ↓	-
Serine	18% ↓	
Tyrosine	-	28% ↓[b]
Phenylalanine	-	25% ↓[b]
Energy metabolites		
Lactate	38% ↑[a]	44% ↓
Fumarate	35% ↑	-
Succinate	Not found	12% ↑
Glucose	-	52% ↑[c]
Glycogen	Not found	-
Osmolytes		
Taurine	52% ↓[a]	38% ↓[b]
Glycerophosphocholine	32% ↓[a]	23% ↑
Nucleosides and derivatives		
Inosine	18% ↓	16% ↓
Uracil	25% ↑[a]	32% ↓
Hypoxanthine	-	45% ↓[c]
Miscellaneous metabolites		
Glutathione	38% ↓[a]	56% ↑[b]
Choline	48% ↑[a]	Not found
Phosphocholine	19% ↓	93% ↑[a]

Table 6.2. Relative changes in gill and liver metabolite concentrations between LAR and SJ golden grey mullets ($p < 0.05^a$; $p < 0.01^b$; $p < 0.005^c$; Student's *t* test). (- indicates no changes in metabolite level).

6.3. Discussion

The environmental characterization of water column and surface sediments reported in Chapter III of this thesis confirmed that LAR is a highly Hg contaminated area in comparison with SJ. Accordingly, both liver and gills of fish from LAR showed higher levels of tHg and MeHg than those from SJ, clearly evidencing an Hg exposure. However, it is important to highlight that an organ-specific bioaccumulation of Hg was detected in fish liver and gills, attributable to the different structural and functional properties of the two organs. Noteworthy, accumulation of tHg and MeHg were respectively 10-fold and 17-fold higher in concentration in mullet liver than in gills. Therefore, liver showed to play a crucial role in storage and detoxifiaction of Hg, being the organ that accumulated the highest amounts of Hg in respect to gills. Liver demonstrated to be a good indicator regarding Hg accumulation, and this finding reinforces the important function of liver on protecting the other tissues and organs in relation to Hg accumulation, as previously documented by Mieiro et al. (2014). In accordance, the high Hg burden in liver is indicative of the high susceptibility of liver to Hg toxicity.

As previously recognized, a similar perturbation in the environment can induce different responses in an organisms depending on the organ evaluated (Mieiro et al., 2011). Accordingly, the occurrence of high concentrations of Hg detected in both liver and gills of *L. aurata* from LAR in respect to samples from the unpolluted SJ site, which were in line with the environmental contamination levels of water and sediments, are the basis of differential toxicity of Hg in the two organs investigated, as demonstrated by the differential interferences of Hg with several metabolic pathways. Indeed, a complete picture of Hg toxicity in fish liver and gills was obtained by the application of the environmental metabolomics approach. In fact, a considerable amount of metabolites from different categories, including amino acids, osmolytes, carbohydrates and nucleotides were found to display different

levels in mullet liver and gills, allowing to uncover tissue-specific effects of Hg at different biochemical levels. In detail, Hg accumulation provoked severe disturbances in ion-osmoregulatory processes, as well as disorders in energy-producing metabolic pathways and protein metabolism. In addition, alteration in membrane stabilization processes and perturbation of antioxidant defence system were also pointed out by the metabolomics approach, with differential mechanisms observed in liver and gills. Therefore, this study has also the merit of identifying new potential metabolite biomarkers of Hg toxicity in fish.

6.3.1. Disturbances in osmoregulation and ionoregulation

As discussed in Chapter V, the largest change in the gill metabolic profile of mullets collected from the two sites occurred in the concentration of taurine, which decreased significantly in fish from LAR in respect to fish from SJ. Taking into account that taurine is mainly involved in the maintenance of cellular homeostasis, its reduction in gills can be associated to interferences in osmotic balance both due to differences in salinity recorded in the two areas investigated (salinity decreased in LAR) and to Hg accumulation. Further evidence of osmoregulatory disturbances in gills of *L. aurata* was provided by the concomitant depletion at LAR of glycerophosphocholine, which plays a major role as organic osmolyte (Burg and Ferraris, 2008).

It should be noted that a significant decrease in taurine level was also recorded in liver of the same mullets (Chapter IV), though liver is an internal organ mainly involved in detoxification processes and not directly challenged by the external medium. In this regard, it has been widely reported on taurine heavy metal detoxifying effects due to the electrical interaction between the taurine sulfonate ion and metal cations, resulting in a chelation product protective against metal-induced toxicity (Sears, 2013; Sinha et al., 2009).

Treatment with taurine in goldfish *C. auratus* after exposure to cadmium demonstrated that, with the increases in taurine levels, depletion in the content of cadmium was recorded in muscle, gill and bone tissues of goldfish (Choi et al., 2013). Taurine hepatoprotective effects were also observed in Hg-intoxicated rats after dietary taurine supplementation (Jagadeesan and Sankarsami Pillai, 2007). Interestingly, in Manila clam *Ruditapes philippinarum* exposed to Hg under controlled laboratory conditions, depletion in taurine levels was recorded in gills (Liu et al., 2011). Therefore, the reduction of taurine in gills could be also attributable to the metal-chelating property of taurine, as discussed above.

Moreover, in gills of *L. aurata* from LAR, an increase in creatine content was also observed (Chapter IV), and it is supportive of alteration in ion-osmoregulatory processes. It is noteworthy to point out that the hepatic creatine level in *L. aurata* from LAR was found to be unaltered with respect to fish from SJ. Taking into account that liver, along with kidney, are usually the sites of synthesis of creatine, which is then transported to the blood to be taken up by the teleost gills (Kültz and Somero, 1995), it is reasonable to hypothesize an increase in creatine production and continuous removal from liver, and successive utilization of creatine in gills to cope with changes in osmotic balance, likely related to both lower salinity in LAR and Hg accumulation.

6.3.2. Changes in energy metabolism

Impairments in energy-producing metabolic pathways were also found in gills of fish collected from the Hg-contaminated site. Specifically, the rise in the level of lactate, accompanied by the concomitant increase of fumarate, may indicate an enhancement in anaerobic metabolism as strategy to replenish insufficient energy supply, attributable to Hg contamination as well as lower

oxygen availability recorded at LAR, as described in detail in Chapter V. Contrarily to what observed in gills, a different pattern of metabolites involved in energy metabolism was affected by Hg exposure in liver of the same organisms (Chapter IV). In detail, hepatic depletion, although not statistically significant, of lactate was detected and might be ascribed to its utilization as a substrate for gluconeogenesis. This explanation was further supported by the increase in hepatic glucose along with the unaltered glycogen level recorded in the liver of mullets from LAR, as reported in Chapter IV. Similarly, up-regulation of gluconeogenesis was found in liver of mercury-treated zebrafish (Ung et al., 2010). Therefore, Hg-induced toxicity may affect differentially energy-related metabolic pathways in fish gills and liver, and this may be considered as a tissue-specific toxicological effect of Hg.

6.3.3. Interferences with protein metabolism

Changes in the pool of amino acids were also observed in mullet gills presumably in response to Hg contamination. Indeed, the concentrations of three important BCAAs, namely isoleucine, leucine, and valine, were found increased, though not significantly. Because BCAAs are essential proteinogenic amino acids, but also play a crucial role in the regulation of protein turnover process, the increased levels of BCAAs in gills might thus suggest an enhancement of protein catabolism, attributable to the Hg-induced formation of reactive oxygen species that could attack proteins provoking their damage and consequent fragmentation (Liu et al., 2015). Conversely, the liver of the same mullets from LAR displayed decreased levels (not significant) of BCAAs that may be attributable to an augmented protein synthesis, to be linked to the need to repair proteins damaged by a Hg-induced oxidative conditions or to the activation of cytoprotective mechanisms to counteract Hg

toxicity. This assertion was reinforced by the observed hepatic decrease (not significant) of uracil, which suggests an enhancement in the ongoing transcriptional activities (Chapter IV), and that instead was found significantly increased in gills. Interestingly, opposite effects of toxicity were documented for butachlor, an herbicide, which induced depletion and marked increased of BCAAs in gills and liver, respectively, of intoxicated goldfish (Xu et al., 2015). These different toxicity effects may be explained by the fact that butachlor is an organic compound and not a metalloid as Hg.

