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Sofia Isabel Antunes Gomes Guilherme

Indicadores químicos, bioquímicos e citogenéticos em *Liza aurata* após exposição ambiental a mercúrio

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia, realizada sob a orientação científica do Prof. Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Prof. Doutora Maria Ana Dias Monteiro Santos, Professora Catedrática do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave Mercúrio; *Liza aurata*; genotoxicidade; biotransformação, stresse oxidativo

resumo

A presença de mercúrio no ambiente aquático é, actualmente, considerada como um problema à escala global, com repercussão generalizada em todo o biota. A conhecida contaminação por mercúrio numa área particular da Ria de Aveiro (Portugal) - Largo do Laranjo - serviu como motivação para o presente estudo. Foram definidos como objectivos centrais (i) a avaliação do estado da população de *Liza aurata* (Tainha-garrento) nessa área e (ii) contribuir para um melhor conhecimento da toxicidade do mercúrio e mecanismos subjacentes em peixes, clarificando a relação entre a contaminação no ambiente e efeitos químicos, bioquímicos e citogenéticos.

Deste modo, o presente trabalho encontra-se divido em duas componentes, sendo a primeira baseada na amostragem sazonal de espécimes selvagens de *L. aurata*. Em paralelo, foi levada a cabo uma segunda componente pondo em prática uma experiência *in situ* com animais da mesma espécie engaiolados durante 3 dias no Largo do Laranjo, em três locais diferentes (LAR1, LAR2 e LAR3, por ordem crescente de proximidade à fonte de contaminação). Para avaliar a influência da distância ao sedimento, em cada local foi colocada uma gaiola próxima do fundo e outra a alguns centímetros da superfície. Os resultados obtidos no Largo do Laranjo foram comparados, em ambas as componentes, com resultados correspondentes relativos a uma área pouco contaminada - S. Jacinto.

O mercúrio total (Hg_t) foi quantificado na coluna de água e no sedimento, assim como em três diferentes tecidos alvo – sangue, fígado e músculo. Ao nível bioquímico/metabólico, foram estudadas respostas de stresse oxidativo no fígado com a análise de enzimas antioxidantes como a catalase (CAT) e a glutationa-*S*-transferase (GST), do conteúdo total de glutationa (GSH_t) e ainda da peroxidação lipídica (LPO). A interferência do mercúrio com o metabolismo hepático de biotransformação associado ao citocromo P450 (P450) foi também analisada através da actividade da etoxiresorufina-*O*-desetilase (EROD), assim como da determinação do conteúdo em P450 e da sua degradação (conteúdo em P420). Ao nível citogenético, foi avaliada a acção genotóxica do mercúrio por intermédio do teste das anomalias nucleares eritrocíticas (ANE).

Tendo em conta os objectivos gerais anteriormente referidos, foi investigada a correlação entre o nível de Hg_t no fígado e as respectivas respostas metabólicas, assim como o Hg_t no sangue e a frequência de ANE.

Os peixes selvagens capturados no Laranjo não apresentaram alterações significativas nos parâmetros antioxidantes (excluindo um aumento esporádico do conteúdo GSH_t), nem dos níveis de LPO.

Os parâmetros associados ao citocromo P450 também não evidenciaram diferenças significativas. Em paralelo, foi observado um claro aumento dos níveis hepáticos de Hg_t nos peixes desta área contaminada, o que pode ser entendido como uma evidência de que o metal se encontra acumulado numa forma inócua. Os níveis de Hg_t encontrados no músculo dos peixes provenientes do Laranjo foram significativamente elevados em todas as estações do ano, sem, contudo, exceder os limites regulamentados pela UE. Todavia, o potencial risco para a saúde humana não pode ser excluído dada a forte dependência da taxa de consumo de peixe de cada população. Este aspecto assume particular importância para o caso português, uma vez que Portugal é um dos maiores consumidores de peixe da Europa.

Na avaliação da genotoxicidade, os peixes selvagens oriundos do Laranjo, pescados no Verão e Outono, demonstraram uma elevada frequência de ANE, evidenciando também uma correlação com os altos níveis de Hg_t encontrados no sangue. Surpreendentemente, e tendo em conta os níveis determinados no sangue, no Inverno não se observou indução de ANE. Numa tentativa de explicar este facto, foi avaliada a dinâmica hematológica pela determinação da frequência de eritrócitos imaturos (EI), que sinalizou alterações (redução da eritropoiese e/ou o aumento da remoção de eritrócitos) capazes de mascarar a expressão de genotoxicidade.

No que respeita à experiência *in situ*, os peixes engaiolados no Laranjo mostraram um aumento generalizado no Hg_t hepático, sendo acompanhado pelos aumentos da actividade da CAT e de GSH_t, assim como pela inibição da actividade da EROD. As gaiolas de fundo em LAR2 e LAR3 mostraram um aumento dos níveis hepáticos de Hg_t e do conteúdo de GSH_t, respectivamente, quando comparadas com as gaiolas de superfície, sublinhando a importância da proximidade ao sedimento. Foi ainda demonstrada a relevância da absorção do mercúrio a partir da água, uma vez que a absorção via alimento foi restringida nesta componente. Analisando o efeito genotóxico do mercúrio, observou-se uma indução de ANE no local mais próximo da fonte de contaminação (LAR3). Deste modo, foi possível observar uma correlação entre o Hg_t acumulado no sangue e a genotoxicidade expressa pela frequência de ANE.

No geral, quer os peixes selvagens, quer os engaiolados, revelaram maior acumulação de Hg_t no fígado, quando comparado com os níveis encontrados no músculo e no sangue. Assim, o fígado revelou-se como o órgão com maior capacidade de bioacumulação, provavelmente devido ao seu activo papel na desintoxicação de xenobióticos. A ausência de dano peroxidativo neste órgão poderá ser atribuída a esta capacidade de desintoxicação e/ou a uma eficaz defesa antioxidante.

Globalmente, o mercúrio mostrou ser um agente genotóxico, tendo evidenciado a capacidade de inibir algumas respostas enzimáticas, não se podendo, com base nos dados obtidos, considerar um indutor de stresse oxidativo.

A estratégia adoptada, baseada na análise integrada de diferentes indicadores aplicados ao mugilídeo *L. aurata*, demonstrou a sua aplicabilidade na monitorização ambiental de contaminação por metais em águas costeiras.

keywords Mercury, *Liza aurata*; genotoxicity, biotransformation, oxidative stress

abstract

Mercury contamination of aquatic ecosystems became a worldwide environmental problem, representing a worrying threat to biota. In this context, the intense mercury contamination historically observed at Laranjo basin, in Ria de Aveiro (Portugal), was the motivation for the present study. Hence, the main goals of this research were (i) to evaluate the health status of the local *Liza aurata* (gold grey mullet) population, and (ii) to improve the knowledge about mercury toxicity and its subjacent mechanisms, clarifying the relation between the environmental contamination and chemical, biochemical and cytogenetic effects in fish.

The present investigation is divided in two components, being the first based on a wild *L. aurata* seasonal sampling at the contaminated area (Laranjo basin = LAR), and the second, an *in situ* experiment where *L. aurata* specimens were caged, during 3 days, at three different sites (LAR1, LAR2 and LAR3, increasing proximity to the mercury contamination source). To assess the influence of the sediment distance on fish responses, at each site, one cage was placed closed to the bottom and other in the surface. For both components, the results obtained at Laranjo basin were compared with the corresponding results in a low contaminated area – S. Jacinto.

Total mercury (Hg_t) concentration was quantified in the water column and sediment, as well as in three different target tissues – blood, liver and muscle. Biochemical/metabolic effects were assessed by measuring the following hepatic oxidative stress indicators: catalase (CAT) and glutathione-*S*-transferase (GST) activities, total glutathione content (GSH_t) and lipid peroxidation (LPO). In addition, the mercury interference on the hepatic cytochrome P450 (P450) dependent biotransformation was evaluated through ethoxyresorufin-*O*-deethylase (EROD) activity, total P450 content and its degradation (P420 content). In a cytogenetic level, the mercury genotoxicity was determined, using the erythrocytic nuclear abnormalities (ENA) assay. Taking into account the previous objectives, the correlation between Hg_t levels in liver and blood and respective responses was also investigated.

Wild fish from Laranjo showed no alterations in antioxidant parameters (excluding a sporadic GSH_t increase), LPO or P450 parameters. Considering the concomitant increment on hepatic Hg_t levels, these results were regarded as an indication that metal was accumulated in a detoxified form.

Muscle Hg_t concentration was significantly increased in *L. aurata* captured at Laranjo for all sampling seasons, although the EU regulatory limit was not exceeded. However, the potential risk to human health can not be excluded, taking into account the high fish consumption rate observed in Portugal.

Wild *L. aurata* from Laranjo displayed elevated ENA frequency in summer and autumn in concomitance with increased blood Hg_t levels. Surprisingly, no ENA induction was observed in winter, where the highest blood Hg_t level was measured. This particular result may be explained by an altered haematological dynamics related to high blood Hg_t levels, as supported by a decreased immature erythrocytes frequency, affecting the ENA generation.

Considering the *in situ* experiment, fish caged at Laranjo showed a general hepatic Hg_t increase, followed by an elevation of CAT activity and GSH_t content, as well as an EROD activity inhibition. Bottom cages from LAR2 and LAR3 revealed higher hepatic Hg_t levels and GSH_t content, respectively, when compared with surface cages, underlining the importance of sediment proximity. This caging experiment also demonstrated the relevance of aqueous mercury uptake, since food intake was restricted due to caging. Analyzing the mercury genotoxicity, an ENA frequency induction was observed in LAR3, the closest site to the contamination source. Moreover, a correlation between blood Hg_t concentration and ENA frequency was found.

Globally, both wild and caged fish revealed that liver accumulates higher mercury concentrations, when compared with blood and muscle. Thus, liver was found to be the organ with the most bioaccumulation capacity, probably due to its role in xenobiotics detoxification. The absence of mercury-induced hepatic peroxidative damage can be attributed to an effective detoxification and antioxidant defense. Mercury showed to be a genotoxic agent, being also responsible by enzyme inhibition. However, based on the obtained results, this metal was not confirmed as an oxidative stress inducer.

The adopted strategy based on the integrated evaluation of different indicators, using the mugilide *L. aurata* as biosensor, demonstrated its usefulness on the assessment of metal pollution in coastal ecosystems.

NOTA PRÉVIA

A presente tese de Mestrado está organizada em três partes distintas. Uma primeira, representada pela Introdução Geral, em que se estabelece o contexto do trabalho e respectivos objectivos. A segunda parte compreende a apresentação de dois artigos científicos submetidos para publicação, e que correspondem aos capítulos I e II. A terceira e última parte, corresponde a uma Discussão Geral relativa aos dois capítulos precedentes, numa perspectiva global e integradora.

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"Metals are perhaps one of the earliest medicines and poisons known to humankind"
(Chang, 1996)

INTRODUÇÃO GERAL

CONSIDERAÇÕES PRÉVIAS

O desenvolvimento tecnológico originou um aumento considerável do número de produtos químicos que têm vindo a ser introduzidos no ambiente. Estes são, por vezes, perniciosos para os organismos e, consequentemente, para os ecossistemas. Os poluentes bioacumulados nos organismos causam, primeiramente, efeitos a níveis molecular e celular, podendo, ao longo do tempo, provocar mudanças tanto ao nível da população, como da comunidade (Arinç *et al.*, 2000).

Os organismos aquáticos mostraram ser sensíveis a efeitos tóxicos produzidos por contaminantes, principalmente desde que estes começaram a ser utilizados em grande escala para fins industriais e agrícolas (Arinç *et al.*, 2000). Os peixes, bem como a generalidade dos organismos vivos, quando confrontados com as constantes modificações no ambiente (frequentemente hostis aos processos vitais), desencadeiam mecanismos adaptativos, com o intuito de assegurarem a sobrevivência. Esta adaptabilidade baseia-se num apurado controlo homeostático que resulta da manutenção do equilíbrio interno, face a alterações exteriores, através de diversas respostas fisiológicas (Pacheco, 1999).

Neste contexto, uma vez que as espécies piscícolas podem ser encontradas virtualmente por todo o ambiente aquático, desempenhando um papel importante nas cadeias alimentares, elas têm atraído sobre si um interesse considerável. Apesar de, aparentemente, a sua elevada mobilidade os limitar como bioindicadores, os peixes são geralmente considerados os organismos mais fiáveis para monitorizar a poluição em sistemas aquáticos (van der Oost *et al.*, 2003).

Os metais pesados ocorrem frequentemente no ambiente e no biota. Alguns destes como o ferro, o cobre, e o zinco, são considerados essenciais ao desenvolvimento dos organismos, enquanto outros, não essenciais como o arsénico, o cádmio, o chumbo e o mercúrio, são marcadamente tóxicos e bioacumuláveis na cadeia trófica. O excesso de metais essenciais ou a presença dos não essenciais poderá desencadear efeitos tóxicos nos peixes (Chang *et al.*, 1996).

Os metais pesados geralmente ocorrem em baixas concentrações no ambiente aquático, mas a bioconcentração destes através de processos metabólicos nos tecidos dos peixes é importante, podendo originar reacções crónicas, tais como alterações na reprodução e mudanças na estrutura da população.

A toxicidade induzida pelos metais pesados é atribuída à reactividade do metal livre, muitas vezes observada nos tecidos envolvidos no seu transporte (Ballatori, 2000)

armazenamento e tentativa de eliminação. As diferenças específicas na toxicidade desses metais podem estar relacionadas com as diferenças na solubilidade, absorção, transporte, actividade química e complexos formados.

Assim, no âmbito da toxicologia ambiental, o mercúrio assume uma enorme importância uma vez que, sendo um metal não essencial, está frequentemente presente no ambiente. Esta ocorrência está intimamente relacionada com fontes antropogénicas, sendo observada à escala global. A zona de Estarreja (Largo do Laranjo – Ria de Aveiro), é uma das regiões mais afectadas por este problema em Portugal Continental, o que motivou a sua escolha como área de estudo da presente dissertação.

1. TOXICOLOGIA DO MERCÚRIO

1.1. O Mercúrio no Ambiente Aquático

O interesse científico na presença de mercúrio nos ecossistemas aquáticos tem sido motivado, principalmente, pelos potenciais riscos para a saúde pública. O mercúrio apresenta-se como um elemento que, ao contrário de outros metais, não tem qualquer função biológica. Encontra-se no estado líquido à temperatura ambiente e a sua massa é 13,6 vezes superior à da água. As suas propriedades tóxicas têm sido exploradas tanto na medicina, como na desinfecção e formulação de pesticidas (Gochfeld, 2003).

O desgaste da crosta terrestre é a principal fonte natural de mercúrio no ambiente, embora este metal seja usado em larga escala na indústria para produção de cloro e soda cáustica, através da electrólise da salmoura, assim como na incineração de resíduos e queima de hulha, e produção de electricidade. É também utilizado na produção de instrumentos de medição como termómetros e barómetros, assim como em tubos de fluorescência, pilhas alcalinas e amálgamas na medicina dentária (WHO, 1991). A exploração mineira, principalmente a extracção de ouro, utiliza também grandes quantidades de mercúrio líquido que acaba por contaminar os ecossistemas aquáticos (Tchounwou *et al.*, 2003) dando, deste modo, início ao seu ciclo no ambiente.

É possível encontrar este metal em três formas distintas, com solubilidade, reactividade e toxicidade características (Clarkson, 2002) sendo, no entanto, todas elas tóxicas. Nos ecossistemas aquáticos pode ocorrer na forma elementar (Hg^0), apresentando-se dissolvido sob a forma de iões particulados (Hg^{2+} e Hg^+), ou ainda como metilmercúrio ($MeHg^+$) dissolvido ou particulado (Mason e Fitzgerald, 1993). Devido à

elevada afinidade com halogéneos, revela uma facilidade relativa em formar cloro-complexos (Sadiq, 1992).

No ambiente, o provável destino do Hg^0 é a oxidação a Hg^{2+}, pela reacção com o oxigénio, ozono ou cloro. Uma vez na forma oxidada, o mercúrio torna-se muito mais solúvel e mais susceptível a ser removido da atmosfera, por precipitação. Assim, quando assume a forma inorgânica, atinge os sistemas aquáticos, fixa-se à superfície do sedimento ou às algas, onde é convertido pelas bactérias na sua forma mais tóxica, o $MeHg^+$. Esta espécie química torna-se facilmente biodisponível, dando-se a sua biomagnificação ao longo das cadeias tróficas (Gochfeld, 2003), o que pode vir a reflectir-se na saúde pública (Tan e Perkin, 2000).

A especiação do mercúrio torna-se relevante em estudos ambientais devido à variação na toxicidade das diferentes formas e respectivas implicações biológicas. No entanto, atendendo ao facto da especiação não ser um processo analítico simples, considera-se que a quase totalidade do mercúrio presente nos organismos se encontra na sua forma metilada. Noutras matrizes, como o sedimento e a água, a percentagem de $MeHg^+$ é muito baixa (cerca de 1%), assumindo, contudo, relevância na análise de risco (Gochfeld, 2003; Storelli *et al.*, 2005).

1.2. Toxicocinética do Mercúrio

O campo da toxicologia ambiental é complexo e multidisciplinar, pois envolve a compreensão a nível físico, químico e biológico, na tentativa de interpretar as actuais perturbações causadas pelos tóxicos, assim como prever as futuras. É, portanto, necessário conhecer o fluxo de elementos químicos entre o ambiente e os peixes, assim como os mecanismos que o controlam. A fonte de contaminação, o transporte e a transformação, por processos bióticos ou abióticos, assumem-se como factores determinantes nos movimentos do mercúrio no ambiente (McKim *et al.*, 1976) e na sua distribuição no organismo.

1.2.1. Absorção

A indução de respostas ao mercúrio pressupõe que este se encontre biodisponível, entrando assim, em contacto com o sistema biológico. A exposição dos peixes ao mercúrio pode ocorrer a partir do sedimento ou da coluna de água (dissolvido ou na

forma particulada). No entanto, a maior via de exposição está intimamente relacionada com a dieta (Livingstone, 1998).

O processo de absorção de metais pelos peixes assenta em vários mecanismos, ocorrendo através das membranas semi-permeáveis das guelras e/ou epitélio do intestino. No caso particular do mercúrio, estudos com modelos de membranas sugeriram como forma de absorção, a difusão passiva através das membranas de clorocomplexos ($HgCl_2$) e compostos inorgânicos neutros (Newman e Jagoe, 1996; Klinck *et al.*, 2005). O seu catião (Hg^{2+}) poderá ser absorvido através de processos de transporte próprios de iões essenciais, como é o caso do Ca^{2+} (Klinck *et al.*, 2005). O mercúrio possui ainda a capacidade de estabelecer ligações com os grupos sulfidrilo (-SH) membranares envolvidos na absorção de outros elementos metálicos (Newman e Jagoe, 1996). As guelras constituem o primeiro local de absorção de metais dissolvidos na água. A pele, por sua vez, representa uma barreira quase contínua, pouco permeável, permitindo assim uma absorção lenta (Timbrell, 1992). Os peixes absorvem o Hg^{2+} com muito menor eficácia através do intestino e das guelras, comparativamente com a forma metilada. Niimi e Kisoon (1994) realçaram a importância da especiação química do mercúrio na sua absorção, bem como num elevado número de mecanismos biológicos envolvidos na sua regulação.

1.2.2. Transporte, Distribuição e Acumulação

Os órgãos com um papel preponderante na absorção de metais tornam-se também importantes alvos da sua toxicidade, uma vez que tendem a concentrá-los, exibindo níveis de bioacumulação relativamente altos (Malins e Ostrander, 1993). Harrison *et al*. (1990) encontraram maiores concentrações de mercúrio nas guelras, quando se constituíam como o principal órgão de absorção, e no intestino quando a entrada do mesmo se dava através do alimento. Após a absorção através do epitélio das guelras ou do sistema gastrointestinal, o mercúrio atinge o sistema circulatório. Uma vez no sangue, é rapidamente transportado para os diferentes órgãos, podendo ainda sofrer uma redistribuição, e acumular-se maioritariamente em tecidos específicos, devido à sua afinidade com determinadas moléculas.

O subsistema vascular entero-portal assume uma relevância particular no transporte de xenobióticos para o fígado. O sangue tem, assim, um duplo papel: o de transportador e redistribuidor (Malins e Ostrander, 1993). Dados anteriores demonstraram o papel preponderante da dieta na acumulação de mercúrio (Francesconi e Lenanton, 1992)

contribuindo, provavelmente, para mais de 90% do total de metal acumulado (Rodgers, 1994). É assim, importante examinar, tanto no campo como em laboratório, a bioacumulação de mercúrio em peixes expostos a concentrações realistas, quer via água, quer através da dieta. Em águas temperadas, a acumulação de mercúrio nos peixes parece ser mais rápida no Verão, quando a sua alimentação e taxa metabólica, assim como a produção microbiana de $MeHg^+$ no sedimento estão maximizadas (Bodaly *et al.*, 1993; Ramlal *et al.*, 1993). No entanto, estudos laboratoriais revelaram que os peixes conseguem igualmente acumular elevadas concentrações de mercúrio directamente a partir da água (Niimi e Kisoon, 1994).

De acordo com testes laboratoriais (Ribeyre e Boudou, 1984) e estudos de campo (Niimi e Kisoon, 1994), as concentrações de $MeHg^+$ elevam-se no sangue, baço, rim e fígado, diminuindo à medida que cessa a exposição, independentemente da via de absorção (Harrison *et al.*, 1990).

O mercúrio acumulado no músculo liga-se aos grupos -SH das proteínas, apesar das concentrações aqui serem geralmente mais baixas que em outros tecidos (Ribeyre e Boudou, 1984; Harrison *et al.*, 1990). A quase totalidade do mercúrio encontrado no tecido muscular dos peixes (cerca de 60 a 100%) é $MeHg^+$ (Joiris *et al.*, 1999; Storelli *et al.*, 2003, 2005), devido ao facto de ser a forma mais facilmente absorvida, sendo por isso particularmente bioacumulada neste tecido (Klinck *et al.*, 2005). Os peixes não metilam o mercúrio inorgânico no interior dos seus tecidos, mas são afectados pela metilação que ocorre no lúmen intestinal através da participação activa do seu conteúdo microbiano (Rudd *et al.*, 1980). Assim, a produção de $MeHg^+$ primariamente por metilação microbiana do mercúrio inorgânico Hg^{2+} no ambiente, é o mecanismo-chave que afecta a quantidade de metal acumulado nos organismos (Gilmour e Henry, 1991; Bodaly *et al.*, 1993).

A concentração de mercúrio nos tecidos dos peixes aumenta, geralmente, com a idade e com o tamanho destes, em virtude da baixa taxa de eliminação do $MeHg^+$, quando comparada com a sua absorção (Ribeyre e Boudou, 1984).

1.2.3. Desintoxicação

Embora os peixes se deparem frequentemente com stresse ambiental, o conhecimento das suas respostas adaptativas é ainda limitado, nomeadamente no que concerne à manutenção do equilíbrio interno face a um contaminante.

Compreendendo a desintoxicação como o decréscimo na quantidade de contaminante no interior do organismo, esta pode ter lugar a partir da sua transformação num produto menos tóxico através de actividades biológicas tal como a biotransformação, ou do seu sequestro sob formas inócuas, como quando complexadas com metalotioninas (MTs) (Newman e Jagoe, 1996).

Os peixes, na incapacidade de biotransformar metais, desenvolveram uma estratégia particular de desintoxicação, mantendo-os sequestrados de modo a que não se façam sentir os seus efeitos adversos (Newman e Jagoe, 1996). Este sequestro ocorre em vários tecidos e órgãos, podendo o mesmo ser temporário, ou constituir uma forma de armazenamento a longo prazo. O armazenamento compartimentado dos metais assume extrema importância, uma vez que condicionará os indesejáveis efeitos tóxicos (Malins e Ostrander, 1993).

As MTs são, nos peixes, as principais estruturas intracelulares envolvidas no sequestro e armazenamento de metais. Representam uma família de proteínas de baixo peso molecular que se ligam a metais, com um papel na regulação dos metais essenciais e, na desintoxicação, tanto destes últimos como dos não essenciais (Newman e Jagoe, 1996). O papel das MTs no sequestro de qualquer metal e, posterior redução da quantidade disponível causadora de toxicidade, está bem definido. A indução da sua síntese, provocada pela exposição a metais, está associada à protecção contra a toxicidade metálica. Estudos da expressão genética das MTs revelaram que a sua indução é uma resposta directa ao aumento intracelular da concentração dos metais, sendo a capacidade de induzir MTs maior em tecidos onde existe absorção, armazenamento ou excreção (Filipović e Raspor, 2003).

As estratégias de acumulação diferem, de organismo para organismo, resultando em diferentes dependências do uso destas proteínas para a respectiva desintoxicação. Assim, através dessa protecção o organismo não sucumbe tão prontamente à toxicidade e, considerando a elevada taxa de renovação das MTs, maiores quantidades de metais poderão vir a ser acumuladas. O sequestro temporário do metal por estas proteínas de alta afinidade, providencia indubitavelmente, um reservatório não reactivo de metais, representando uma forma de desintoxicação essencial para optimizar a disponibilidade dos metais tóxicos (Newman e Jagoe, 1996).

No caso particular do mercúrio, considera-se o comportamento das MTs semelhante ao estudado para a generalidade dos metais. Segundo Langston *et al*. (2002), o mercúrio

é encarado como um dos metais com maior afinidade com os resíduos de cisteína, não sendo a indução da síntese de MTs uma consequência obrigatória desta afinidade.

1.2.4. Eliminação e Excreção

A eliminação de xenobióticos é, obviamente, um factor determinante no possível efeito biológico causado. Quanto mais rápida for a eliminação, menor é a probabilidade do contaminante exercer um efeito tóxico. Se, por outro lado, a retenção deste for prolongada, o potencial tóxico aumenta (Timbrell, 1992). A taxa de excreção do mercúrio é, em geral, muito baixa, sendo no caso da forma organometálica muito mais reduzida quando comparada com as formas inorgânicas (Nepomuceno *et al.*, 1997).

A determinação de mercúrio na bílis permite considerá-la como uma via de eliminação de metais pesados, mas com a libertação para o intestino, poderá ocorrer uma reabsorção, relançando o metal no organismo. Apesar da possibilidade de excreção através das vias biliar e intestinal, o rim assume o principal papel (Malins e Ostrander, 1993).

A excreção presume-se como o passo final desejável após a absorção de compostos mercuriais, dado que corresponde à sua remoção do organismo. Variados efeitos adversos poderão surgir, caso esta excreção não venha a ter lugar, tal como se descreve no ponto seguinte.

1.3. Efeitos Tóxicos do Mercúrio

Neste ponto, os efeitos e mecanismos descritos dizem respeito fundamentalmente a peixes. No entanto, dado o facto de o consumo de peixe constituir a principal fonte de mercúrio para o Homem, é também abordado o impacte do mercúrio na saúde humana.

Os metais pesados quando incorporados nos organismos, sendo indestrutíveis nos tecidos biológicos, produzem efeitos tóxicos pela reacção com ligandos específicos. Em solução, são altamente reactivos e combinam-se prontamente com várias moléculas biológicas e constituintes das membranas celulares, como as proteínas e os fosfolípidos. A compreensão do modo como o mercúrio reage com as membranas e as atravessa, é fundamental para perceber a sua acção tóxica. Esta deve-se, em particular, à sua capacidade de combinação com os grupos -SH, resultando assim, em mudanças na conformação das proteínas e alterando o seu funcionamento (Quig, 1998).

O mercúrio, na sua forma inorgânica, afecta as respostas celulares, afectando assim os processos homeostáticos, provocando mudanças no estado fisiológico (Morimoto e

Georgopoulous, 1994). A toxicidade deste metal pode ser modulada pelo desenvolvimento de tolerância ou resistência, sendo, no entanto, da maior relevância a via e o modo como o organismo incorpora o metal.

1.3.1. Alterações Metabólicas

As constantes alterações do ambiente celular exigem um grau considerável de adaptabilidade por parte das células, nomeadamente em termos da regulação de funções metabólicas (Burkitt *et al*., 1996).

A toxicidade induzida pelos metais pesados pode envolver vários mecanismos, sendo a formação de espécies reactivas de oxigénio (ROS, do inglês "reactive oxygen species") um dos mais bem estudados (Di Giulio *et al*., 1989; Halliwell e Gutteridge, 1999). O anião superóxido ($O_2^{\bullet -}$), o peróxido de hidrogénio (H_2O_2) e o radical hidroxil ($OH^{\bullet}$) são exemplos de produtos de redução do oxigénio molecular que possuem poder oxidante. As defesas antioxidantes exercem, assim, um papel crucial na manutenção da homeostase celular, mas quando estas são deprimidas ou ultrapassadas, poderá ocorrer stresse oxidativo (Halliwell e Gutteridge, 1989). Os ROS têm capacidade de reagir com macromoléculas celulares, podendo originar inactivação de enzimas, peroxidação lipídica e mesmo a morte celular (Winston e Di Giulio, 1991). Todavia, segundo Ercal *et al*. (2001), são as reacções do tipo Fenton que parecem desempenhar o papel principal no stresse oxidativo. Neste contexto, a depleção das reservas de grupos -SH nas células parece ser um mecanismo indirecto importante para o stresse oxidativo induzido pelo mercúrio. Este metal tem afinidades electrónicas que permitem estabelecer ligações covalentes principalmente com grupos -SH das proteínas, revelando-se a sua interacção com o metabolismo da glutationa de extrema importância para a compreensão dos seus efeitos. A glutationa, na sua forma reduzida (GSH), está envolvida na formação de conjugados GSH-S com formas iónicas de mercúrio, dando origem a complexos lineares e covalentes (Rabenstein, 1989). Uma vez dentro da célula, tanto na forma orgânica como na inorgânica, estabelece ligações covalentes com a glutationa e com os resíduos de cisteína das proteínas. A glutationa, um antioxidante intracelular da primeira linha de defesa, evita deste modo, que o mercúrio exerça a sua toxicidade. Um ião deste metal pode ligar-se a duas moléculas de GSH (Quig, 1998). No entanto, a libertação deste pode causar uma enorme actividade, interferindo assim com o metabolismo da glutationa, e causando dano celular. Contudo, a interacção dos compostos mercuriais com a GSH sugere a ocorrência de

stresse oxidativo (Sarafian, 1999). Com a diminuição do conteúdo em glutationa, os sistemas de síntese desta entram em acção, produzindo cisteína a partir do ciclo γ-glutamil. No entanto, quando a exposição é crónica, a concentração de glutationa acaba por ser substancialmente diminuída. A deplecção de glutationa acabará assim por reduzir a capacidade que a célula tem de destruir os ROS, aumentando deste modo o potencial oxidativo, o qual irá dar origem a um consequente desequilíbrio intracelular.

Paralelamente, e por intermédio da enzima glutationa-*S*-transferase (GST), poderá dar-se a conjugação de compostos electrofílicos com a GSH. No entanto, esta família de isoenzimas poderá ter também um papel relevante na desactivação dos produtos da peroxidação lipídica e seus derivados, tornando-a um interveniente importante na defesa contra o stresse oxidativo (Cnubben *et al*., 2001). Assim, perante uma intoxicação por mercúrio, e uma vez que os peixes não biotransformam metais, a GST terá uma acção preponderantemente antioxidante.