Among the changes in amino acids in response to Hg-exposure, it must be pointed out the significant increase in alanine, recorded both in mullet gills and liver. Considering that nitrogen is mainly carried to the liver by alanine, its arise might be an adaptive response to deal with an excess of free ammonia to prevent intoxication resulting from the protein catabolism occurring in gills.

Finally, from the gill metabolome of mullets from the Hg-contaminated area, it is worth to be noted the increased level of isobutyrate, which is a marker of anoxia. Thus, its elevation in gills is attributable to the onset of a hypoxic condition (Giannetto et al., 2015), that may be a direct consequence of the lower dissolved oxygen content measured at LAR relatively to SJ, or be indirectly related to Hg toxicity. As expected, isobutyrate did not appear in the metabolome of the fish liver, more likely because of its different anatomical localization and physiological functions in respect to gills (Chapter IV).

6.3.4. Alteration in membrane stabilization processes

In gills of *L. aurata* from LAR, the significant depletion of glycerophosphocholine, besides being attributable to its involvement in the mechanisms of osmoregulation as above discussed, may be associated to occurring membrane stabilization/repair processes, a supported by the concomitant reduction in level of glycerophosphocholine and phosphocholine.

This explanation gains plausibility from the significant decrease in lipid peroxidation (LPO) level observed in gills, as reported in Chapter V. Therefore, this finding is suggestive of adaptive cell-protecting antioxidant mechanisms elicited by mullets to prevent peroxidation of membrane lipids in gills. Furthermore, the increase in choline found in gills is in compliance with the decreased levels of glycerophosphocholine and phosphocholine, which are the main storage forms for choline within the cytosol (Rohlfs et al., 1993).

Interestingly, an opposite behaviour of the metabolites involved in the membrane stabilization processes was observed in the liver of the same mullets from the Hg-contaminated area. In fact, the largest change in the liver metabolome occurred in the level of phosphocholine, significantly elevated by 93% in fish from LAR relative to those from SJ, in concomitance with the elevated (not significantly) content of glycerophosphocholine. Taken together, these data may be attributable to a breakdown of phosphatidylcholine and/or membrane turnover. However, as previously reported in Chapter IV, no increase in LPO was found in liver of the same mullets, demonstrating hepatic protective efficiency against oxidative stress induced by Hg. Therefore, it is plausible that liver cells suffered from an oxidative insult, but lipid peroxidation was prevented.

6.3.5. Perturbation in antioxidant defence system

The NMR-based metabolomics approach revealed also a significant decrease in reduced glutathione (GSH) and its constituent amino acids (i.e. glycine and glutamate) in the gills of mullets from the Hg-contaminated area. Therefore, this results in the formation of Hg-GSH complexes and in a consequent reduction of the intracellular pool of GSH molecules available (Sears, 2013). Taking into account the increase in GSHt content explaining a

massive GSH oxidation under Hg stress, along with the activity of GR found unaltered, the decrease in GSH constituent amino acids may suggest in gills an inability to efficiently carry out the regeneration or *de novo* synthesis of GSH, as described in Chapter V. Overall, the depletion of GSH indicates some vulnerability in mullet gills to Hg toxicity, as it enhances the risk of oxidative stress due to the accumulation of ROS.

Contrarily to the gills, liver of fish collected at LAR exhibited a significant elevation of GSH, concomitant with an increase (not significant) in glutamate. Since GSH has been shown to act as the first line of defence against Hg (Ung et al., 2010), the hepatic elevation in GSH content indicates an adaptive response to Hg bioaccumulation, resulting in the activation of antioxidant defence mechanisms in mullets naturally Hg-exposed. The hepatic increase in GSH observed by NMR spectroscopy was in line with the significant rise in the content of GSHt measured by conventional biochemical assays (Chapter IV) and, as a consequence of the unaltered hepatic GR activity, the increased GSH was attributed to its *de novo* synthesis. In fact, it is documented that liver is the main organ for GSH synthesis, from which is then exported to other tissues (Giannetto et al., 2014; Monteiro et al., 2010). Overall, the differential antioxidant defence responses triggered by Hg in mullet gills and liver, may be explained taking into account that liver is the major site of storage and detoxification of toxicants, and the organ in which higher Hg levels were recorded in respect to the gills.

6.4. Conclusions

The ^{1}H NMR-based metabolomics approach enabled an integrated description of the Hg-induced metabolic responses in liver and gills of the golden grey mullet *L. aurata*. Moreover, metabolomics also allowed the

discernment of tissue-specific toxicological mechanisms of Hg, attributable to differential structural properties and physiological functions of the two organs under examination.

Specifically, it was demonstrated that Hg accumulation in fish tissues provoked severe disturbances in ion-osmoregulatory processes, mainly in gills, as highlighted by the depletion of osmolytes, namely taurine and glycerophosphocholine. However, the decrease in taurine level recorded in liver was associated to the metal-chelating property of taurine, thus resulting in the occurrence of hepatic detoxification processes. Differential disorders in energy-producing metabolic pathways were found between gills and liver, resulting in an enhancement of anaerobic metabolism as strategy to replenish insufficient energy supply in gills and promotion of gluconeogenesis in liver. Interferences with protein metabolism were also detected in both organs, depicting an ongoing protein catabolism in gills and, conversely, augmented protein synthesis in liver for the repair of Hg-damaged proteins or activation of cytoprotective mechanisms to counteract Hg toxicity. Additionally, differential alteration in membrane stabilization/repair processes and perturbation of antioxidant defence system were also pointed out between mullet gills and liver by the metabolomics approach.

Therefore, this study provides evidence that environmental metabolomics can be successfully applied for direct evaluation of biological effects of a given contaminant in fish under field conditions. Overall, the environmental metabolomics approach demonstrated its effectiveness in the elucidation of the mechanisms of toxicity of Hg in wild fish, and allowed to discern tissue-specific toxicological effects of Hg in fish gills and liver, attributable to their differential physiological functions, and potentially observable in humans.

CHAPTER VII

Conclusions

7. Conclusions

Findings from this thesis demonstrated that metabolomics is a sensitive and effective tool for unravelling subtle alterations in various biological pathways and for discovery of novel metabolite biomarkers, thus suitable to be applied in mechanistic research. Indeed environmental metabolomics, in conjunction with conventional and well-established oxidative stress biomarkers, provided a comprehensive assessment of the organismal health status, allowing to elucidate the mechanisms of toxicity of Hg in wild fish, and to discern tissue-specific toxicological effects of Hg in fish liver and gills, attributable to their differential physiological functions, and potentially observable in humans.

Indeed, data reported in this thesis revealed that environmental exposure to Hg induces marked changes both in the metabolic profile and antioxidant responses in the liver, as well as in gills, of golden grey mullet *L. aurata*, pointing out a compromised health status of fish.