Enzimas como a superóxido dismutase (SOD) e a catalase (CAT) entram em acção, no sentido de neutralizar ROS. A SOD, um grupo de metaloenzimas, catalisa a conversão de aniões $O_2^{\bullet -}$ em H_2O_2. O H_2O_2 é depois transformado em oxigénio molecular e água por dois tipos de enzimas, a CAT e a glutationa peroxidase (GPx) assumindo, assim, um papel antioxidante importante, na remoção do H_2O_2. No entanto, a maioria destas enzimas torna-se inactiva aquando da ligação do mercúrio aos seus locais activos, mais especificamente, aos seus grupos -SH, sendo este um dos mecanismos mais importantes na indução de dano oxidativo por este metal (Stegeman *et al*., 1992; Filho, 1996).

Estudos efectuados por Elia *et al*. (2000) evidenciaram que a GSH e enzimas dependentes desta, presentes no fígado de *Ictalurus melas*, foram afectadas pelo mercúrio na forma iónica. Os mesmos autores sugeriram que as altas concentrações do metal são responsáveis pela inibição da GST e GPx, assim como pelo aumento do conteúdo total de glutationa. Contudo, a deplecção de glutationa em organismos expostos a metais parece ser, geralmente, acompanhada por variações na actividade da GST.

Os metais pesados são conhecidos como inibidores de várias actividades enzimáticas nos organismos marinhos (Viarengo *et al.,* 1997), dentro das quais as ligadas a um sistema de enzimas referidas como oxidases multifunção (MFO). Estas mono-oxigenases pertencem, predominantemente, a um sistema de hemo-proteínas – o citocromo P450 (P450).

Estudos *in vivo* com fígado de peixes revelaram que os metais pesados provocam um decréscimo no conteúdo em citocromo P450 (Tallandini *et al.*, 1987). A actividade de etoxiresorufina-*O*-desetilase (EROD), dependente do sistema P450, foi medida em peixes como um indicador de exposição a determinados compostos, tal como a hidrocarbonetos policíclicos aromáticos (HPAs), uma vez que estes induzem a sua actividade (Stegeman e Hahn, 1994). No entanto, a actividade de EROD em microssomas de peixes mostrou ser inibida *in vitro* pela presença de cobre e mercúrio (Viarengo *et al.*, 1997; Oliveira *et al.*, 2004), podendo assim esta inibição ser adoptada como um indicador da presença destes contaminantes.

Pode considerar-se que o efeito inibidor do mercúrio no sistema P450 se deve à ligação directa a grupos -SH, uma vez que estes são parte integrante das proteínas. Para além de inactivar as enzimas, alterando a sua conformação por meio de ligações covalentes (Quig, 1998), possui também a capacidade de afectar a taxa de renovação destas proteínas (Viarengo, 1989).

Deste modo, é possível afirmar que, havendo inibição deste sistema de enzimas, o peixe tornar-se-á menos capaz de metabolizar xenobióticos, diminuindo a sua capacidade de defesa e, consequentemente, de sobrevivência.

1.3.2. Genotoxicidade

Durante décadas, a contagem de micronúcleos (MN) tem servido como um índice de quebras cromossómicas e da disfunção do fuso mitótico. Trata-se de um método largamente utilizado na avaliação do impacte biológico da poluição aquática, testando assim, a genotoxicidade de determinados compostos, após uma exposição *in vivo* (Sanchez-Galan *et al.*, 2001; Rodriguez-Cea *et al.*, 2003; Çavas e Ergene-Gözükara, 2005). O teste de MN apresenta diversas vantagens, quando comparado com outros métodos citogenéticos. No entanto, foram encontrados alguns obstáculos na sua utilização, uma vez que nem todas as espécies de peixes são sensíveis à indução destas alterações. Assim, e de modo a ultrapassar estas barreiras, passou a incluir-se a análise de pequenas deformações - as anomalias nucleares eritrocíticas (ANE), as quais foram reportadas pela primeira vez por Carrasco *et al.* (1990), sendo presentemente consideradas por muitos autores como um sinal de dano genético. O teste de ANE revelou-se de extrema importância, uma vez que estas anomalias são visíveis mesmo quando não se observa a indução de MN. Deste modo, diversos autores alargaram o teste

de MN, quantificando-os em simultâneo com os núcleos com forma anómala (Ayllon e Garcia-Vazquez, 2000; Pacheco *et al*., 2005).

A genotoxicidade dos compostos mercuriais foi avaliada em diferentes grupos de organismos e revista por De Flora *et al*. (1994), revelando a sua capacidade de danificar o ADN. Experiências laboratoriais com peixes demonstraram a indução de MN e outras anomalias em eritrócitos (Al-Sabti, 1994; Nepomuceno *et al*., 1997; Sanchez-Galan *et al*., 1999; Ayllon e Garcia-Vasquez, 2001). Uma elevada taxa de quebras no DNA (cadeia simples e dupla), medida através do teste do cometa, foi também observada em suspensões de células de guelras, em peixes tratados com mercúrio (Arabi, 2004). No entanto, os mecanismos envolvidos na acção genotóxica deste metal são ainda pouco conhecidos, tal como a relação com os níveis de mercúrio encontrados nos tecidos alvo.

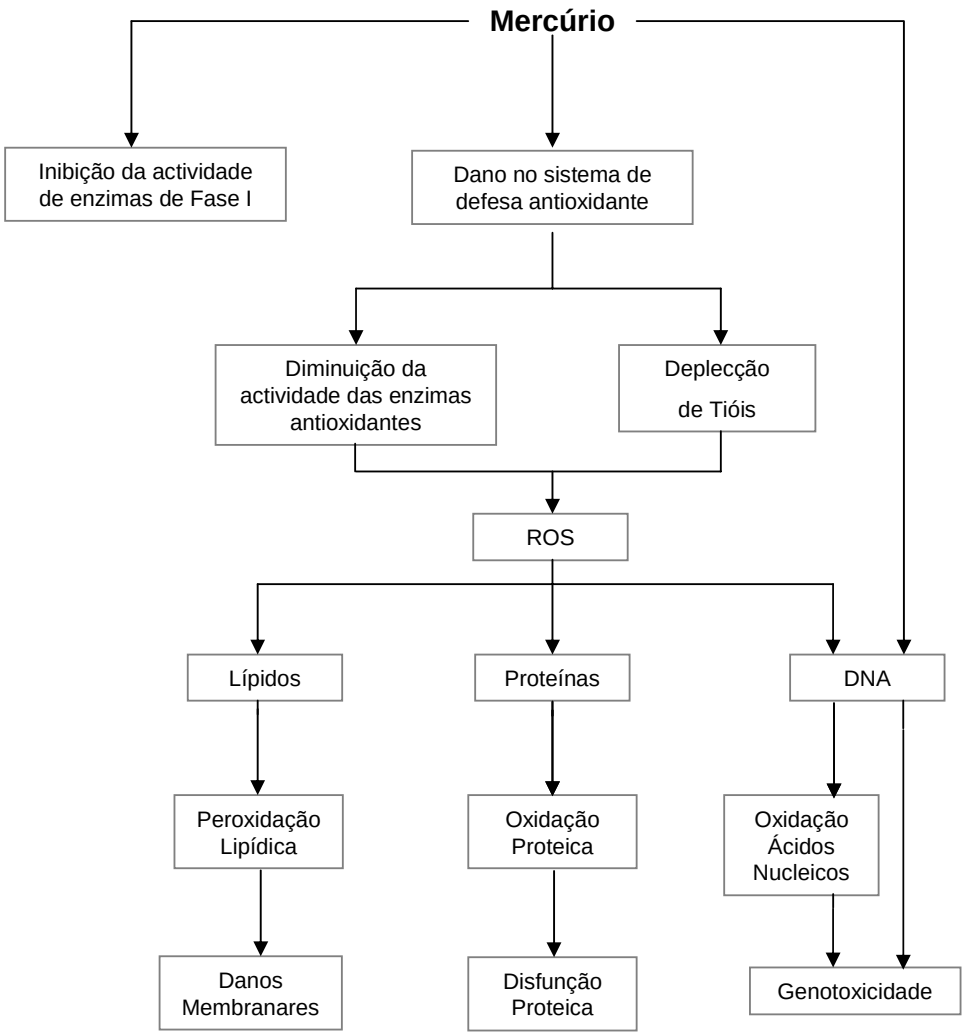

Figura 1: Representação esquemática dos efeitos tóxicos provocados pelo mercúrio (Adaptado de *Ercal et al.*, 2001)

1.3.3. Efeitos em Humanos

Os problemas associados com o mercúrio e posterior contaminação das cadeias alimentares aquáticas, não eram reconhecidos até ocorrer no Japão o que é hoje conhecida como a Doença de Minamata (Clarkson, 1997). O uso de Hg^{2+} como catalisador provocou uma conversão catalítica do composto, tornando-o organomercurial. Anos mais tarde, a população da Baía de Minamata (Japão) mostrava sintomas de envenenamento, sendo esta intoxicação atribuída às concentrações do composto na sua forma orgânica encontradas em animais marinhos, que faziam parte integrante da dieta das populações humanas locais. Dados epidemilógicos sobre as vias de exposição a mercúrio estimam que os peixes e bivalves tenham tido uma importante contribuição para as intoxicações por $MeHg^{+}$ (Gochfeld, 2003).

Presentemente, é conhecido o risco associado à exposição a este metal pesado, no sentido lato, demonstrando ter potencial teratogénico e causador de danos neurológicos irreversíveis (De Flora *et al.,* 1994). Deste modo, foi necessário estabelecer uma dose limite de exposição, sem risco para a saúde pública. Na Europa, o valor limite estabelecido foi de 0,5 µg/g peso fresco (excepto para algumas espécies, nas quais pode chegar ao 1 µg/g) (Official Journal of the European Communities, 1994), no Japão são permitidas 0,4 µg/g peso fresco, enquanto que a US Food and Drug Administration (FDA) estabeleceu 1 µg/g peso fresco como a quantidade máxima permitida (Storelli *et al.*, 2005).

2. ORIENTAÇÕES DA DISSERTAÇÃO

O Largo do Laranjo na Ria de Aveiro (litoral centro de Portugal), representa um local com uma elevada contaminação por mercúrio (Lucas *et al.,* 1986; Pereira *et al.*, 1998). Esta área, situada perto de Estarreja, recebeu durante décadas descargas contínuas de uma indústria de soda cáustica, originando um gradiente de contaminação ambiental. Apesar das emissões terem sido drasticamente reduzidas, devido a melhoramentos nas técnicas de produção, o sedimento retém ainda grandes quantidades de mercúrio, cuja ressuspensão, por acção das fortes correntes sentidas principalmente durante os meses de Inverno, aumenta a sua biodisponibilidade para os organismos aquáticos (Pereira *et al.*, 1997, 1998).

Deste modo, e perante o cenário de uma severa contaminação dos sedimentos existente neste local, coloca-se a necessidade de avaliar o estado da comunidade íctica autóctone. Em simultâneo, o Largo do Laranjo surge como um "laboratório de campo", proporcionando uma excelente oportunidade para aprofundar o conhecimento da toxicidade do mercúrio sob condições realistas.

A Tainha-garrento, *Liza aurata,* foi o bioindicador seleccionado para a realização do presente estudo, por se tratar de uma das espécies de teleósteos mais abundantes neste ecossistema, apresentando ainda uma vasta distribuição geográfica ao longo do Atlântico Oriental, desde o Golfo da Gasconha até Marrocos, mares Mediterrâneo e Negro. Este mugilídeo demonstra também uma interessante dicotomia, pois apesar de sensível, dá mostras de possuir bastante resistência aquando da exposição ambiental a contaminantes. Adapta-se a diferentes graus de salinidade, revelando-se de fácil manuseamento e adaptação a experiências laboratoriais. A sua alimentação à base de organismos bentónicos e pequenos anelídeos faz com que esteja em contacto com o sedimento, não deixando, contudo, de se mover ao longo de toda a coluna de água. Por toda esta versatilidade, *L. aurata* assume-se como uma boa candidata a espécie sentinela, sendo a validação deste estatuto também um dos objectivos da presente investigação. Adicionalmente, os mugilídeos têm vindo a tornar-se um grupo emergente, ocupando cada vez mais um lugar de destaque na alimentação dos europeus, principalmente nos países mediterrâneos.

O presente trabalho tem como objectivos centrais (i) a avaliação do estado da população de *L. aurata* na área de estudo e dos riscos associados à contaminação por mercúrio e (ii) contribuir para um melhor conhecimento da toxicidade deste metal e mecanismos subjacentes em peixes.

Assim, foi adoptada uma estratégia baseada no estudo integrado da acumulação de mercúrio em tecidos alvo e de respostas bioquímicas/metabólicas e citogenéticas. Pretende-se avaliar o risco de stresse oxidativo (respostas antioxidantes e peroxidação lipídica), a interferência com o metabolismo de biotransformação dependente do citocromo P450 e a genotoxicidade decorrentes da exposição ambiental a mercúrio, clarificando a relação com os níveis teciduais.

Com o intuito de avaliar os riscos para a saúde humana, foi quantificado o teor de mercúrio na porção edível de L. *aurata*, que é também assumido como uma importante ferramenta de monitorização.

O trabalho desenvolvido conjuga duas componentes, correspondendo a uma amostragem sazonal de espécimes selvagens e a uma exposição *in situ* envolvendo animais engaiolados.

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CAPÍTULO I

Respostas antioxidantes e de biotransformação em *Liza aurata* após exposição ambiental a mercúrio – relação com a distribuição do mercúrio e sua acumulação

Antioxidant and biotransformation responses in *Liza aurata* under environmental mercury exposure – relationship with mercury distribution and accumulation

Antioxidant and biotransformation responses in *Liza aurata* under environmental mercury exposure – relationship with mercury distribution and accumulation

ABSTRACT

Increased loadings of mercury to the aquatic environment have resulted in greater environmental concern mainly due to the accumulation of this metal in fish tissues. However, the mercury effects on fish metabolism have received relatively little attention. This work was implemented to provide information on hepatic oxidative stress and biotransformation responses in golden grey mullet (*Liza aurata*) environmentally exposed to mercury, assessing the relationship with liver total mercury (Hg_t) bioaccumulation. The potential risk to human health associated to fish consumption was also evaluated by the measurement of Hg_t in the muscle. The study was carried out in a particular area of the Aveiro lagoon (Portugal) – Laranjo basin - impacted by mercury discharges during several decades, and the responses were compared with a reference site (S. Jacinto). A seasonal wild fish survey was carried out revealing no alterations in antioxidants (excluding a sporadic increase on total glutathione - GSH_t), lipid peroxidation or cytochrome P450 parameters. Considering the clear increment on hepatic Hg_t levels, the results were regarded as an indication that metal is accumulated in a detoxified form. Hg_t concentration in the muscle was significantly high in *L. aurata* captured at Laranjo for all sampling seasons, although the EU regulatory limit was not exceeded. Complementarily, fish were caged for 3 days within Laranjo basin at three locations (LAR1, LAR2 and LAR3) differing on their distances to the contamination source. In order to evaluate the influence of the sediment proximity, at each site one cage was placed close to the bottom and another on the water surface. Caged fish displayed a general increment on Hg_t hepatic levels accompanied by increases on GSH_t content and catalase activity, as well as an ethoxyresorufin *O*-deethylase activity inhibition. The bottom group displayed higher hepatic Hg_t concentration (LAR2) and GSH_t (LAR3) comparing with surface group, underlining the importance of the sediment proximity. This caging experiment also demonstrated the relevance of aqueous mercury uptake since food intake was restricted due to caging. Globally, both wild and caged fish revealed that liver accumulates higher Hg_t concentrations than muscle and thus better reflecting the environmental contamination levels. The absence of peroxidative damage in liver can be attributed to an effective detoxification and antioxidant defence. The adopted strategy based on the evaluation of wild and *in situ* caged animal, allied to the use of *L. aurata* as biosensor, demonstrated its usefulness on the assessment of metal pollution in coastal ecosystems.