In particular, the metabolomics analysis applied to mullet liver displayed a clear differentiation between the two sites with different contamination levels, revealing that accumulation of Hg at hepatic level has multiple levels of impact, interfering with membrane stabilization/degradation/repair processes, osmoregulation, energy metabolism, gene expression and antioxidant protection. Moreover, the research on oxidative stress biomarkers in liver revealed that Hg triggers adaptive responses of antioxidant system depicted in the increase of GST and CAT activities as well as GSHt content, compensating GPx activity depletion. The GPx inhibition revealed some vulnerability and provides evidences of the role of selenohydryl groups as a primary target of Hg. Therefore, the combined approach of metabolomics and oxidative stress endpoints allowed a better understanding of Hg toxicity, namely the identification of GSH as a first line of defence against Hg and evidences of oxidative insults in cell membranes recognized through the increase of phosphatidylcholine degradation products, namely phosphocholine and

glycerophosphocholine. Nonetheless, the induction of lipid peroxidation in mullet liver was efficiently prevented.

The ^{1}H NMR-based metabolomics approach applied on mullet gills enabled an integrated description of a number of metabolites involved in different metabolic pathways. In regard to metabolic responses to Hg, in gills of fish from the Hg-contaminated area interferences with the ion-osmoregulatory processes were revealed, seen as decreases in taurine and glycerophosphocholine, along with increased creatine level. Impairments in energy-producing metabolic pathways were also observed, as well as the occurrence of protein catabolism supported by the increased levels in amino acids and alanine, the latter mainly involved in nitrogenous waste excretion. Moreover, some vulnerability in the respiratory gas exchange activity of mullets from the Hg-polluted area was suggested by augmentation of isobutyrate, a recognized biomarker of anoxia. The combination of the metabolomics and pro-oxidant status evaluation indicated the occurrence of massive GSH oxidation under Hg stress, and an inability to carry out its regeneration (GR activity was unaltered) or *de novo* synthesis (depletion in GSH constituent amino acids, glycine and glutamate). The prevention of lipid peroxidative damage may be associated with the enhancement of membrane stabilization/repair processes, resulting from depletion in precursors of phosphatidylcholine (phosphocholine and glycerophosphocholine). Though the exposure to Hg in gills led to a state of vulnerability due to limitations of antioxidant system towards a physiological accommodation, the membranes were able to answer to oxidative insult through the enhancement of stabilization/repair processes.

Finally, this thesis has also the merit to provide a detailed comparative analysis of the differential bioaccumulation of Hg and metabolic responses elicited in fish liver and gills, with the aim to achieve a more comprehensive assessment of the mechanisms of Hg toxicity in fish. Indeed, the ^{1}H NMR-based metabolomics approach herein applied, has allowed to discern tissue-

specific toxicological mechanisms of Hg, attributable to differential structural properties and physiological functions of the two organs under examination.

Specifically, it was demonstrated that Hg accumulation in fish tissues provoked severe disturbances in ion-osmoregulatory processes, mainly in gills, as highlighted by the depletion of osmolytes, namely taurine and glycerophosphocholine. However, the decrease in taurine level recorded in liver was associated to the metal-chelating property of taurine, thus resulting in the occurrence of hepatic detoxification processes. Differential disorders in energy-producing metabolic pathways were found between gills and liver, resulting in an enhancement of anaerobic metabolism as strategy to replenish insufficient energy supply in gills and promotion of gluconeogenesis in liver. Interferences with protein metabolism were also detected in both organs, depicting an ongoing protein catabolism in gills and, conversely, augmented protein synthesis in liver for the repair of Hg-damaged proteins or activation of cytoprotective mechanisms to counteract Hg toxicity. Additionally, differential alteration in membrane stabilization/repair processes and perturbation of antioxidant defence system were also pointed out between mullet gills and liver by the metabolomics approach.

Therefore, the novel concurrent use of metabolomics and conventional oxidative stress endpoints, coupled with the Hg tissue burdens characterization, demonstrated to be a sensitive and effective tool towards a mechanistically based assessment of Hg toxicity, providing novel insights into the mechanisms underlying the oxidative damage induced by Hg. Overall, the environmental metabolomics approach demonstrated its effectiveness in the elucidation of the mechanisms of toxicity of Hg in wild fish, and allowed to discern tissue-specific toxicological effects of Hg in fish gills and liver, attributable to their differential physiological functions, and potentially observable in humans.

CHAPTER VIII

References

8. References

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CHAPTER IX

Appendix

9.1. Curriculum Vitae of Dr. Tiziana Cappello

Dept. of Chemical, Biological, Pharmaceutical and Environmental Sciences
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Education

2014 - to date	**PhD-student** in Applied Biology and Experimental Medicine, Curriculum Experimental Medicine, XXIX cycle (first classified with the highest score; without bursary), University of Messina, IT. PhD Thesis entitled "^{1}H NMR-based metabolomics: a powerful tool to unravel the mechanisms of toxicity of mercury". Supervisor: Prof. Maria Maisano, University of Messina, IT; Co-supervisor: Dr. Patrícia Pereira, University of Aveiro and CESAM, PT.
2010 - 2013	**PhD** in Biology and Cellular Biotechnologies, XXV cycle (first classified with the highest score, with bursary), University of Messina, IT. Mention of ***Doctor Europaeus***. PhD Thesis entitled "Metabolome changes in mussel (*Mytilus* spp.) from natural environments. Discovery of novel biomarkers for environmental monitoring". Supervisor: Prof. Salvatore Fasulo, University of Messina, IT; Co-supervisor: Prof. Mark Viant, University of Birmingham, UK.
2006 - 2008	**Master's Degree** in Biology and Ecology of the Coastal Marine Environment, with the voting of **110/110** ***cum laude***. University of Messina, IT. M.Sc. Thesis entitled "Metallothioneins in tissues of aquatic organisms". Supervisor: Prof. Salvatore Fasulo, University of Messina, IT.

2003 - 2006 **Bachelor's Degree** in Biology and Marine Ecology, with the voting of **110/110 *cum laude*.** University of Messina, IT. B.Sc. Thesis entitled "Spatial structure and biodiversity of marine bacterial communities in an area of the Western Mediterranean Sea (Strait of Gibraltar – Sardinia Channel)". Supervisor: Prof. Mariolina De Francesco, University of Messina, IT.

Research Interests

Dr. Cappello's dissertation research is mainly focused on **ecocytotoxicology** and **biomonitoring** of the natural aquatic environments, by using both invertebrates and lower vertebrates as bioindicators. The target organs primarily investigated to assessing the organisms' recovery capacities and the strategies elicited against the various environmental stresses are the gills, mainly involved in nutrient uptake, gas exchange and neurotransmission, and first organ to suffer from pollutant toxic effects due to their surface at direct and continuous contact with the external medium, and the digestive gland (in invertebrates) and liver (in lower vertebrates) as the major sites for xenobiotic bioaccumulation and detoxification processes. The **biomarkers** applied in order to assess the effects of standard (i.e. metals, hydrocarbons) and emerging (i.e. nanoparticles, pharmaceuticals, microplastics) environmental contaminants on aquatic biota are both of **histological** type, through morphological, histochemical and immunohistochemical investigations; **molecular** type, as RNA extraction, RT-PCR, cloning, sequencing and synthesis of oligonucleidic probes for fluorescent *in situ* hybridation (FISH) technique on histological sections; and **metabolomics** type, by applying proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, as well as associated bioinformatics approaches and chemometrics. Environmental metabolomics, based on the simultaneous identification of low molecular weight metabolites (*metabolite biomarkers*) involved in various metabolic pathways, is a cutting edge approach to assessing the health of organisms and discover novel biomarkers, since it allows to detect changes in the organismal metabolome profile in response to environmental stressors, diseases or exposure to toxicants, thus providing an overview of the metabolic status of a biological system.

Professional Experiences

2015 **Visiting PhD-student** for a six-month formative stage at the **IAMC-CNR** of **Capo Granitola, Campobello di Mazara, IT**, within the second PhD. Research project on "Evaluation of metabolic changes induced by anthropogenic acoustic pollution on aquatic invertebrates and lower vertebrates by environmental

metabolomics and biochemical/genetics tools", supervised by Dr. Francesco Filiciotto, IAMC-CNR of Capo Granitola, IT.

2013 **One-year Post-Doctoral Research Fellowship** funded by the **IAMC-CNR** of **Messina, IT**. Research activity on "Research experience on monitoring by the use of bioindicators (vertebrates and invertebrates), with particular focus on novel biomarkers, such as molecular and -omics, and application of Nuclear Magnetic Resonance (NMR)-based environmental metabolomics". Supervisor: Dr. Simone Cappello, IAMC-CNR of Messina, IT; Co-supervisor: Prof. Salvatore Fasulo, University of Messina, IT.

2012 **Visiting PhD-student** for a three-month formative stage at the School of Biosciences of the **University of Birmingham, UK**, within the first PhD, for advanced training in Environmental Metabolomics. Research project on "Seasonal variation of metabolism in wild mussel *Mytilus edulis* populations over an annual cycle", supervised by Prof. Mark Viant, University of Birmingham, UK.

2009 **Visiting Student** for a six-month formative stage at the Department of Biology of the **Virginia Commonwealth University (VCU, Richmond, USA)**, supported by a Bonino-Pulejo Fellowship. Research project on "The employment of novel bioindicators and biomarkers to assess the environmental health status", supervised by Prof. Leonard Smock and Dr. Rima Franklin, Virginia Commonwealth University, USA

2008 **Formative stage** at the Laboratory of Biomonitoring and Ecocytotoxicology of the University of Messina (Messina, IT), within the **M.Sc.** Laboratory experience supervised by Prof. Salvatore Fasulo, University of Messina, IT.

2006 **Formative stage** at the Laboratory of Ecology of the Marine Bacterial Communities of the University of Messina (Messina, IT), within the **B.Sc.** Laboratory experience supervised by Prof. Mariolina De Francesco, University of Messina, IT.

2005 **Formative experience** on board of the N/O Urania, for the oceanographic campaign organized by CIESM (Project SUB II).

2005 **Formative experience** within a project on the demographic rate of marine benthonic species (*Aristeus antennatus*; *Aristaemorpha fogliacea*) in an area of the Central Mediterranean Sea.

Workshops and Practical Courses

2016 Participation, after selection, to the **2nd Metabolomics Sardinian Scientific School** on "How to bridge metabolomics and genomics", organized by the **University of Cagliari**, held in a Pula, Cagliari (**Italy**), 12 - 16 September

2014 Participation to the **International Workshop** on "Plastics: resource or environmental risk?", at the Department of Sciences and Technological Innovation (DISIT) at the **University of the Eastern Piedmont**, Alessandria (**Italy**), 10 February, sponsored by **SETAC Italian Branch**

2012 Participation, after selection, to the **EMBO Practical Course** on "Mass Spectrometry and Proteomics", at the Department of Biochemistry and Molecular Biology of the **University of Southern Denmark**, Odense (**Denmark**), 18 - 25 April

2011 Participation, after selection, to the **2th PRIMO's Next – "Advanced Students Workshop** on Fundamentals of Science, Environment and Health", organized by the Biophysics Institute of the **Rio de Janeiro Federal University,** held in Bùzios, Rio de Janeiro (**Brazil**), 20 - 30 October, sponsored by **PRIMO**

2011 Participation to the **International Workshop** on "The Endocrine Disruptors: what scientists, physicians, politicians and people should know about what's going on in the environment", held in Montalbano Elicona, Messina (**Italy**), 7 - 9 September, sponsored by **The Endocrine Society**, **Society for Reproduction and Fertility** (UK), **European Medical Association** (B)

Didactic activities

2016 **Teaching activity** within the Doctoral School in Agronomy and Environment at the **University of Sousse**, Tunisia, to training PhD-students on "Metabolomics analysis of biological extracts"

as well as providing Lectures on "Metabolomics evaluation of the effects of xenobotics on marine organisms"

2016 **Teaching activity** within the Protocol of Agreement between the High School "Leonardo da Vinci" in Reggio Calabria and the **University of Messina**, with specific didactic activities on the enhancement of knowledge in **Biology** for the access to University courses for high school students, with a task of n° 21 hours.

2016 **Informative tutoring activity** at the Struttura Interdipartimentale di Raccordo (SIR), Faculty of Sciences and Technologies, **University of Messina,** with Front-Office activities for university students, with a task of n° 54 hours.

2016 **Specialized didactic tutoring activity** within the project for the "Enhancement of knowledge to access the University courses" in **Biology** for high school students, at the Struttura Interdipartimentale di Raccordo (SIR), Faculty of Sciences and Technologies, **University of Messina,** with a task of n° 120 hours.

2016 **Teaching activity** within the **Summer School** organized by the Centro Orientamento e Placement (COP) of the **University of Messina**, aimed at the enhancemnet of **Biology** for high school students by classes, lectures, didactic laboratories, and elaboration and evaluation (individual and collective) of simulated tests for access to medical-scientific University courses.

2015 **Teaching activity** within the **Basic Course of Gemmology**, organized by Centro Analisi Gemmologiche, affiliated to the Department of Biological and Environmental Sciences of the **University of Messina,** for the modules on organic materials: Pearls and Corals. In both modules, the potential effects of environmental pollution on the normal biological activities of aquatic organisms were addressed.

2014 - to date **Member of the Evaluation Committee** for the discipline "Developmental Biology" (S.S.D. BIO/06) as **Connoisseur of the subject.**

2010 - 2011 **Tutoring activity** for undergraduates of the B.Sc. in Biological Sciences, with a task of n° 20 hours.

2010 - 2011 **Laboratory tutoring activity** for undergraduates attending the courses of "Fundamental of Cell Biology" and "Cytochemistry and Histochemistry" of the M.Sc. in Biology, with a task of n° 90 hours.

2011 - 2014 **Member of the Evaluation Committee** for the discipline "Fundamentals of Cell Biology" (S.S.D. BIO/06) as **Connoisseur of the subject.**

P.I. in Research Projects

2009 "The employment of novel bioindicators and biomarkers to assess the environmental health status". Uberto Bonino e Maria Sofia Pulejo Foundation **(FBP) - XXXI Fellowship 2008. Principal Investigator:** Dott.ssa Tiziana Cappello, University of Messina, IT. **Tutor:** Prof. Leonard Smock, Virginia Commonwealth University (USA)

Participation to Research Projects

To be submitted "Induction and reversibility of sensory systems disruption and behaviour syndromes in fish upon mercury exposure – SENSEMER". Fundação para a Ciência e a Tecnologia **(FCT) - Investigator Programme**. **Scientific Coordinator**: Dr. Patricia Pereira, University of Aveiro (PT)

2013 - 2016 "Systems Biology in the study of xenobiotic effects on marine organisms for evaluation of the environmental health status: biotechnological applications for potential recovery". Funded by **MIUR, PRIN 2010-2011** (2010ARBLT7_001/008). **Scientific Coordinator**: Prof. Salvatore Fasulo, University of Messina, IT. **Responsibles of Unit**: Prof. Claudio Leonzio, University of Siena, IT; Prof. Marco Colasanti, Univeristy of Roma 3, IT; Prof. Trifone Schettino, University of Salento, IT; Prof. Rosalba Gornati, University of Insubria, IT; Prof. Giulia Guerriero, University of Naples, IT; Dr. Giuseppe Mancini, University of Catania, IT; Dr. Cappello Simone, IAMC-CNR of Messina, IT.