1. INTRODUCTION

Mercury is a toxic and hazardous metal that occurs naturally in the earth's crust. Natural phenomena such as erosion and volcanic eruptions, as well as anthropogenic activities may lead to substantial contamination of the environment (Tchounwou *et al.*, 2003). Increased loadings of mercury to the aquatic environment have resulted in greater

accumulation of this metal in tissues of fish and fish consumers (Gilbertson and Carpenter, 2004).

Fish populations can be adversely affected by mercury within their tissues, being then important to assess its distribution and subsequent retention. Mercury quantifications in tissues are, generally, the best way to gain a better understanding of the dynamics of this contaminant in fish body. Liver is targeted due to its role in the detoxification and importance to individual fish health (Cizdziel *et al*., 2003). Most of the mercury resides in fish muscle are present as methylmercury ($MeHg^{+}$), the most toxic form, which is recognized as the major via of mercury human exposure (Tchounwou *et al*., 2003). Despite the existence of many studies about mercury quantification in fish tissues, the scientific knowledge on the relation between those levels and the effects in fish health is still scarce.

Studies on fish have demonstrated that the antioxidant systems could provide relevant indices in explaining the sensitivity of some fish species to xenobiotics (Di Giulio *et al*., 1993; Lemaire and Livingstone, 1993). Antioxidants play a crucial role in maintaining cell homeostasis and when these defences are impaired or overcome, oxidative stress products, namely reactive oxygen species (ROS), may induce DNA damage, enzymatic inactivation and peroxidation of cell constituents (Halliwell and Gutteridge, 1989). To prevent damage caused by ROS, fish usually cope by increasing the levels of protective antioxidants enzymes, namely catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), as well as non-enzymatic free radical scavengers like reduced glutathione (GSH). Hence, the modulation of antioxidants enzymes and alterations on GSH content, may serve as biomarkers of exposure to heavy metals (Doyotte *et al.,* 1997), including mercury, since it was recognised as a pro-oxidant that exerts oxidative stress (Stohs and Bagchi, 1995).

Several reports have implicated metals as modifiers of cytochrome P450 (P450) function (Vakharia *et al*., 2001). Mercury seems to present the same property (Ke *et al*., 2002) but a few articles reported this effect on fish. Thus, ethoxyresorufin *O*-deethylase (EROD) activity, a functional and specific assessment of CYP1A, was found to be inhibited by low concentrations of mercury (Viarengo *et al.,* 1997; Oliveira *et al*., 2004). Mercury can alter the activity of enzymes by binding to their functional groups or by displacing the metal associated with the enzyme (Viarengo, 1985). In addition, mercury binds directly to sulfhydryl (-SH) groups by forming disulfide bridges (-S-S-) in proteins, the primary mechanism of cellular toxicity. These reactions may result in protein conformational

changes and thereby preventing their normal functions (Quig, 1998). The physiological implications of P450 activities inhibition have not been fully established (Arukwe *et al.*, 1997); however, is assumed that may reduce fish ability to metabolize and excrete xenobiotics, causing alterations at different biological levels.

Laranjo basin is a confined area of Ria de Aveiro, a coastal lagoon in the northern west region of Portugal, where a well defined gradient of mercury was identified because of chlor-alkali plant discharges (Pereira *et al.*, 1998). Due to the absence of other important sources of contaminants, this area can be regarded as a "field laboratory" offering to researchers a unique opportunity for the assessment of mercury toxicity under realistic conditions. Thus, the study conducted at Laranjo basin, using *Liza aurata* as biosensor, had five primary objectives: (1) to investigate the relationship between mercury levels in liver and induction of antioxidant defences as well as P450 inhibition; (2) to assess the relative importance of water uptake route provided by the conjugation of wild and caged fish analysis; (3) to evaluate *L. aurata* as target fish species to carry out toxicity tests; (4) to determine the environmental risk of mercury contamination to native ichthyofauna; (5) to assess the potential risk to human health associated to fish consumption by the measurement of mercury in fish muscle.

2. MATERIAL AND METHODS

2.1. Characterization of study area

Laranjo basin, near Estarreja (Figure 1), is the most contaminated area in the Ria de Aveiro coastal lagoon, north-western coast of Portugal. This area received chlor-alkali plant discharges continuously during five decades, generating a mercury contamination gradient. Recently (approximately 15 years ago) this industry improved the production process, leading to a considerable decrease of mercury released. Nevertheless, high mercury levels are still present in sediments due to its progressive deposition, which resuspension, manly during periods of stronger tidal currents, is responsible by its exportation and increased bioavailability (Pereira *et al.*, 1997, 1998). Therefore, moderate mercury levels were observed in fish inhabiting this area (Abreu *et al.*, 2000).

S. Jacinto area was selected as reference for comparison purposes because of the lagoon entrance proximity (Figure 1), the distance to the main polluting sources and low-contamination load (Pacheco *et al.*, 2005).

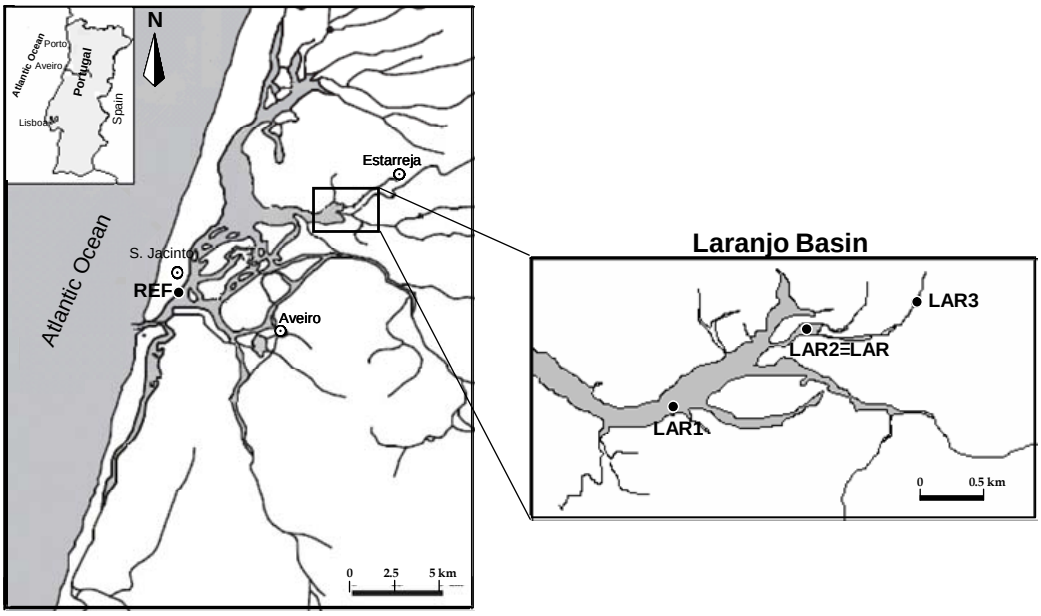

Figure 1 - Map of Ria de Aveiro (Portugal) with locations of fish-capture and caging sites (dark circles). The respective coordinates are: reference site (REF) - 40º40'26''N, 8º43'17''W; LAR1 - 40º43'24''N, 8º37'55''W; LAR2 - 40º43'49"N, 8º36'53"W; LAR3 - 40º44'04"N, 8º36'02"W. The LAR2 site adopted in the caging experiment coincides (≡) with LAR site selected for sampling in the wild fish survey.

2.2. Fish and sampling

Golden grey mullet (*Liza aurata*) is a pelagic species that frequently contacts with sediments, feeding on small benthic organisms, detritus and occasionally on insects and plankton. Juvenile specimens were used to minimize the interference of variables such as gender and contaminants accumulation. Fish with an average weight of 13.5± 0.1 g and length of 12.1 ± 0.1 cm were caught in reference and contaminated areas during low tide, using a traditional beach-seine net named "chincha". At each sampling point, fish were killed and their liver and muscle were collected. These tissues were immediately frozen in liquid nitrogen, being muscle stored at –20 ºC and liver at -80 ºC until homogenization. Fish were weighed and measured in the laboratory.

2.3. Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB), 2,5-dithiobis-tetranitrobenzoic acid (DTNB), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA) and NADPH were purchased from

Sigma (Spain) and other routine chemicals and reagents (analytical grade) were purchased from local sources.

2.4. Experimental Design

This study includes two different components corresponding to a seasonal analysis of wild mullets and a field-caging experiment. At each sampling site, hydrological parameters were measured at sub-surface (a few centimetres below the water surface), in low and high tide, including temperature, dissolved oxygen, salinity, and pH. For caged experiment, the previous parameters were also assessed at bottom level. Water column depth was also evaluated. Water turbidity was measured using a 20 cm black and white Secchi disc. A 3 L van Dorn bottle was used to collect water samples from the bottom. Besides water samples, sediment samples were also collected at the same sites for total mercury (Hg_t) analysis.

2.4.1. Wild fish survey

Fish were caught according to the procedure previously described, during winter (December 2004), spring (March 2005), summer (July 2005) and autumn (September 2005) at Laranjo basin and S. Jacinto (reference site) (Figure 1). Ten fish of approximately similar weight and length were analysed in each sampling moment.

Hg_t concentration in liver was determined and correlated with the biochemical responses. Additionally, Hg_t concentrations in muscle, water (dissolved and in suspended particulate material - SPM) and sediment were measured.

2.4.2. Caged fish experiment

Fish were caught in a low-contaminated site (S. Jacinto, Figure 1) and then transported to laboratory in aerated saltwater and allowed to stabilize for 2 weeks prior to experimentation. This stabilization period aimed the reduction of inter-individual differences among the experimental fish group, also providing the elimination of previously taken up chemicals and the adaptation to confinement. During stabilization, fish were kept in 80 L aquaria at room temperature, in aerated (dissolved oxygen level = 8.4±0.2 mg/L) and filtered artificial seawater Sera Premium (Germany) (23±0.1 g/L salinity), under natural photoperiod, being daily fed with polychaete worms (*Nereis* sp.) collected in a clean area of Ria de Aveiro.

The experiment was conducted by caging mullets, during 3 days, in three precise sites of Laranjo basin differing on their distances to the metal contamination source (LAR1, LAR2 and LAR3 - Figure 1). Reference groups were caged in S. Jacinto. In the same day, a group of 10 fish was sacrificed in the laboratory to constitute t_0 group. In order to assess the effect of fish position in the water column on mercury uptake and subsequent responses, two 80 L cages were placed at each site, one in the surface and the other close to sediment. Surface cages were maintained in a submerged position (30-cm depth) by a buoy-anchor system. Bottom cages were set at 15 cm from the sediment to avoid the direct contact. When transferred to cages, all fish were visually examined and found to be in adequate condition. During field exposure, fish were kept without any food supply. The study was performed in December 2004. After the exposure and liver sampling, oxidative stress and biotransformation parameters were evaluated. Hg_t content was determined in liver and muscle, as well as in water (dissolved and SPM) and sediment.

2.5. Mercury analyses

2.5.1. Total mercury (Hg_t) in the water column and in sediment

Water samples were filtered with 0.45 µm Millipore filters. The filtrate was then acidified with HNO_3 (Merck, mercury-free) to pH < 2 and stored at 4 ºC until analysis. SPM collected in the filters was oven dried at 60 ºC until constant weight. Procedure blanks were always run with samples and its contribution corrected when necessary.

Total dissolved mercury concentrations were measured by Cold Vapour Atomic Fluorescence Spectrometry (CV-AFS; PSA model Merlin 10.023 equipped with a detector PSA model 10.003), using tin(II) chloride as reducing agent, after addition of 500 µL of a saturated solution of potassium persulfate to 50 mL of filtered water and irradiation by a UV lamp (1000 W) for 30 min. Following irradiation, the excess of oxidant was reduced with 37.5 µL of hydroxylamine solution 12% (w/v), prior to analysis (Mucci *et al.*, 1995). The equipment was calibrated every day with acidified (HNO_3 mercury-free) standard solutions prepared from a 1000 mg/L solution (BDH). The detection limit of CV-AFS technique was 0.5 ng/L.

Mercury in SPM was also determined by CV-AFS, after digestion of the filters in glass reactors with 50 mL of a solution 4 mol/L HNO_3, at 60 ºC for 4 h (Pereira *et al.*, 1995, 1998). Results presented for Hg_t concentrations in water column (total water), are always

the sum of dissolved and SPM metal concentrations, expressed in µg/L, taking into account the mass of SPM and the volume of filtered water.

Sediment samples were oven-dried to constant weight at 60 ºC homogenized and sieved through a 1 mm sieve, prior to analysis. Samples were analysed by Atomic Absorption Spectrometry (AAS) with thermal decomposition of the sample using the equipment LECO AMA-254 (Advanced Mercury Analyser), with no pre-treatment of samples (Costley *et al.*, 2000). Accuracy was assessed with certified reference materials (CRMs) from the National Research Council of Canada (NRCC). The CRMs used were MESS-3 (0.091±0.009 mg Hg/Kg) and PACS-2 (3.04±0.20 mg Hg/Kg) (both for sediments).

2.5.2. Total mercury (Hg_t) in tissues

Muscle and liver samples were also analysed by AAS with thermal decomposition of the samples. As in sediment analysis, accuracy was assessed using certified reference materials, namely TORT-2 (0.27±0.06 mg Hg/Kg) and DORM-2 (4.64±0.26 mg Hg/Kg) (both for biological samples).

2.6. Biochemical analysis in liver

2.6.1. Liver homogenization and fractioning

Liver was homogenized in a 1:11 w/v ratio (liver weight:buffer volume) of 0.1 mol/L Tris–HCl (pH 7.4) containing 0.15 mol/L KCl and 20% glycerol, using a Potter–Elvehjem glass–Teflon homogenizer, at 2500 rpm. Microsomes were obtained by differential centrifugation, at 4°C, in a Beckman Optima TL Ultracentrifuge (TLA-100.4 fixed angle rotor), according to the methods of Lange *et al.* (1993) and Monod and Vindimian (1991). The homogenate was first centrifuged at 15000 rpm for 20 min to remove cell debris, nuclei, mitochondria, and lysosomes. The supernatant was collected and recentrifuged at 50000 rpm for 75 min. The resulting microsomal pellet was resuspended in 200 µL of the previous buffer. Both cytosolic and microsomal fractions were frozen in liquid nitrogen, and stored at −80 °C until use.

2.6.2. Antioxidant responses and peroxidative damage

. Total glutathione (GSH_t) content

GSH_t content was determined in the cytosolic fraction, adopting the enzymatic recycling method using glutathione reductase (GR) excess, whereby the sulfhydryl group

of GSH reacts with DTNB (Ellman's reagent) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample (Tietze, 1969; Baker *et al.*, 1990). Protein content was precipitated with TCA (12%). Formation of TNB was measured by spectrophotometry at 412 nm. It should be noted that oxidized glutathione is converted to GSH by GR in this system, which consequently measures total GSH. The results were expressed as nmol TNB formed/min/mg protein using a molar extinction coefficient of 14.1×10^3 M^{-1} cm^{-1}.