2013 - 2015 "Neurotoxicity of mercury in fish and association with morphofunctional brain alterations and behavior shifts – NEUTOXMER". Funded by Fundação para a Ciência e a Tecnologia **(FCT)**. **Scientific Coordinator**: Prof. Mário Pacheco, University of Aveiro (PT)

2012 - 2013 "Characterization of the Daphnid metabolome to discover novel biomarkers for environmental monitoring." Funded by **Bio-NMR** (BIO-NMR-00141). **Scientific Coordinator:** Prof. Salvatore Fasulo, University of Messina (IT). **Scientific Responsible:** Prof. Mark Viant, University of Birmingham (UK)

2009 - 2011 "Biomonitoring of coastal marine environments: development and application of novel integrated cytochemical and molecular methodologies". Funded by **MIUR, PRIN 2007** (prot. 20079FELYB). **Scientific Coordinator**: Prof. Salvatore Fasulo, University of Messina (IT). **Responsibles of Unit**: Prof. Laura Canesi, Università di Genova (IT); Prof. Claudio Leonzio, Università di Siena (IT)

2013 - not funded "Transcriptomic Response to Polychlorinated Biphenyls in Mussel, *Mytilus edulis*". **Scientific Coordinator**: Prof. John Stegeman, Woods Hole Oceanographic Institution (MA, USA). **Participants:** Dr. Afonso Bainy, Federal University of Santa Catarina (Brazil); Prof. Maria Bebianno, University of Algarve (Portugal); Prof. Miren Cajaraville, University of the Basque Country (Spain); Dr. Ibon Cancio, University of the Basque Country (Spain); **Dr. Tiziana Cappello**, University of Messina (Italy); Prof. Kevin Chipman, University of Birmingham (United Kingdom); Dr. John Craft, Glasgow Caledonian University (United Kingdom); Dr. Francesco Dondero, University of the Piedmont (Italy); Prof. Salvatore Fasulo, University of Messina (Italy); Prof. Hisato Iwata, Ehime University (Japan); Dr. Matthew Jenny, University of Alabama (AL, USA); Prof. Mauro Rebelo, Federal University of Rio de Janeiro (Brazil); Dr. David Sheehan, University College, Cork (Ireland); Dr. Shinsuke Tanabe, Ehime University (Japan); Dr. Juliano Zanette, University of the Rio Grande (Brazil); Dr. Jed Goldstone, Woods Hole Center for Oceans and Human Health (MA, USA).

On-going scientific collaborations

Dr. Tiziana Cappello has devoting considerable amount of time to disseminate worldwide the results of her researches through participation to national and international scientific meetings, which are for her great opportunities to introduce herself to the scientific communities**,** as well as actively undertake, thanks to her friendly personality and dedication to research, new collaborations with researchers worldwide.

2015 - to date	**Prof. Jérôme Cachot** - EPOC, UMR CNRS, University of Bordeaux, **FR** "Use of environmental metabolomics approaches to detect alteration in Japanese medaka larvae after exposure to imidacloprid, a insecticide neonicotinoid".
2015 - to date	**Prof. Cinta Porte** - IDAEA-CSIC, Barcelona, **ES** "Metabolic effects induced by drospirenone on livers of sea bass *Dicentrarchus labrax* and digestive glands of mussel *Mytilus galloprovincialis*".
2015 - ad oggi	**Dott. Francesco Filiciotto** - IAMC-CNR Capo Granitola, **IT** "Application of environmental metabolomics and biochemical/genetics tools for evaluation of metabolic changes induced by anthropogenic acoustic pollution on aquatic invertebrates and lower vertebrates".
2013 - ad oggi	**Prof. Maria João Bebianno** - University of Algarve, **PT** "Metabolomic and proteomic responses to active pharmaceutical ingredients, i.e. ibuprofen, fluoxetine, diclofenac, and their mixture, on mussel *Mytilus galloprovincialis*".
2013 – to date	**Prof. Mário Pacheco; Dr. Patricia Pereira** - University of Aveiro, **PT** "Correlation of mercury bioaccumulation, oxidative stress and metabolomics responses in wild golden grey mullet *Liza aurata*".
2012 – to date	**Prof. Mark Viant** - University of Birmingham, **UK** "Seasonal variation of metabolism in wild mussel *Mytilus edulis* populations over an annual cycle using NMR-based metabolomics".

Academic Awards and Distinctions

2016	**Highly Cited Research Award** for the article "**Cappello T.**, Maisano M., D'Agata A., Natalotto A., Mauceri A., Fasulo S., 2013. Effects of environmental pollution in caged mussels (*Mytilus galloprovincialis*). *Marine Environmental Research,* 91: 52-60"
2016	**Qualification** for the position of **Researcher**, S.S.D. BIO/06 - University of Messina, IT
2016	Selection (25 vacancies for PhD students) and participation to the **2nd Metabolomics Sardinian Scientific School** – "How to bridge metabolomics and genomics", organized by the University of Cagliari, held in Pula, Cagliari (**Italy**), from 12th to 16th September
2016	Brandão F., **Cappello T.*,[1]**, Raimundo J., Santos M.A., Maisano M., Mauceri A., Pacheco M., Pereira P., 2015. Unravelling mechanisms of mercury hepatotoxicity in wild fish (*Liza aurata*) through a triad approach: bioaccumulation, metabolomic profiles, and oxidative stress. *Metallomics*, 7: 1352-1363 added in ***Metallomics*** **Metals in Marine Biochemistry online collection** <http://rsc.li/29KfqlX>
2015	**Conference Student Travel Award** at the 18th International Symposium on Pollutant Responses in Marine Organisms (**PRIMO**)
2015	**Conference Registration and Travel Grant** at the 25th SETAC Europe Annual Meeting (**SETAC**)
2015	Classified among the **first top 10 candidates** for the fellowship "**L'Oréal Italia** – per le donne e la Scienza", 2014-2015
2014 - 2017	**Connoisseur of the subject** "Developmental Biology" (S.S.D. BIO/06), as acknowledged by the Council of Professors at the University of Messina, Faculty of Mathematical, Physical and Natural Sciences
2014	**Conference Travel Grant** at the 24th Congress of the Italian Society of Ecology (**S.It.E.**)
2013	Mention of ***Doctor Europaeus*** (PhD European Label), awarded with the title of *Philosophiae Doctor* (**PhD**)

2012	Selected for the **Erasmus Student Mobility** 2012/2013 to carry out research at the University of Algarve, Faro (Portugal)
2012	Selection (16 vacancies for PhD students) and participation to the **EMBO Practical Course** – "Mass Spectrometry and Proteomics" at the University of Southern Denmark, Odense (**Denmark**), from 18th to 25th April
2011	**Connoisseur of the subject** "Fundamentals of Cell Biology" (S.S.D. BIO/06), as acknowledged by the Council of Professors at the University of Messina, Faculty of Mathematical, Physical and Natural Sciences
2011	Selection (15 vacancies for PhD students) and participation to the 2nd **PRIMO's Next** – "Advanced Students Workshop on Fundamentals of Science, Environment and Health" organized by the Biophysics Institute of the Rio de Janeiro Federal University and the PRIMO conference, held in Bùzios, Rio de Janeiro (**Brazil**) from 20th to 30th October
2011	**Qualification** to exercise the profession of **Biologist** - University of Messina, IT
2009	**Bonino-Pulejo Fellowship**, awarded in occasion of the XXXI Grant Announcement of the Bonino-Pulejo Foundation, for a six-month formative stage abroad, spent at the Virginia Commonwealth University (**VCU; Richmond, USA**)