. Glutathione-S-transferase (GST) activity

GST activity was determined in the cytosolic fraction, using CDNB as substrate, according to the method of Habig *et al.* (1974). The assay was carried out, at 25 ºC, in a quartz cuvette with a 2 mL mixture of 0.2 mol/L phosphate buffer (pH 7.4), 0.2 mmol/L CDNB and 0.2 mmol/L GSH. The reaction was initiated by the addition of 10 µL sample, and the increase in absorbance was recorded at 340 nm during 3 min and enzyme activity calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

. Catalase (CAT) activity

CAT activity was assayed by the method of Claiborne (1985) as described by Giri *et al.* (1996). Briefly, the assay mixture consisted of 1.95 mL phosphate buffer (0.05 mol/L, pH 7.0), 1 mL hydrogen peroxide (0.019 mol/L) and 50 µL of cytosolic fraction in a final volume of 3 mL. Change in absorbance was recorded at 240 nm and CAT activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

. Lipid peroxidation (LPO)

The determination of LPO was carried out on the previously prepared tissue homogenate, based in the measurement of thiobarbituric acid reactive substances (TBARS), according to Ohkawa (1979) and Bird and Draper (1984), as adapted by Filho *et al.* (2001) and Torres *et al.* (2002). The absorbance of each sample was measured at 535 nm. The rate of LPO was expressed as nmol of TBARS formed per milligram of fresh tissue, using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. This parameter elucidates the damage that occurs in membrane lipids as a result of ROS intervention.

2.6.3. Phase I biotransformation

. Ethoxyresorufin-*O*-deethylase (EROD) activity

EROD activity was measured in microsome suspension as described by Burke and Mayer (1974). The reaction was carried out, at 25 ºC, in the fluorometer cuvette containing 1 mL 0.5 µmol/L ethoxyresorufin (in homogenization buffer, pH 7,4) and 50 µL of microsomal suspension. The reaction was initiated by adding 10 µL of NADPH (10 mmol/l) and the progressive increase in fluorescence, resulting from the resorufin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). EROD activity was expressed as pmol/min/mg protein.

. Cytochrome P450 (P450) content

Microsomal P450 content (extinction coefficient 91 $mM^{-1}.cm^{-1}$) was determined using the dithionite-reduced carbon monoxide difference spectrum between 450 and 490 nm, as described by Stegeman *et al*. (1979). The conversion of the native total spectral P450 to P420 was measured as an estimation of P450 degradation. P420 content (extinction coefficient 111 $mM^{-1}.cm^{-1}$) was determined and expressed as the ratio P420 and the sum P420 and P450 [P420/(P420+P450)].

2.6.4. Protein measurement

Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornal *et al*., 1949) using bovine serum albumin (E. Merck-Darmstadt) as a standard.

2.7. Statistical analysis

SigmaStat software (SPSS Inc.) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between fish groups, followed by Tukey test (Zar, 1996). Differences between means were considered significant when $P < 0.05$. The relationship between biochemical parameters and Hg_t in tissues was explored using linear regression analyses. The correlation coefficient (r) was calculated and its statistical significance (p) was determined from table of Critical Values for Correlation Coefficient, r in Zar (1996).

3. RESULTS

3.1. Wild fish survey

The hydrological parameters including water temperature (T), dissolved oxygen (DO), salinity, pH, turbidity and depth, for each sampling season, are depicted in table I.

Table I

Hydrological characteristics determined seasonally on reference (REF) and contaminated (LAR) sites at Ria de Aveiro: water temperature (T), dissolved oxygen (DO), salinity, pH, turbidity and depth.

Season	Site	Tide	Position	T (ºC)	DO (mg/l)	Salinity (‰)	pH	Turbidity (m)	Depth (m)
Winter	REF	Low	Surface	13.5	9.89	35	8.48	1.85	2.9
			Bottom	14.5	9.78	33	8.47		
		High	Surface	14.6	9.77	35	8.46	2.5	7.7
			Bottom	13.6	9.9	37	8.48		
	LAR	Low	Surface	13.5	8.79	15	7.48	0.4	2.05
			Bottom	13.5	10.5	14	7.62		
		High	Surface	13.8	8.62	21	7.75	0.8	1.8
			Bottom	13.8	8.61	23	7.76		
Spring	REF	Low	Surface	12	10.69	31	8.39	2	2.5
			Bottom	11.8	10.33	29	8.34		
		High	Surface	11.2	10.33	25	7.77	2.9	7
			Bottom	11	9.98	33	8.29		
	LAR	Low	Surface	11.9	8.67	16	7.76	0.5	2
			Bottom	11.8	8.56	18	7.83		
		High	Surface	12.3	9.88	24	8.28	0.8	2
			Bottom	11.9	9.93	27	8.32		
Summer	REF	Low	Surface	23.5	8.37	36	8.39	0.7	1.5
			Bottom	22.8	8.67	35	7.99		
		High	Surface	19.1	9.47	35	8.05	2.6	7
			Bottom	19.7	9.17	35	8.04		
	LAR	Low	Surface	27.7	5.37	35	7.052	0.7	1
			Bottom	27.4	6.12	35	7.93		
		High	Surface	23	6.7	36	8.05	1.2	2.8
			Bottom	24.1	6.86	25	7.75		
Autumn	REF	Low	Surface	18.1	7.52	35	7.42	0.9	1.2
			Bottom	18.3	6.65	35	8.04		
		High	Surface	16.8	7.07	35	7.88	7	7.5
			Bottom	16.9	6.99	35	7.32		
	LAR	Low	Surface	19.3	4.02	35	7.67	0.5	1
			Bottom	20.2	3.77	35	7.82		
		High	Surface	18.6	5.49	37	7.29	1.3	2.7
			Bottom	18.5	5.6	35	7.52		

3.1.1. Total mercury (Hg_t) levels in the environment

Hg_t levels determined in the water column (dissolved mercury and associated with suspended particulate matter) at two tide conditions (high and low tide) and in surface and bottom waters were always low in the two sampling stations (Table II).

Table II

Environmental total mercury (Hg_t) concentrations determined seasonally in water (sum of dissolved and particulate fraction) from reference (REF) and contaminated (LAR) sites at Ria de Aveiro.

Season	Site	Tide	Position	Hg_t in Water (µg/L)
Winter	REF	Low	Surface	0.6272
			Bottom	0.8245
		High	Surface	0.2580
			Bottom	mv
	LAR	Low	Surface	1.5917
			Bottom	3.3948
		High	Surface	1.4788
			Bottom	3.2966
Spring	REF	Low	Surface	1.9255
			Bottom	3.3845
		High	Surface	0.5131
			Bottom	1.1205
	LAR	Low	Surface	1.8848
			Bottom	1.4974
		High	Surface	0.9525
			Bottom	0.7297
Summer	REF	Low	Surface	0.3893
			Bottom	0.5906
		High	Surface	0.3469
			Bottom	0.2666
	LAR	Low	Surface	0.7365
			Bottom	0.5620
		High	Surface	0.3514
			Bottom	0.1774
Autumn	REF	Low	Surface	0.1666
			Bottom	0.3238
		High	Surface	0.2463
			Bottom	0.5150
	LAR	Low	Surface	0.8356
			Bottom	2.7331
		High	Surface	1.0655
			Bottom	0.3620

mv = missed value

Most of the times, the higher mercury concentrations occurred at deep layers of the water column during low tide conditions and mainly in autumn and winter surveys. This is

probably due to the higher volumes of the water discharged from the upstream channels, enriched in mercury, into the Laranjo Basin at this time of the year. In the reference site, higher concentrations were found in the bottom water sample, both in high and low tide conditions; at this site no relevant differences were found in Hg_t concentrations in water column, during the surveyed seasons.

As expected, higher Hg_t concentrations were found at LAR site near the anthropogenic source of the metal. The differences observed along the seasons are mainly due to the high hydrodynamic variability of the studied area. The higher concentrations found in the bottom can be related with remobilisation processes due to tidal dynamics.

3.1.2. Total mercury (Hg_t) levels in liver and muscle

Fish captured at LAR site revealed, in all seasons, a significant liver Hg_t increase when compared with reference site (REF), exhibiting increments between 2.5 (spring) and 3.7 times (autumn) (Figure 2A).

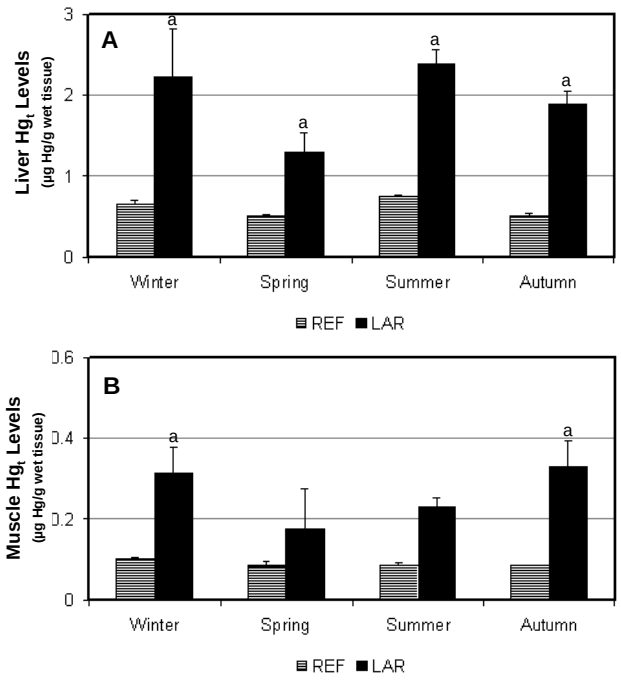

Figure 2 - Mean total mercury concentrations in the liver (A) and muscle (B) of indigenous *Liza aurata* collected seasonally from Ria de Aveiro. The letter (a) denotes statistically significant differences ($p< 0.05$) between mercury-contaminated (LAR = Laranjo basin) and reference (REF) sites within each season. Error bars represent the standard error.

A significant Hg_t increase was also determined in the muscle of fish captured at LAR site, comparing with REF site, in winter and autumn (Figure 2B), though all seasons exhibited Hg_t muscle increments ranging from 2.1 (spring) to 3.9 times (autumn).

Fish captured at LAR site showed different ratios liver Hg_t/muscle Hg_t along the year. This ratio was 1.8 in autumn, 2.2 in winter and spring, reaching the highest value - 3.2 - in summer.

3.1.3. Antioxidant responses and peroxidative damage in liver

Liver GSH_t levels (Figure 3A) exhibited a significant increase in LAR site (1.3 times) in spring, relative to REF site. On the other hand, enzymatic antioxidants GST (Figure 3B) and CAT (Figure 3C) displayed no statistical differences comparing LAR and REF sites, despite a general tendency to their increase.

The evaluation of peroxidative damage (Figure 3D) showed a significant LPO decrease at LAR site in summer, comparing with REF site.

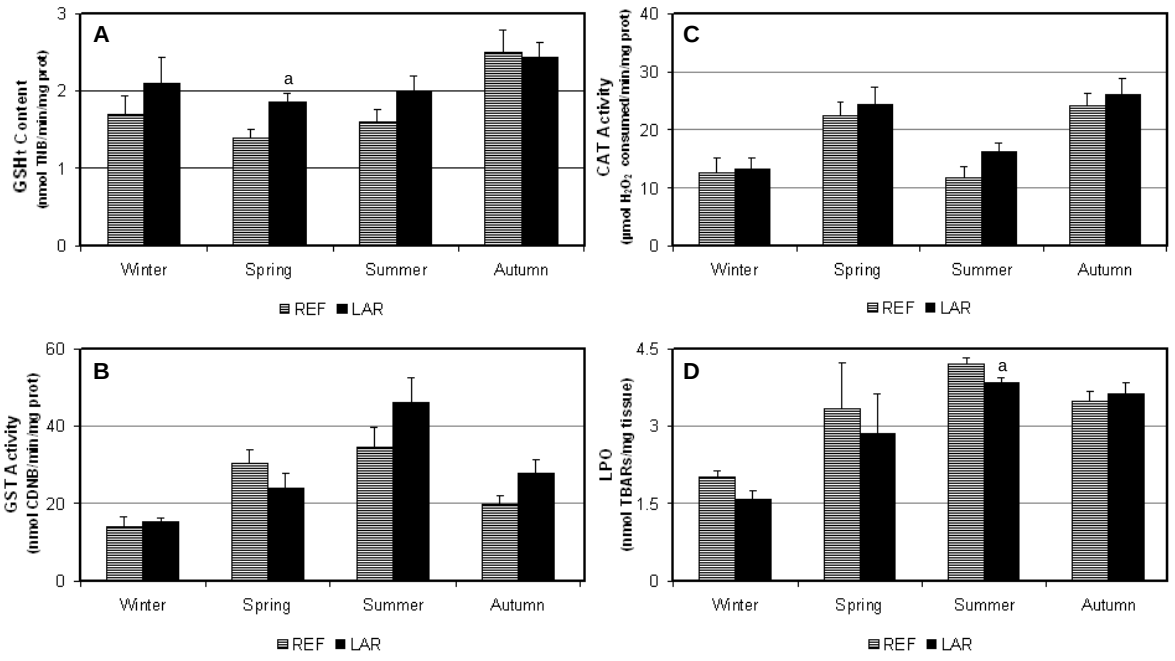

Figure 3 - Mean hepatic total glutathione (GSH_t) content (A), glutathione-*S*-transferase (GST) activity (B), catalase (CAT) activity (C), and lipid peroxidation (LPO) (D) in indigenous *Liza aurata* collected seasonally from Ria de Aveiro. The letter (a) denotes statistically significant differences ($p< 0.05$) between mercury-contaminated (LAR = Laranjo basin) and reference (REF) sites within each season. Error bars represent the standard error.

3.1.4. Liver phase I biotransformation

Fish captured at LAR in spring revealed a significant increase in liver EROD activity compared with the REF site (7.9 times increment) (Figure 4A). A considerable liver EROD activity increase was also observed in summer, though not significant.

On the other hand, concerning quantification of total P450 content (Figure 4B), only in autumn significant differences were detected, increasing 2 times. Relatively to the ratio P420/(P420+P450) (Figure 4C), no differences were observed between LAR and REF groups.

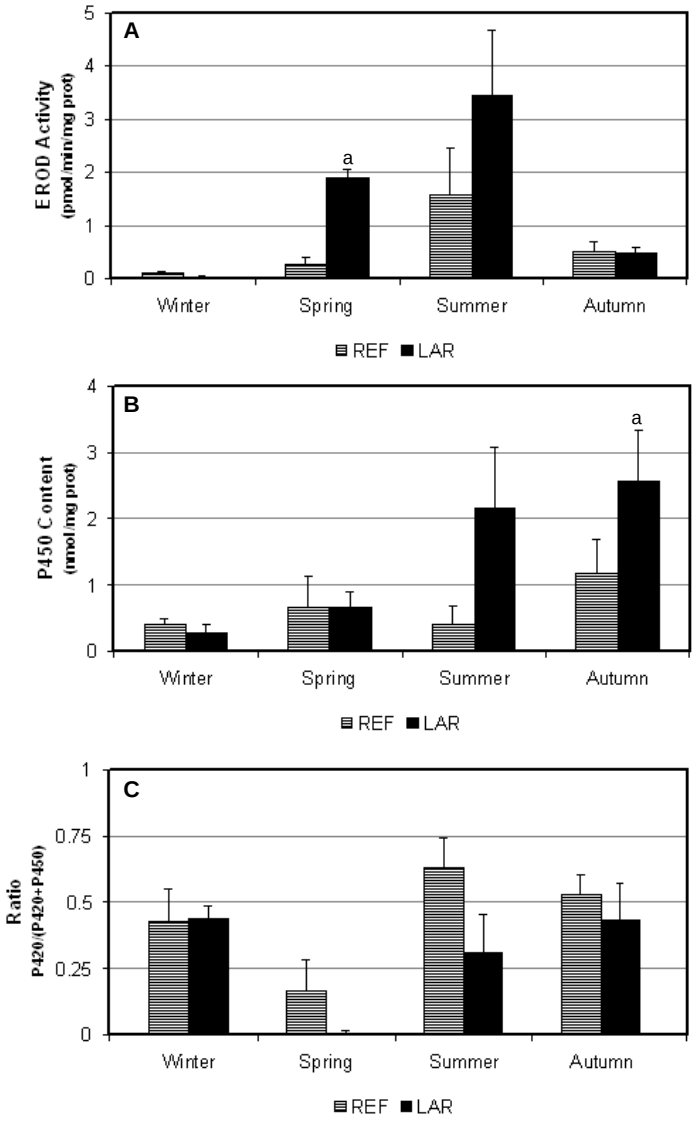

Figure 4 - Mean hepatic ethoxyresorufin *O*-deethylase (EROD) activity (A), cytochrome P450 (P450) content (B) and ratio P420/(P420+P450) (C) in indigenous *Liza aurata* collected seasonally from Ria de Aveiro. The letter (a) denotes statistically significant differences ($p < 0.05$) between mercury-contaminated (LAR = Laranjo basin) and reference (REF) sites within each season. Error bars represent the standard error.

3.1.5. Correlations between biological parameters

The relationship between liver and muscle Hg_t (Figure 5) was statistically tested, demonstrating a significant correlation when all sampling seasons were considered.

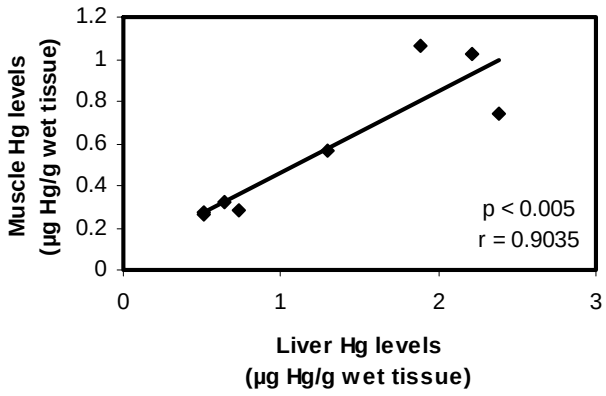

Figure 5 - Correlation between total mercury concentrations in the liver and muscle of *Liza aurata* collected in Ria de Aveiro. Statistical significance and correlation coefficient are represented by p and r, respectively.

3.2. Caged fish experiment

The inter-sites comparisons were carried out separately for surface or bottom groups. The biological responses corresponding to surface group at LAR1 were not measured due to cage disappearance. Nevertheless, data concerning hydrological parameters and Hg_t environmental levels are presented. The initial condition of fish was evaluated at moment of transplantation from laboratory to field (t_0).

The hydrological parameters previously mentioned were also measured for this component in all caging sites (REF, LAR1, LAR2 and LAR3) and presented in table III.

Table III

Hydrological characteristics of reference (REF) and contaminated (LAR1, LAR2, LAR3) sites on the caging experiment at Ria de Aveiro: water temperature (T), dissolved oxygen (DO), salinity, pH, turbidity and depth.

Site	Tide	Position	T (ºC)	DO (mg/l)	Salinity (‰)	pH	Turbidity (m)	Depth (m)
REF	Low	Surface	13.5	9.89	35	8.48	1.85	2.9
		Bottom	14.5	9.78	33	8.47		
	High	Surface	14.6	9.77	35	8.46	2.5	7.7
		Bottom	13.6	9.9	37	8.48		
LAR1	Low	Surface	13.8	8.64	9	7.42	0.35	2.7
		Bottom	13.8	8.9	17	7.69		
	High	Surface	13.8	8.83	23	7.78	0.85	1.8
		Bottom	13.5	9.66	25	7.95		
LAR2	Low	Surface	13.5	8.79	15	7.48	0.4	2.05
		Bottom	13.5	10.5	14	7.62		
	High	Surface	13.8	8.62	21	7.75	0.8	1.8
		Bottom	13.8	8.61	23	7.76		
LAR3	Low	Surface	13	10.3	2	7.06	0.25	2.15
		Bottom	13	10.13	6	7.29		
	High	Surface	14.5	8.5	18	7.58	0.5	2.7
		Bottom	14.2	8.43	20	7.69		

3.2.1. Total mercury (Hg_t) levels in the environment

Hg_t concentrations in water column (Table IV) at the reference site and at the three caging sites at Laranjo basin were always very low (less than 10 µg/L in most of the samples and only with one value higher than 40 µg/L). All the values are less than 1000 µg/L, the permitted value by law for mercury concentrations in water column of aquatic systems.

During high and low tide conditions Hg_t concentrations in the water column are of the same order of magnitude. The higher Hg_t concentrations were found at the bottom almost in all studied stations located in the Laranjo basin. The differences observed between mercury concentrations in bottom and surface layers are attenuated as we move far away from the contamination source.

Hg_t concentrations in sediments (Table IV) revealed the pronounced human-induced environmental mercury gradient in the lagoon with the highest concentrations found near the contamination source. This way, the results obtained for mercury concentrations in

the sediments are mostly related with distance to the industrial source of the metal, with concentrations as high as 37 ng/mg measured in the most contaminated area, whereas in the reference site concentrations were very low (0.001 ng/mg).

Table IV

Environmental total mercury (Hg_t) concentrations in water (sum of dissolved and particulate fraction) and sediment of reference (REF) and contaminated (LAR1, LAR2, LAR3) sites on the caging experiment at Ria de Aveiro.

Site	Tide	Position	Hg_t in Water (µg/L)	Hg_t in Sediment (ng/mg)
REF	Low	Surface	0.6272	0.001
		Bottom	0.8245	
	High	Surface	0.258	
		Bottom	mv	
LAR1	Low	Surface	0.6833	3.0
		Bottom	0.7542	
	High	Surface	0.6876	
		Bottom	0.4056	
LAR2	Low	Surface	1.5917	7.1
		Bottom	3.3948	
	High	Surface	1.4788	
		Bottom	3.2966	
LAR3	Low	Surface	6.541	36.9
		Bottom	49.5915	
	High	Surface	2.585	
		Bottom	15.2026	

mv = missed value

3.2.2. Total mercury (Hg_t) levels in the liver and muscle

Liver Hg_t levels revealed significant increases in LAR2 and LAR3 compared with REF site, in both surface and bottom groups, showing about 1.5 times increments (Figure 6A). Taking into account the bottom groups, LAR2 and LAR3 displayed Hg_t levels significantly higher than LAR1.

Differences between surface and bottom groups were only detected in LAR2 site.

Muscle Hg_t levels showed no significant differences between LAR and REF sites (Figure 6B).

Fish captured at LAR sites showed an increase in the ratio liver Hg_t/muscle Hg_t with the proximity to the contamination source. This ratio was 6.1, 8.9 and 9.4 respectively for LAR1, LAR2 and LAR3.

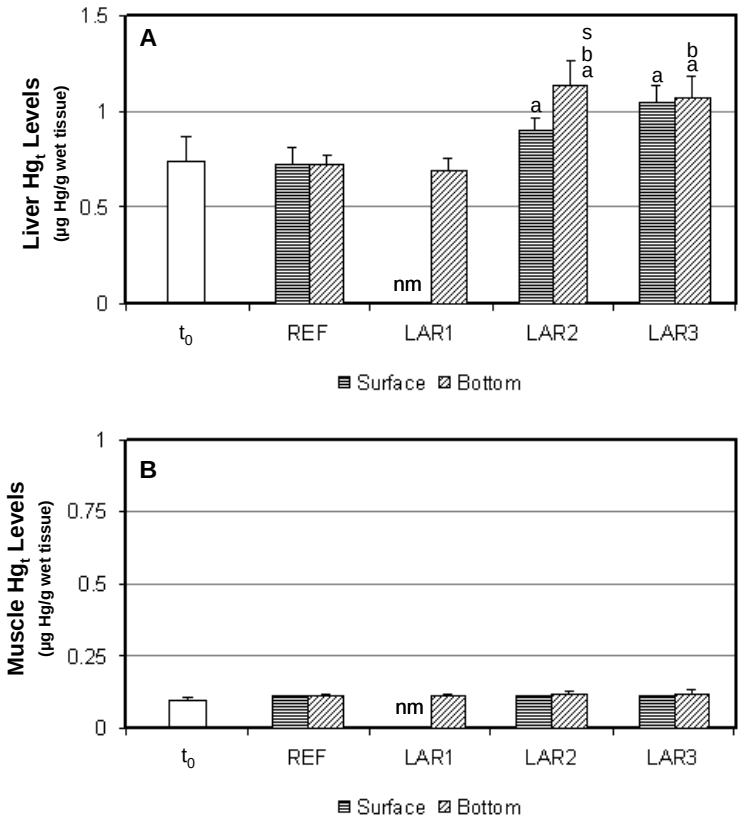

Figure 6 - Mean total mercury concentrations in the liver (A) and muscle (B) of *Liza aurata* caged during 3 days at different Ria de Aveiro locations, i.e. within a mercury-contaminated area (Laranjo basin - LAR1, LAR2 and LAR3) and a reference site (REF). t_0 represents the value at the beginning of the experiment. Letters denote statistically significant differences ($p< 0.05$): (a) *vs* REF; (b) *vs* LAR1; (s) *vs* Surface. Error bars represent the standard error. nm = not measured.

3.2.3. Antioxidant responses and peroxidative damage in liver

GSH_t content showed a highly significant increase (5 times) at the LAR3 bottom group when compared with the correspondent REF group, as well as when compared with LAR2 bottom group. A significant GSH_t difference between surface and bottom groups was found at LAR3, representing a 2.8 times increment (Figure 7A). GST activity revealed no alterations in fish caged at LAR sites comparing to REF (Figure 7B).

Liver CAT activity demonstrated a single significant elevation (1.4 times) detected in LAR3 bottom group in comparison with the correspondent REF group (Figure 7C).

No peroxidative damage was observed in fish caged at LAR sites (surface and bottom) in comparison with REF groups (Figure 7D).

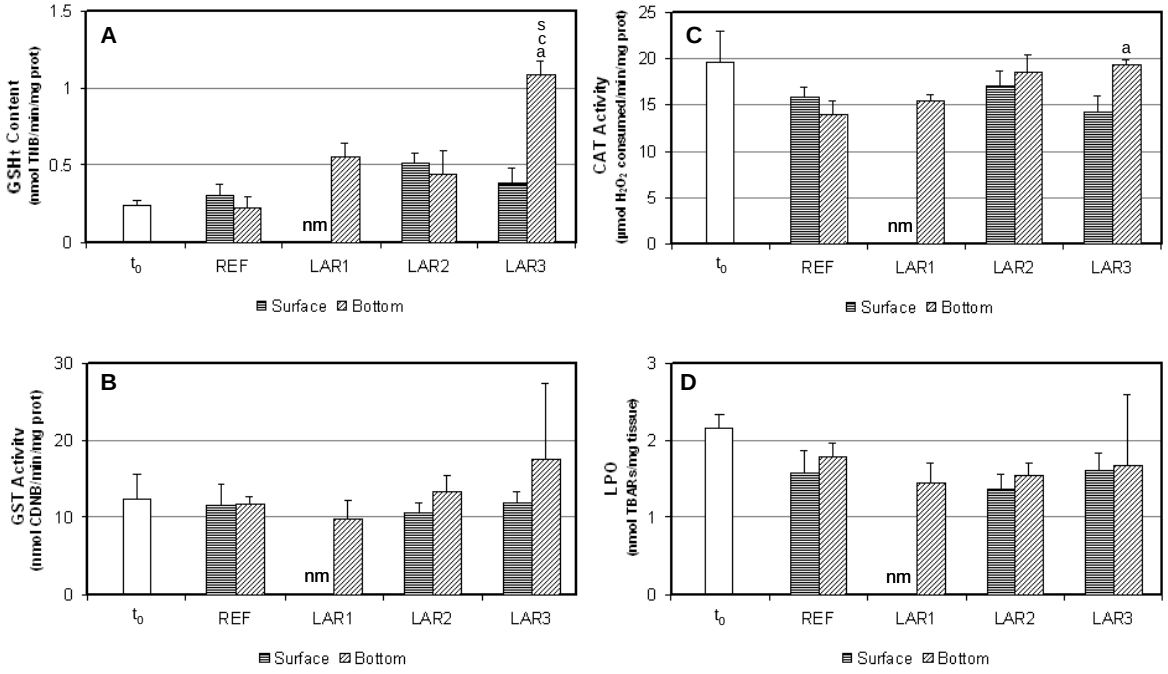

Figure 7 - Mean hepatic total glutathione (GSH_t) content (A), glutathione-*S*-transferase (GST) activity (B), catalase (CAT) activity (C), and lipid peroxidation (LPO) (D) in *Liza aurata* caged during 3 days at different Ria de Aveiro locations, i.e. within a mercury-contaminated area (Laranjo basin - LAR1, LAR2 and LAR3) and a reference site (REF). t_0 represents the value at the beginning of the experiment. Letters denote statistically significant differences ($p< 0.05$): (a) *vs* REF; (b) *vs* LAR1; (c) *vs* LAR2; (s) *vs* Surface. Error bars represent the standard error. nm = not measured.

3.2.4. Liver phase I biotransformation

The analysis of EROD activity revealed a significant decrease in LAR2 bottom fish in comparison with the REF fish group, exhibiting a 12 times decline (Figure 8A).

Relatively to P450 (Figure 8B) and the ratio P420/(P420+P450) (Figure 8C), no differences were observed among surface or bottom groups.

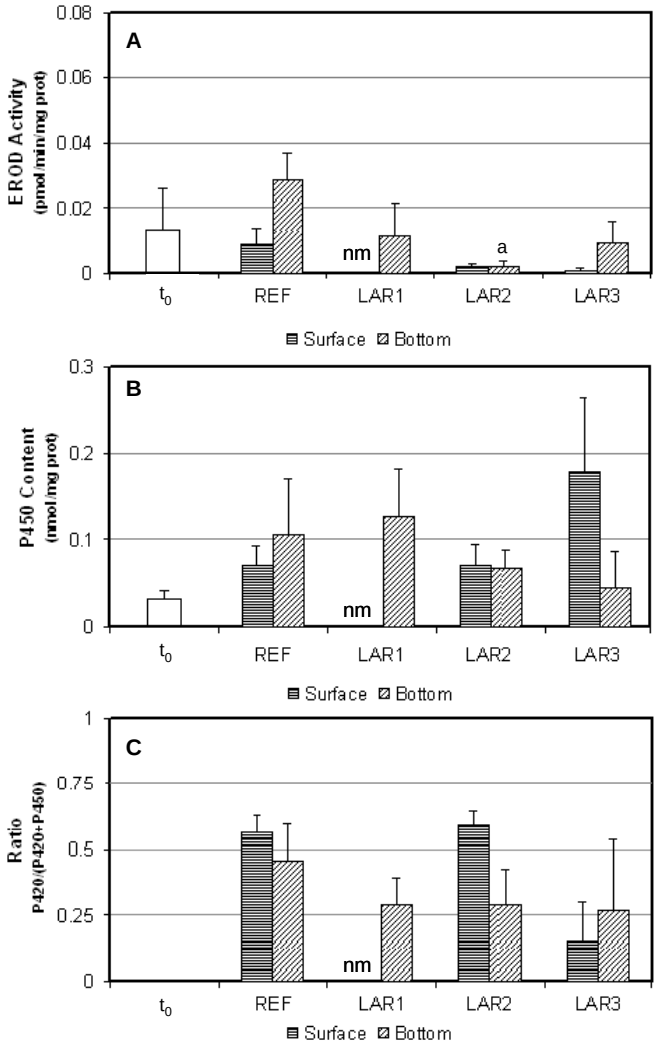

Figure 8 - Mean hepatic ethoxyresorufin *O*-deethylase (EROD) activity (A), cytochrome P450 (P450) content (B) and ratio P420/(P420+P450) (C) in *Liza aurata* caged during 3 days at different Ria de Aveiro locations, i.e. within a mercury-contaminated area (Laranjo basin - LAR1, LAR2 and LAR3) and a reference site (REF). t_0 represents the value at the beginning of the experiment. The letter (a) denotes statistically significant differences ($p< 0.05$) between LAR and REF sites. Error bars represent the standard error. nm = not measured.