Society Memberships

2015 - to date	Society of Environmental Toxicology and Chemistry (**SETAC**) and **SETAC Italian Branch**
2014 - to date	Società Italiana di Ecologia (**S.It.E.**)
2010 - to date	Unione Zoologica Italiana (**U.Z.I.**)

Reviewer Activity

2016 - to date	Reviews in Aquaculture (**RAQ**; IF = 4.769)
2016 - to date	Ecotoxicology and Environmental Safety (**EES**; IF = 3.130)
2016 - to date	Comparative Biochemistry and Physiology – Part D (**CBP-D**; IF = 2.254)
2016 - to date	PeerJ – Life, Bio, & Health Sciences (**PeerJ**; IF = 2.180)
2016 - to date	Archives of Environmental Contamination and Toxicology (**AECT**; IF = 2.039)
2016 - to date	Continental Shelf Research (**CSR**; IF = 2.011)
2016 - to date	Aquaculture Research (**ARE**; IF = 1.606)
2016 - to date	Fish Physiology and Biochemistry (**FISH**; IF = 1.442)
2016 - to date	International Journal of Environmental Analytical Chemistry (**GEAC**; IF = 1.411)
2016 - to date	Chinese Journal of Natural Medicines (**CJNM**; IF = 1.114)
2015 - to date	Science of the Total Environment (**STOTEN;** IF = 4.099)
2015 - to date	Environmental Science and Pollution Research (**ESPR**; IF = 2.828)
2015 - to date	Comparative Biochemistry and Physiology – Part C (**CBP-C**; IF = 2.301)

Editorial Board Member

2016 - to date	**Lead Guest Editor** for the Special Issue on "Toxic Effects of Metals and Metalloids in Aquatic Organisms" in BioMed Research International (IF = 2.134) – Hindawi
2016 - to date	**Editorial Board Member** of International Journal of Aquaculture and Fishery Sciences (**IJAFS**) – Peertechz

2016 - to date	**Editorial Board Member** of SM Journal of Environmental Toxicology (**SMJET**) – SMGroup
2016 - to date	**Editorial Board Member** of Fishery and Aquaculture Journal (**FAJ**) – OMICS International
2016 - to date	**Editorial Board Member** of Journal of Aquatic Pollution and Toxicology (**IPJAPT**) – iMedPub
2015 - to date	**Review Board Member** of Modern Environmental Science and Engineering (**MESE**) – Academic Star
2015 - to date	**Review Board Member** of Applied Ecology and Environmental Sciences (**AEES**) – Science and Education

Complementary Qualifications

Computer Skills	Good knowledge of Windows and MacOS X operating systems; MS Office (Word, Excel, PowerPoint, Entourage), iWork (Pages, Numbers, Keynote), Adobe Acrobat, Adobe Photoshop; Web Browsers (Internet Explorer, Safari, Firefox, Google Chrome, Opera) Knowledge of the Axio Vision Release 4.5 software, furnished with the motorized microscope Zeiss Axio Imager Z1; GelDoc software, for analysis of band intensity; GraphPad Instat and Prism softwares for statistical analysis Good knowledge of Chenomx NMR Suite, for characterization of metabolomic profiles; MatLab; ProMetab 3.3 software in MatLab; The Unscrambler X for statistical multivariate analysis; Origin 6.1 software
Technical Skills	Histochemical investigation (i.e. H/E, AB/PAS, Masson, Mallory); Immunohistochemical analysis; Molecular analyses (RNA extraction, PCR, sequencing, probes for FISH); Spectrophotometric and spectrofluorimetric analyses for enzymatic activities; Genotoxic analyses (Micronuclei and Comet Assays); Metabolomic analysis (metabolite extraction, NMR spectrum acquisition, identification and quantification of metabolites); uni- and multi-variate statistical analysis (Student's *t*-test, Mann-Whitney U, ANOVA, PCA)
Languages	Fluent speaker of Italian (native language) and English

Courses	- Core Laboratory Safety Training Course, completed with the score of 99.9 out of 99.9. Virginia Commonwealth University (VCU), Richmond, USA (2009); - Physical Hazards Course, completed with the score of 100 out of 100. Virginia Commonwealth University (VCU), Richmond, USA (2009); - Carcinogen Course, completed with the score of 90 out of 100. Virginia Commonwealth University (VCU), Richmond, USA (2009); - Course for the acquisition of safety regulation in scientific laboratory. University of Messina, IT (2004); - EJO English Language Course, level Advanced. The Eagle House, Greenwich, England (2004); - English Trinity, Grade 7 - Spoken English for Speakers of Other Languages (2002)
Other	- "Open Water Diver" patent, SSI Level I; - Since the 2007/08 academic year, Dr. Cappello has been collaborating with Professors of Cytology, Histology, Cell Biology and Cellular Biotechnologies in conducting practical lab classes and tutoring students from B.Sc. in Biological Sciences, in Biology and Marine Ecology and in Natural Sciences, and students from M.Sc. in Biology and in Biology and Ecology of the Coastal Marine Environment.

9.2. Scientific Production

To date, Dr. Cappello has co-authored **19 articles** in international peer-reviewed journals (**9 as first author** or **joint co-author[1],** and **3 as corresponding author***), other 2 are currently under submission (**2 as first author[1]** and **2 as corresponding author***), 2 *proceedings* and 1 Editor Note.

Moreover, to date, Dr. Cappello has co-authored **12 communications/posters in International Conferences** (5 as oral communications and 3 as poster **delivered by herself***), and **11 communications/posters in National Conferences** (4 as oral communications and 1 as poster **delivered by herself***).

H-index: 10 in S; 10 in WoS; 10 in RG; 11 in GS

Tot. cit.: 261 in S; 240 in WoS; 295 in RG; 345 in GS

(Scopus: S; Web of Science: WoS; ResearchGate: RG; Google Scholar: GS)

Peer-reviewed manuscripts

21. **Cappello T.***, Maisano M., Mauceri A., Fasulo S. ^{1}H NMR-based metabolomics investigation on the effects of petrochemical contamination in posterior adductor muscles of caged mussel *Mytilus galloprovincialis***.** *Ecotoxicology and Environmental Safety,* submitted. [IF = 3.130]

20. **Cappello T.***, Fernandes D., Maisano M., Casano A., Bonastre M., Mauceri A., Fasulo S., Porte C. Sex steroids and metabolic responses in mussels *Mytilus galloprovincialis* exposed to drospirenone. *Ecotoxicology and Environmental Safety,* submitted. [IF = 3.130]

19. **Cappello T.**, Vitale V., Oliva S., Villari V., Mauceri A., Fasulo S., Maisano M.. Alteration of neurotransmission and skeletogenesis in sea urchin *Arbacia lixula* embryos exposed to copper oxide nanoparticles. *Comparative Biochemistry and Physiology – Part C,* in press. [IF = 2.546]
doi.org/10.1016/j.cbpc.2017.02.002

[cited by 0 in S, 0 in WoS, 0 in RG, 0 in GS]

18. Maisano M., **Cappello T.**, Natalotto A., Vitale V., Parrino V., Giannetto A., Oliva S., Mancini G., Cappello S., Mauceri A., Fasulo S., 2016. Effects of petrochemical contamination on caged marine mussels using a multi-biomarker approach: histological changes, neurotoxicity and hypoxic stress. *Marine Environmental Research,* in press. [IF = 2.762]
doi.org/10.1016/j.marenvres.2016.03.008