3.2.5. Correlations between biological parameters

The relationships between parameters were statistically tested analysing the data obtained in all sampling sites. A negative correlation was found when P450 and P420 were considered (Figure 9).

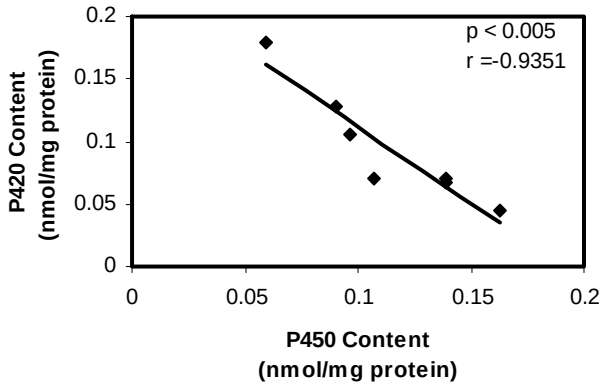

Figure 9 - Correlation between hepatic cytochrome P450 (P450) content and cytochrome P420 (P420) content in caged *Liza aurata*. Statistical significance and correlation coefficient are represented by p and r, respectively.

4. DISCUSSION

4.1. Wild fish survey

4.1.1. Total mercury (Hg_t) levels in fish tissues

The increased Hg_t levels observed in the liver and muscle of fish from Laranjo (LAR), with respect to those from the reference site (S. Jacinto), clearly indicated exposure to mercury and, consequently, its uptake. Liver always demonstrated higher levels (from 5 to 10 times, approximately) in comparison with muscle, which is in agreement with previous investigations on fish exposure to mercury, either in field (Cizdziel *et al.*, 2003) or in laboratory under two modes of exposure, i.e. via water (Serra *et al.*, 1996) or via food (Elia *et al.*, 2003). This tissue differentiated mercury accumulation is related to their physiological role namely metabolic capabilities together with its anatomic location, determining liver as a main target, keeping the muscle in a second line. Moreover, the correlation observed between liver and muscle Hg_t levels suggests that mercury accumulation in the muscle is associated with an exhaustion of metal retention by the liver.

Currently, the health authorities are concerned with the risk associated with mercury exposure mainly due to its teratogenic effects and irreversible neurological damage cause on humans (De Flora *et al.*, 1994; Gilbertson and Carpenter, 2004). Previous studies on mercury speciation reported a percentage between 60 and 100% of organic mercury in the muscle of different fish species (Joiris *et al.*, 1999; Storelli *et al.*, 2003, 2005). Thus,

seafood consumption appears as a major route of methylmercury exposure for humans. The definition of the maximum permitted limit of total mercury concentration in fish edible tissues resulted in a variety of values. In Europe, the limit value is set at 0.5 µg/g wet wt (except for some species, in which it is raised to 1 µg/g wet wt) (Official Journal of the European Communities, 1994), in Japan at 0.4 µg/g wet wt, whereas US Food and Drug Administration (FDA) set a level of 1 µg/g wet wt (Storelli *et al.*, 2005). Therefore, the Hg_t concentrations found in the current study in *L. aurata* muscle did not exceed the previous regulatory limits.

Nevertheless, it must be highlighted that statistical differences in mercury intake were found between sub-populations with high and low fish consumption (Holsbeek *et al.*, 1996; Nakagawa *et al.*, 1997). In recognition of that fact, Canadian authorities redefined the mercury levels recommended in edible portions of fish from 0.5 to 0.2 µg total mercury/g wet wt in the case of people who consume more fish than the population average (Health and Welfare Canada, 1979). Concerning Portugal, where this study was carried out, it should be further considered that it presents the highest fish consumption rate *per capita* in the European Union (EU). Due to its piscatorial tradition and geographic localization, each person consumes approximately 62 kg of fish per year (≈170 g/day or 1.200 g/week) (Lourenço *et al.*, 2006).

The joint Food and Agriculture Organisation/World Health Organisation (FAO/WHO) expert committee on food additives has established regulatory guidelines regarding the dietary mercury intake. It was recommend a provisional tolerable weekly intake (PTWI) of 300 µg of total mercury per person, amount equivalent to 5 µg/kg body wt of total mercury (WHO, 2003). Interpreting the current results in terms of the PTWI defined by FAO/WHO, by using the means of fish weekly consumption on the Portuguese population (1.200 g) (Lourenço *et al.*, 2006), the mean mercury concentrations in *L. aurata* from Laranjo (0.26 µg/g wet wt), and a human body weight of 60 kg, the weekly intake calculated would be 5.2 µg/kg body wt for total mercury. Consequently, the estimated weekly intake of total mercury exceeds the established PTWI.

In addition, US EPA, based on an independent study by the National Academy of Sciences (NRC, 2000), defined the reference dose (RfD) (the highest possible level of daily oral mercury exposure) as 0.1 µg/person kg/day. Adopting the previous premises, the RfD calculated for *L. aurata* will be 0.78, which is almost 8 times higher than the EPA safety limit.

Hence, in view of these statements, the assumption of fish from Laranjo as suitable for human consumption must be carefully evaluated. In addition, is important to consider that only juvenile fish were analyzed in the current study, which point out the increased risk to human consumers related with the ingestion of larger specimens presenting, supposedly, higher Hg_t levels as a consequence of a longer exposure time (Abreu *et al.*, 2000; Storelli and Marcotrigiano, 2000).

4.1.2. Antioxidant responses and peroxidative damage in liver

Both redox-active and redox-inactive metals may cause an increase in production of ROS such as hydroxyl radical ($HO^{\bullet}$), superoxide radical ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2). Enhanced ROS generation can overwhelm cells' intrinsic antioxidant defenses and result in a condition known as "oxidative stress" (Ercal *et al.*, 2001). Mercury, as a redox-inactive metal, does not undergo redox cycling and thus, depletion of cells' major antioxidants, particularly thiol-containing antioxidants and enzymes, seems to be an important indirect mechanism for oxidative stress induced by this metal (Stohs and Bagchi, 1995).

On the other hand, the organism makes important adaptive changes in response to sulfhydryl-reactive metals such as mercury. Studies in rats illustrated the importance of GSH metabolism in short and long-term exposure to $MeHg^+$, revealing an up-regulation of mRNA encoding for γ-glutamylcysteine synthetase (Woods and Ellis, 1995), which is the rate-limiting enzyme in GSH synthesis. Concomitantly, there was a similar magnitude of increase in the steady state levels of GSH, and the activities of GR and GPx.

In agreement with these findings, current data revealed an increment on liver GSH_t (spring) in fish captured at mercury contaminated area. These data illustrate a protective and adaptive response to mercury exposure in hepatic cells, highlighting the central role of GSH as an antioxidant. Reduced GSH is involved in the formation of GSH-S conjugates with the ionic mercury forms, forming linear II covalent complexes (Rabenstein, 1989). It acts as an intracellular chelator, preventing the nucleophilic interaction of metals with the main cellular structures (Maracine and Segner, 1998).

In contrast, no alterations were observed in the antioxidant enzymes assessed (CAT and GST). According to Stohs and Bagchi (1995), mercury is a pro-oxidant that exerts oxidative stress via H_2O_2 production. Hence, assuming that CAT provides a first line of defence against H_2O_2, current data do not confirm the previous statement. Alternatively, it

can be hypothesised that GPx may have played the main role against H_2O_2, as previously suggested by Ahmad *et al*. (2006).

Globally, current antioxidants profile demonstrated that fish protection against ROS production was not compromised, neither confirming the mercury damaging action over antioxidant enzymes, nor the adaptive reaction expressed as an antioxidant activity induction. Considering the elevated Hg_t accumulated in the LAR fish liver and the sporadic activation of the assayed antioxidants, a second protective response to toxic metal exposure - metallothionein (MT) synthesis - may be suggested. These intracellular proteins, rich in cysteine (about 30%), "scavenge" sulfhydryl-reactive metals that enter the cell, providing an additional protective mechanism against mercury-induced toxicity. Given the known capacity of mercury to induce MTs in fish (Langston *et al*., 2002), we hypothesized that the regulatory strategy used by fish was mainly one of detoxification by the sequestration of this metal with MTs. This hypothesis is supported by a previous study where fish (*Perca flavescens*) environmentally exposed to cadmium (a non-essential and redox-inactive metal, like mercury) accumulated 75 % of the metal in the heat-stable protein cellular fraction, which includes low molecular weight peptides such as MTs (Kraemer *et al*., 2005). As stated by Kraemer *et al*. (2005), metals sequestered by MTs are considered detoxified, and thus are not bioavailable to more sensitive cellular fractions.

The absence LPO increases in *L. aurata* captured at Laranjo corroborates the effectiveness of the overall antioxidants mechanisms. Moreover, in summer a LPO decrease was observed in fish from that mercury contaminated site. In the presence of moderate ROS levels, fish adaptive mechanisms might have been activated providing to exposed fish a better protection against ROS in comparison with fish from the reference site. However, antioxidant responses observed in summer did not provide clear evidences in this direction.

4.1.3. Liver phase I biotransformation

Heavy metals are recognized as potent P450 inhibitors in fish (Fent and Stegeman, 1993) and various mechanisms have been proposed for metal-induced CYP1A inhibition. In the case of mercury, it can alter the activity of enzymes by binding to their functional groups or by displacing the metal associated with the enzyme (Viarengo, 1985), resulting in the EROD activity impairment (Viarengo *et al*., 1997; Oliveira and Santos, 2003). Thus, P450 parameters were included in the present study in order to evaluate the mercury

inhibitory potential, as well as to clarify the associated processes. Considering the Hg_t levels detected in the liver of LAR fish, it would be expectable a decrease on EROD activity. Surprisingly, neither EROD activity and P450 reflected a decrease tendency, nor P420 content, measured as the ratio P420/(P420+P450), was increased. Moreover, a significant EROD activity increase (spring) and P450 content (autumn) was observed, which might be an indication that mercury is accumulated in a harmless form, corroborating the idea presented above concerning MTs induction. When environmental exposures are under evaluation, it must be considered that EROD activity can reflect a balance between the action of inducers and inhibitors likely to affect fish simultaneously or in a sequential mode. Accordingly, the inability of mercury to inhibit P450 suggested by current findings seems to permit the inducers to exert their action, probably as a result of fish mobility into areas with different contamination patterns.

4.2. Caged fish experiment

4.2.1. Total mercury (Hg_t) levels in fish tissues

Liver Hg_t concentration reached its higher values at LAR2 and LAR3, which was expectable considering their closeness to the mercury contamination source. In addition, LAR2 fish noticed differences between surface and bottom cages, pointing out the importance of the sediment proximity on mercury uptake and liver accumulation. It was also confirmed that mercury released from the sediment could generate layers in water column with different metal levels.

In general, fish hepatic Hg_t levels clearly express its exposure to mercury and its rapid accumulation in this target organ. This is in agreement with Niimi and Kisson (1994) who found that fish can accumulate rapidly waterborne mercury. In contrast, no significant alterations were detected in muscle Hg_t levels, reinforcing the primary role of liver on mercury distribution and accumulation, as previously presented for wild fish survey. This is also in agreement with Giguère *et al.* (2004), who stated that the liver is typically important for metal accumulation and storage in fish, especially in metal contaminated environments.

The results demonstrated that 3 days of continuous exposure into an mercury contaminated area is not enough to increase Hg_t levels accumulated in the muscle. Comparing with the corresponding levels observed in wild fish, caged fish displayed liver and muscle Hg_t concentrations 3 and 10 times lower, respectively. Besides the exposure duration, an additional factor must be considered to explain this difference concerning the

impossibility of confined fish to contact directly with the sediment and feed on benthic invertebrates or detritus.

4.2.2. Antioxidant responses and peroxidative damage in liver

The increment on GSH_t content and CAT activity observed in LAR3 bottom group should be regarded as a *L. aurata* adaptive response to mercury exposure. Thus, CAT induction in particular represents a reaction to H_2O_2 overproduction, which was expected to occur following mercury exposure as stated by Stohs and Bagchi (1995). The results evidenced the crucial role of GSH on the protection against mercury-induced oxidative stress, since the increment of GSH_t levels increases the cellular ability to destroy free radicals and ROS. An incremented hepatic GSH_t content was previously observed in *Mugil cephalus* (Thomas and Wofford, 1984) and *Ictalurus melas* (Elia *et al*., 2000) laboratory exposed to Hg^{2+}. Nevertheless, in *Channa punctatus* a decrease of the thiol content was observed following mercur exposure (Rana *et al*., 1995).

GSH_t content at LAR3 exhibited a significantly higher level in bottom group when compared with surface group, underlining the importance of the sediment proximity. Nonetheless, this result cannot be explained by a higher Hg_t level accumulated in liver.

According to Elia *et al*. (2000) the GSH_t increase, observed in *I. melas,* was related with its reduced utilization, as a consequence of a GST activity reduction. This correlation is not perceptible in current study, since no evidence of GST inhibition was found. Hence, the increase on hepatic GSH_t content in *L. aurata*, under mercury exposure, is probably due to the enhanced hepatic uptake of amino acid substrates and the activities of biosynthetic enzymes, as previously suggested by Gallagher *et al*. (1992). Current findings also suggest that hepatic GST activity is not in the first line of defence against ROS, likely to be generated by mercury exposure. Previous fish studies on GST activity associated with mercury exposure provided contrasting results, since for the same species it was observed either GST activity inhibition (Elia *et al*., 2000) or induction (Elia *et al*., 2003), depending on the mercury concentration and exposure length.

Considering the similar elevation of hepatic Hg_t levels observed for LAR2 and LAR3 groups, it seems surprising that LAR2 groups did not display any significant alteration on antioxidant responses. This may be an indication of the involvement of other antioxidant agents besides those evaluated in the present study.

Despite the evidences of ROS production in LAR3 bottom group, no LPO was observed in the liver, indicating a successful ROS neutralization by hepatic antioxidants.

In contrary to wild fish, the induction of MTs cannot be cogitated in the current caged experiment due to the short exposure length carried out.

4.2.3. Liver phase I biotransformation

A general tendency to EROD activity reduction was noticed at LAR sites, exhibiting a significant inhibition in LAR2 bottom group. These data represent a confirmation of the mercury potential to inhibit EROD activity, previously demonstrated by *in vitro* incubation of fish liver microsomes in mercury containing media (Viarengo *et al.*, 1997; Oliveira *et al.*, 2004).

Total spectral P450 content was not affected and no significant conversion of P450 to P420 was found in LAR exposed fish, when compared with fish caged in the reference site. This may suggest a specificity of mercury inhibitory action to different P450 forms, since the inactivation of the EROD catalyst, CYP1A1, measured as a significant loss of EROD activity, was detected in the absence of a concomitant total P450 loss or degradation. Similar results were previously obtained by Fent and Bucheli (1994) and Fent *et al.* (1998) for *in vitro* exposure of fish microsomes to organotins.

The hepatic P450 dependent monooxygenase system plays a crucial role on the metabolism of a variety compounds. Current results indicate that fish exposure to mercury may impair its capability to detoxify environmental pollutants, as CYP1A dependent activity was clearly inhibited. On the other hand, the risk of endogenous compounds impaired biotransformation, including hormones, could not be demonstrated on the basis of current findings since no evidences of total P450 protein degradation were found.

Given the widespread adoption of EROD activity induction as biomarker of aquatic pollution, it must be highlighted that mercury may mask the contamination with polycyclic and polychlorinated aromatic hydrocarbons, if only CYP1A catalytic activity is monitored, as it was previously pointed out by Viarengo *et al.* (1997) and Oliveira and Santos (2003) *in vitro* observations.