[cited by 0 in S, 0 in WoS, 3 in RG, 4 in GS]

17. **Cappello T.***, Pereira P., Maisano M., Mauceri A., Pacheco M., Fasulo S., 2016. Advances in understanding the mechanisms of mercury toxicity in wild golden grey mullet (*Liza aurata*) by ^{1}H NMR-based metabolomics. *Environmental Pollution,* 219: 139-148. [IF = 4.839]
doi.org/10.1016/j.envpol.2016.10.033

[cited by 0 in S, 0 in WoS, 0 in RG, 0 in GS]

16. Maisano M., **Cappello T.**[1], Oliva S., Natalotto A., Giannetto A., Parrino V., Battaglia P., Romeo T., Salvo A., Spanò N., Mauceri A., 2016. PCB and OCP accumulation and evidence of hepatic alteration in the Atlantic bluefin tuna, *Thunnus thynnus*, from the Mediterranean Sea. *Marine Environmental Research,* 121: 40-48. [IF = 2.762]
doi.org/10.1016/j.marenvres.2016.03.003

[cited by 1 in S, 0 in WoS, 1 in RG, 1 in GS]

15. Maisano M., Natalotto A., **Cappello T.**, Giannetto A., Oliva S., Parrino V., Sanfilippo M., and Mauceri A., 2016. Influences of environmental variables on neurotransmission, oxidative system and hypoxia signaling on two clam species from a Mediterranean coastal lagoon. *Journal of Shellfish Research,* 35(1): 41-49. [IF = 0.791]
doi.org/10.2983/035.035.0106

[cited by 1 in S, 0 in WoS, 1 in RG, 1 in GS]

14. **Cappello T.***, Brandão F., Guilherme S., Santos M.A., Maisano M., Mauceri A., Canario J., Pacheco M., Pereira P., 2016. Insights into the mechanisms underlying mercury-

induced oxidative stress in gills of wild fish *Liza aurata* combining ^{1}H NMR metabolomics and conventional biochemical assays. *Science of the Total Environment*, 548-549: 13-24. [IF = 4.099]
doi.org/10.1016/j.scitotenv.2016.01.008

[cited by 9 in S, 5 in WoS, 10 in RG, 13 in GS]

13. Giannetto A., Maisano M., **Cappello T.**, Oliva S., Parrino V., Natalotto A., De Marco G., Barberi C., Romeo O., Mauceri A., Fasulo S., 2015. Hypoxia-inducible factor α and Hif-prolyl hydroxylase characterization and gene expression in short-time air-exposed *Mytilus galloprovincialis*. *Marine Biotechnology*, 17(6): 768-781. [IF = 3.269]
doi.org/10.1007/s10126-015-9655-7

[cited by 8 in S, 6 in WoS, 6 in RG, 10 in GS]

12. Maisano M., **Cappello T.**[1], Catanese E., Vitale V., Natalotto A., Giannetto A., Barreca D., Brunelli E., Mauceri A., Fasulo S., 2015. Developmental abnormalities and neurotoxicological effects of CuO NPs on the black sea urchin *Arbacia lixula* by embryotoxicity assay. *Marine Environmental Research,* 111: 121-127. [IF = 2.762]
doi.org/10.1016/j.marenvres.2015.05.010

[cited by 3 in S, 3 in WoS, 5 in RG, 5 in GS]

11. Brandão F., **Cappello T.**[1,*], Raimundo J., Santos M.A., Maisano M., Mauceri A., Pacheco M., and Pereira P., 2015. Unravelling mechanisms of mercury hepatotoxicity in wild fish (*Liza aurata*) through a triad approach: bioaccumulation, metabolomic profiles, and oxidative stress. *Metallomics*, 7: 1352-1363. [IF = 3.585]
doi.org/10.1039/C5MT00090D

[cited by 14 in S, 12 in WoS, 16 in RG, 17 in GS]

10. **Cappello T.**, Maisano M., Giannetto A., Parrino V., Mauceri A., Fasulo S., 2015. Neurotoxicological effects on marine mussel *Mytilus galloprovincialis* caged at petrochemical contaminated areas (eastern Sicily, Italy): ^{1}H NMR and immunohistochemical assays. *Comparative Biochemistry and Physiology – Part C ,* 169: 7-15. [IF = 2.301]
doi.org/10.1016/j.cbpc.2014.12.006

[cited by 18 in S, 15 in WoS, 20 in RG, 23 in GS]

9. D'Agata A., **Cappello T.**, Maisano M., Parrino V., Giannetto A., Brundo M.V., Ferrante M. and Mauceri A., 2014. Cellular biomarkers in the mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae) from Lake Faro (Sicily, Italy). *Italian Journal of Zoology,* 81(1): 43-54. [IF = 0.791]
doi.org/10.1080/11250003.2013.878400

[cited by 13 in S, 13 in WoS, 13 in RG, 15 in GS]

8. Giannetto A., Fernandes J.M.O., Nagasawa K., Mauceri A., Maisano M., De Domenico, E., **Cappello T**., Oliva S., Fasulo S., 2014. Influence of continuous light treatment on expression of stress biomarkers in Atlantic cod. *Developmental & Comparative Immunology,* 44(1): 30-34. [IF = 2.815]
doi.org/10.1016/j.dci.2013.11.011

[cited by 11 in S, 10 in WoS, 11 in RG, 13 in GS]

7. **Cappello T.**, Mauceri A., Corsaro C., Maisano M., Parrino V., Lo Paro G., Messina G., Fasulo S., 2013. Impact of environmental pollution on caged mussels *Mytilus galloprovincialis* using NMR-based metabolomics. *Marine Pollution Bulletin,* 77(1-2): 132-139. [IF = 2.793]
doi.org/10.1016/j.marpolbul.2013.10.019

[cited by 28 in S, 25 in WoS, 33 in RG, 34 in GS]

6. **Cappello T.**, Maisano M., D'Agata A., Natalotto A., Mauceri A., Fasulo S., 2013. Effects of environmental pollution in caged mussels (*Mytilus galloprovincialis*). *Marine Environmental Research,* 91: 52-60. [IF = 2.328]
doi.org/10.1016/j.marenvres.2012.12.010

[cited by 43 in S, 36 in WoS, 42 in RG, 50 in GS]

5. De Domenico E., Mauceri A., Giordano D., Maisano M., Giannetto A., Parrino V., Natalotto A., D'Agata A., **Cappello T.**, Fasulo S., 2013. Biological responses of juvenile European sea bass *(Dicentrarchus labrax)* exposed to contaminated sediments. *Ecotoxicology and Environmental Safety,* 97: 114-123. [IF = 2.482]
doi.org/10.1016/j.ecoenv.2013.07.015

[cited by 14 in S, 14 in WoS, 16 in RG, 20 in GS]

4. Maisano M., Trapani M.R., Parrino V., Parisi M.G., **Cappello T.**, D'Agata A., Benenati G., Natalotto A., Mauceri A., and Cammarata M., 2013. Haemolytic activity and characterization of nematocyst venom from *Pelagia noctiluca* (Cnidaria, Scyphozoa). *Italian Journal of Zoology,* 80(2): 168-176. [IF = 0.865]
doi.org/10.1080/11250003.2012.758782

[cited by 7 in S, 7 in WoS, 7 in RG, 9 in GS]

3. Ciacci C., Barmo C., Gallo G., Maisano M., **Cappello T.**, D'Agata A., Leonzio C., Mauceri A., Fasulo S., Canesi L., 2012. Effects of sub-lethal, environmentally relevant concentrations of hexavalent chromium in the gills of *Mytilus galloprovincialis*. *Aquatic Toxicology* 120-121: 109-118. [IF = 3.73]
doi.org/10.1016/j.aquatox.2012.04.015

[cited by 33 in S, 31 in WoS, 35 in RG, 50 in GS]

2. Fasulo S., Iacono F., **Cappello T.**, Corsaro C., Maisano M., D'Agata A., Giannetto A., De Domenico E., Parrino V., Lo Paro G., Mauceri A., 2012. Metabolomic investigation of *Mytilus galloprovincialis* (Lamarck 1819) caged in aquatic environments. *Ecotoxicology and Environmental Safety*, 84: 139-146. [IF = 2.203]
doi.org/10.1016/j.ecoenv.2012.07.001

[cited by 42 in S, 41 in WoS, 47 in RG, 53 in GS]

1. Fasulo S., Maisano M., Sperone E., Mauceri A., Bernabò I., **Cappello T.**, D'Agata A., Tripepi S., and Brunelli E., 2012. Toxicity of Foroozan crude oil to ornate wrasse (*Thalassoma pavo*, Osteichthyes, Labridae): ultrastructure and cellular biomarkers. *Italian Journal of Zoology*, 79(2): 182-199. [IF = 0.89]
doi.org/10.1080/11250003.2011.623139

[cited by 16 in S, 15 in WoS, 19 in RG, 18 in GS]

Proceedings

2. Maisano M., Vitale V., **Cappello T**., Oliva S., Natalotto A., Fasulo S., Mauceri A., 2016. Exposure of sea urchin Arbacia lixula embryos to CuO NPs affects larval morphogenesis, neurotransmission and skeletogenesis. *European Journal of Histochemisrty* 60 *(Suppl.* 1), 7. *62nd GEI Congress 2016.* [IF = 2.421]
doi.org/10.4081/ejh.2016.2693

[cited by 0 in WoS, 0 in RG, 0 in GS]

1. Iacono F., **Cappello T.**, Corsaro C., Branca C., Maisano M., Gioffrè G., De Domenico E., Mauceri A., Fasulo S., 2010. Envionmental metabolomics and multibiomarker approaches on biomonitoring of aquatic habitats. *Comparative Biochemistry and Physiology – Part* A: *Molecular & Integrative Physiology* 157 (*Suppl.* 1), S50. *27th ESCPBnew Congress 2010.* [IF = 2.196]
doi.org/10.1016/j.cbpa.2010.06.143

[cited by 7 in WoS, 10 in RG, 9 in GS]

Editor Note

1. **Cappello T.**, 2016. Editor note. *Fisheries and Aquaculture Journal,* 7(2): e126.
doi.org/10.4172/2150-3508.1000e126

Scientific Communications in International Congresses

12. Vignet C., Lajoie K., Maisano M., Mottaz H., Clerandeau C., **Cappello T.**, Schirmer K., Cachot J. Response of zebrafish and Japanese medaka to imidacloprid: a comparative study**.** 27th Europe Annual Meeting of the Society of the Environmental Toxicology and Chemistry (SETAC Europe), submitted; Brussels (Belgium), 7-11 May 2017. (**Poster**, *delivered by VC*)

11. Maisano M., **Cappello T.***, Vitale V., Oliva S., Natalotto A., Mauceri A., Fasulo S. NMR-based metabolomics reveals CuO NP-induced interferences in larval morphogenesis, neurotransmission and skeletogenesis of sea urchin embryos**.** 30th Congress of the newEuropean Society of Comparative Biochemistry and Physiology (ESCPBnew); Barcelona (Spain), 4 - 7 September 2016. (**Oral**, *delivered by **TC***)

10. Brandão F., **Cappello T.**, Albuquerque O., Guilherme S., Santos M.A., Maisano M., Mauceri A., Pacheco M., Pereira P. Effects of mercury bioaccumulation in gills of wild fish (*Liza aurata*) assessed by 1H NMR metabolomics and oxidative stress endpoints. 10th Iberian and 7th Iberoamerican Congress on Environmental Contamination and Toxicology (CICTA), 269-270; Vila Real (Portugal), 14 - 17 July 2015. (**Poster**, *delivered by FB*)

9. Maisano M., **Cappello T.***, Oliva S., Natalotto A., Giannetto A., Battaglia P., Romeo T., Mauceri A. Evidences of changes in hepatic lipid metabolism in the atlantic bluefin tuna, *Thunnus thynnus*, from the Mediterranean Sea. 18th Pollutant Responses In Marine Organisms (PRIMO), PL-055; Trondheim (Norway), 24 - 27 May 2015. (**Oral**, *delivered by **TC***)

8. **Cappello T.*,** Pereira P., Maisano M., Raimundo J., Mauceri A., Pacheco M. 1H NMR-based metabolic profiling reveals tissue-specific responses to mercury toxicity in wild fish *Liza aurata*. 25th Europe Annual Meeting of the Society of the Environmental Toxicology and Chemistry (SETAC Europe), 143; Barcelona (Spain), 3-7 May 2015. (**Oral**, *delivered by **TC***)

7. **Cappello T.*,** Maisano M., Natalotto A., Parrino V., Giannetto A., Mauceri A., Fasulo S. Effects of petrochemical contamination on caged marine mussels using a multi-biomarker approach: histopathology, metabolomics, neurotoxicity and DNA damage. 6th Bilateral Seminar Italy-Japan (BSIJ), 28; Palermo (Italy), 19 - 20 November 2014. (**Oral**, *delivered by **TC***)

6. Maisano M., Catanese E., Vitale V., **Cappello T.***, Natalotto A., Giannetto A., Barreca D., Brunelli E., Mauceri A., Fasulo S. Effects of CuO NPs on first developmental stages of the sea urchin *Arbacia lixula*. 2nd Marine NanoEcoSafety Workshop (MANET), 25; Palermo (Italy), 17 - 18 November 2014. (**Oral**, *delivered by **TC***)

5. **Cappello T.***, Maisano M., Corsaro C., Mauceri A., Lo Paro G. and Fasulo S. Cellular responses to environmental pollution in *Mytilus galloprovincialis*. *17th Pollutant Responses In Marine Organisms (PRIMO),* 11; Faro (Portugal), 5 - 8 May 2013. (**Poster**, *delivered by **TC***)

4. **Cappello T.*** Research activities for assessing the environmental health status using ecotoxicological approaches. *EMBO* Practical Course in Mass Spectrometry and Proteomics, 19-20; Odense (Denmark), 18 - 25 April 2012. (**Poster**, *delivered by **TC***)

3. De Domenico E., D'Agata A., Maisano M., **Cappello T.**, Fasulo S., Mauceri A. Distribution of neurotransmitter in acontia of *Calliactis parasitica* (cnidaria, Anthozoa). *7th Workshop of the Hydrozoan Society (H.S.)*, 23; Porto Cesareo, Lecce (Italy), 10 - 18 September 2010. (**Poster**, *delivered by EDD*)

2. Iacono F., **Cappello T.***, Corsaro C., Branca C., Maisano M,, Gioffrè G., De Domenico E., Mauceri A., Fasulo S. Environmental metabolomics and multibiomarkers approaches on biomonitoring of aquatic habitats. *27th Congress of the newEuropean Society of Comparative Bochemistry and Physiology (ESCPBnew)*, 157 (*Suppl.* 1), S50; Alessandria (Italy), 5 - 9 September 2010. (**Poster**, *delivered by **TC***)

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Scientific Communications in National Congresses

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