4.3. General discussion

According to Chen and Chen (1999), the uptake of sediment-associated contaminants by fish may occur through three pathways: (1) fine particles re-suspended to water column which are taken up by filter-feeders via gill and digestive tract, (2) leaching of sedimentary contaminants to water which accumulated in fish body via respiration, and (3) direct contact and consumption of the sediment by bottom-dwellers/mud-eaters via

skin and intestine. Thus, it is likely that owing to the feeding habit and life style of *L. aurata*, it is likely that wild specimens might accumulate the sediment-associated mercury through all the three routes. On the other hand, in caged *L. aurata* only (1) and (2) pathways should be considered. This fact is probably a basis for the higher levels of metal accumulated, in both liver and muscle, in wild *L. aurata*. In any case, it was demonstrated that uptake of aqueous mercury represents an important route of exposure.

Analysing EROD results in caged and wild fish (winter) a question emerges. How can be explained the absence of EROD inhibition in wild fish presenting 2.25 μg/g Hg_t in the liver while caged fish exhibited such inhibition for a 2.5 times lower Hg_t? This fact seems to represent a rationale to the development of fish adaptation in longer exposure probably based on MTs production as previously stated.

5. CONCLUSIONS

The results of this study demonstrated that:

- Liver accumulates higher mercury levels in comparison with muscle and thus better reflecting environmental levels of contamination by this metal;

- No evidences of peroxidative damage were detected in fish captured or caged at Laranjo basin, which can be attributed to the effectiveness of antioxidant defences, namely GSH and CAT activity as observed in caged fish;

- The mercury potential to inhibit EROD activity was demonstrated in the caged experiment, whereas wild fish captured at Laranjo site exhibited a different response pattern reflecting the balance between CYP1A inducers and inhibitors;

- The uptake of aqueous mercury, mainly via gills, demonstrated its relevance on metals toxicity;

- Based on significant Hg_t bioaccumulation and antioxidant responses, *L. aurata* can be used as a suitable bioindicator of metal pollution in coastal areas.

In addition, it is reinforced the importance of monitoring mercury burden, particularly in the edible tissue of fish species included in the human diet, in order to safeguard public health, as well as the need to define the regulatory tissue thresholds, taking into consideration the fish consumption rate.

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CAPÍTULO II

Genotoxicidade do mercúrio em peixes (*Liza aurata*) selvagens e engaiolados ao longo de um gradiente de contaminação ambiental

Genotoxicity of mercury in wild and caged fish (*Liza aurata*) along an environmental contamination gradient

Genotoxicity of mercury in wild and caged fish (*Liza aurata*) along an environmental contamination gradient

ABSTRACT

Laranjo basin, in the Ria de Aveiro (Portugal), has been the subject of mercury contamination originating from a chlor-alkali plant. This area presents a well-described gradient of mercury contamination and no other important sources of pollutants, thereby offering a unique opportunity on the assessment of mercury toxicity under field conditions. The present study aims to determine the mercury genotoxic potential by measuring erythrocytic nuclear abnormalities (ENA) frequency in *Liza aurata*, a common mugilide. Wild fish were captured and analyzed seasonally, at the same time as fish were also caged for 3 days within Laranjo basin at three locations, differing on their distances to the mercury contamination source. In addition, total mercury (Hg_t) concentrations were determined in sediment, water, suspended particulate matter, and fish blood. For both wild and caged fish, the results from Laranjo were compared with those from an area not significantly polluted (S. Jacinto). Wild fish from Laranjo showed elevated ENA frequency in summer and autumn in concomitance with increased Hg_t levels in blood. Surprisingly, no ENA induction was observed in winter where the highest blood Hg_t level was measured. This particular result may be explained by an altered haematological dynamics related to high Hg_t levels in blood and haematopoietic organs, as supported by a decreased immature erythrocytes frequency, which affected ENA generation. Caged fish showed ENA induction only at the closest site to the pollution source. Moreover, the genotoxicity induction showed a correlation with Hg_t accumulated in blood. The caged fish experiment successfully demonstrated its suitability on the assessment of mercury contamination. The adopted biomarkers, applied to mullet species, can be successfully employed for biomonitoring anthropogenic contamination in both Atlantic and Mediterranean European coastal waters.

1. INTRODUCTION

Mercury, a non-essential element with no biochemical or nutritional function to organisms, is an ubiquitous environmental contaminant spread globally from natural geological emissions as well as anthropogenic activities, such as fossil fuel combustions (Pacyna *et al*., 2001), mining and smelting operations (WHO, 1990) and chlor-alkali industries (Wiener *et al.*, 2003). Inorganic mercury is the major form present in aquatic ecosystems, which may be converted into organic forms, namely methylmercury ($MeHg^+$), by biogeochemical processes. All forms of mercury are extremely toxic but $MeHg^+$ is of great concern especially as an aquatic contaminant because of its lipofilicity and consequent availability to organisms, increasing the risk to aquatic food webs.

In addition to the well-documented toxicity of mercury compounds, a considerable number of studies carried out over the last decades have investigated their genotoxic potential. A revision carried out by De Flora *et al*. (1994) indicated that mercury compounds often exert clastogenic effects in eukaryotes, mainly binding SH groups and acting as spindle inhibitors, thereby causing aneuploidy and/or polyploidy. Moreover, they can induce the appearance of reactive oxygen species (ROS), which can represent an indirect DNA damaging action (De Flora *et al*., 1994; Minissi *et al*., 1996). Despite the relative abundance of investigations carried out on this subject in a large variety of test systems, from bacteria to humans, a scarcity of studies in fish is manifested. Both organic and inorganic mercury compounds (Zoll *et al*., 1988; Al-Sabti, 1994), as well as elemental mercury (Nepomuceno *et al*., 1997), showed to be chromosomal genotoxicants eliciting *in vivo* formation of erythrocytic micronuclei (MN) in fish. A differential sensitivity of fish species towards the induction of erythrocytic MN and other nuclear abnormalities has been reported by Sanchez-Galan *et al*. (1999) and Ayllón and Garcia-Vazquez (2000). *In vitro* experiments with gill cell suspensions exposed to mercuric chloride detected high rate of DNA breaks (single and double stranded) measured as the comet assay (Arabi, 2004; Arabi and Alaeddini, 2005). According with the available literature analysis, it is perceptible that all studies on genotoxicity of mercury compounds in fish have been conducted in the laboratory conditions. The relevance of data concerning fish exposed in laboratory is often compromised by the use of environmentally unrealistic concentrations (Nepomuceno *et al*., 1997; Ayllón and Garcia-Vazquez, 2000), as well as by artificial modes of exposure such as a single exposure route (Zoll *et al*., 1988), intraperitoneal injection (Ayllón and Garcia-Vazquez, 2000) or the use of cell cultures/suspensions (Babich *et al*., 1990; Arabi and Alaeddini, 2005). Hence, in order to elucidate the

mechanisms involved on the mercury genotoxic action there is a need for further studies under more realistic exposure conditions, as well as the assessment of the relationship between genetic damage and mercury levels in target tissues.

The count of piscine MN has been widely accepted as an *in vivo* index of chromosome breaks and mitotic spindle apparatus dysfunction induced by clastogenic or aneugenic agents (Ayllón and Garcia-Vazquez, 2000; Stoiber *et al.*, 2004). More recently, other nuclear abnormalities were described (Carrasco *et al.*, 1990) and interpreted as nuclear lesions analogous to MN (Pacheco and Santos, 1996; Ayllón and Garcia-Vazquez, 2000). Hence, their jointly detection became the basis of the erythrocytic nuclear abnormalities (ENA) assay, successfully applied to different fish species exposed to various classes of environmental genotoxins (Pacheco and Santos, 2001; Gravato and Santos, 2002; Ayllón and Garcia-Vasquez, 2001; Pacheco *et al.*, 2005). Due to its higher responsiveness, ENA assay represents an alternative to MN test overcoming a possible lack of sensitivity related to the low frequency of micronuclei in wild fish. Moreover, considering the proposed mechanisms for mercury genotoxicity, it appears as an appropriate genetic end-point in this context.

Laranjo basin is a confined area of a coastal lagoon (Ria de Aveiro, Figure 1), in the northwestern region of Portugal, where a mercury gradient was identified as a result of a chlor-alkali plant discharges (Pereira *et al.*, 1998). Due to the absence of other important sources of contaminants, this area can be regarded as a "field laboratory" offering to researchers a unique opportunity for the assessment of mercury toxicity under realistic conditions. Thus, the study conducted at Laranjo basin had four main objectives: (1) to assess the mercury genotoxic potential to fish, strengthening the ENA assay sensitivity and suitability as a biological marker for environmental genotoxicants; (2) to investigate the relationship between mercury levels in blood and ENA frequency, as well as the relative importance of water uptake route provided by the conjugation of wild and caged fish analysis; (3) to evaluate *Liza aurata* as target fish species to carry out genotoxicity tests; (4) to determine the environmental risk of mercury contamination to native ichthyofauna.

2. MATERIAL AND METHODS

2.1. Characterization of study area

Laranjo basin, located near Estarreja (Figure 1), is the most contaminated site in the Ria de Aveiro coastal lagoon and one of the most mercury contaminated areas in Europe. This area received chlor-alkali plant discharges continuously during five decades generating a mercury contamination gradient. Recently, the industry improved the production process, leading to a considerable decrease of mercury released. Nevertheless, high mercury levels have been found into the sediments due to its progressive deposition, and resuspension, mainly during periods of stronger tidal currents, being responsible for metal exportation and increased availability to organisms (Pereira *et al.*, 1997, 1998). Hence, high to moderate mercury levels were observed in fish inhabiting this basin (Abreu *et al.*, 2000).

S. Jacinto area was selected as a reference site inside the lagoon for comparison purposes because of its proximity to the lagoon entrance (Figure 1), the distance to the main polluting sources and low-contamination load (Pacheco *et al.*, 2005).

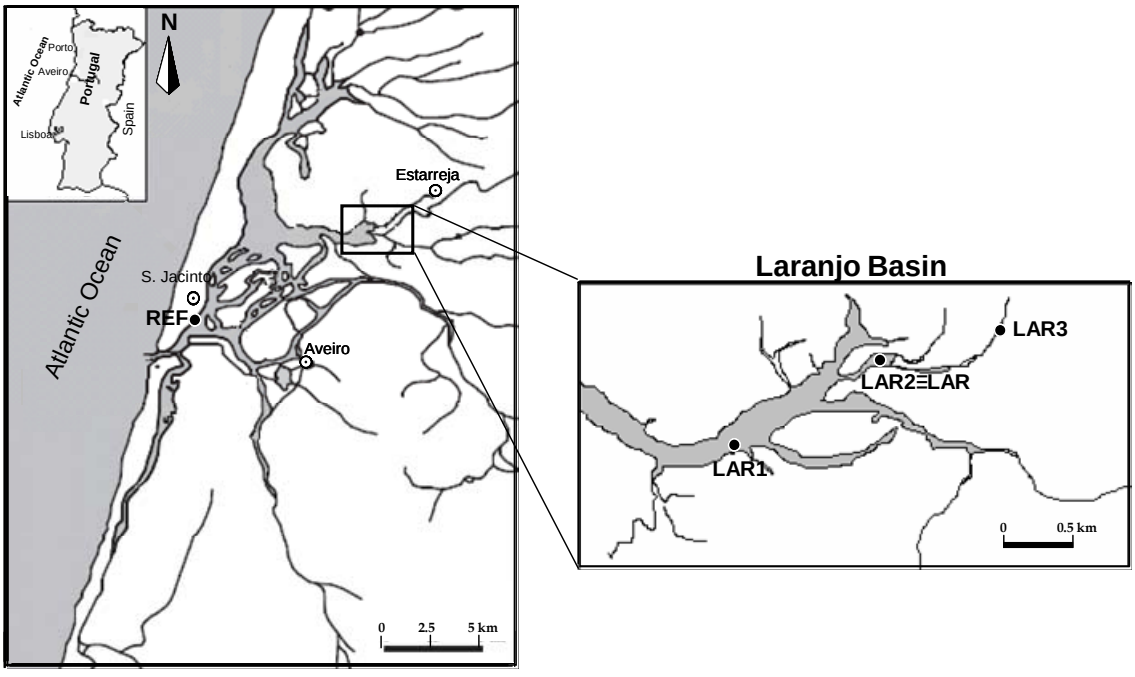

Figure 1 - Map of Ria de Aveiro (Portugal) with locations of fish-capture and caging sites (dark circles). The respective coordinates are: reference site (REF) - 40º40'26''N, 8º43'17''W; LAR1 - 40º43'24''N, 8º37'55''W; LAR2 - 40º43'49"N, 8º36'53"W; LAR3 - 40º44'04"N, 8º36'02"W. The LAR2 site adopted in the caging experiment coincides with LAR site selected for sampling in the wild fish survey.

2.2. Fish and sampling

Golden grey mullet (*Liza aurata*) is a pelagic species that frequently contacts with sediments, feeding on small benthic organisms, detritus and occasionally on insects and plankton. Juvenile specimens were used to minimize the interference of variables such as gender and contaminants accumulation. Fish with an average weight of 13.5±0.1 g and length of 12.1±0.1 cm were caught in reference site and in contaminated areas during low tide, using a traditional beach-seine net named “chincha”. After catching, fish were dissected and blood collected from the posterior cardinal vein by using heparinsed Pasteur pipettes. Blood smears were immediately prepared and the remainder volume stored in Eppendorf tubes and kept on ice. Following blood sampling, fish were sacrificed by decapitation. In the laboratory, blood samples were stored at -80 ºC, until further processing for total mercury (Hg_t) analysis. Fish were weighed and measured in the laboratory.

2.3. Experimental Design

This study includes two different components corresponding to (1) a field survey where wild mullets were seasonally captured at S. Jacinto (Reference site - REF) and Laranjo (LAR), and (2) a field-caging exposure carried out at the same areas.

At each sampling site, hydrological parameters were measured at sub-surface (a few centimetres below the water surface), in low and high tide conditions, including temperature, dissolved oxygen, salinity, and pH. For caged experiment, the previous parameters were also assessed at bottom level. Depth was also evaluated. Water turbidity was measured using a 20 cm black and white Secchi disc. A 3 L van Dorn bottle was used to collect water samples from the bottom. Besides water samples, sediment samples were also collected at the same sites, for Hg_t analysis.

2.3.1. Wild fish survey

Liza aurata was captured during winter (December 2004), spring (March 2005), summer (July 2005) and autumn (September 2005) at Laranjo basin and S. Jacinto, according to the procedure previously described (Figure 1). Ten fish of approximately similar weight and length were analysed in each sampling moment. ENA frequency was determined, to evaluate genetic damage. Immature erythrocytes (IE) frequency was estimated in order to assess alterations on the haematological dynamics. Hg_t concentration in blood was determined and correlated with the previous responses.

Additionally, Hg_t concentrations in water (dissolved and suspended particulate matter - SPM) and sediment were measured.

2.3.2. Caged fish experiment

The study was performed in December 2004. Fish caught at the low-contaminated site (S. Jacinto, Figure 1) were transported to laboratory in oxygenated saltwater and allowed to stabilize for 2 weeks prior to experimentation. This stabilization period aimed the reduction of inter-individual differences among the experimental fish group, also providing the elimination of previously taken up chemicals and the adaptation to confinement. During stabilization, fish were kept in 80 L aquaria at room temperature, in aerated (dissolved oxygen level = 8.4±0.2 mg/L) and filtered artificial seawater Sera Premium (Germany) (23±0.1 g/L salinity), under natural photoperiod, being daily fed with polychaete worms (*Nereis* sp.) collected in a clean area of Ria de Aveiro.

The experiment was conducted by caging mullets, during three days, in three precise sites of Laranjo basin differing on their distances to the metal contamination source (LAR1, LAR2 and LAR3) (Figure 1). Reference groups were caged in S. Jacinto. In the same day, a group of 10 fish were sacrificed in the laboratory to constitute t_0 group. In order to assess the effect of the fish position in the water column on mercury uptake and genotoxicity, two 80 L cages were placed at each site, one in the surface (10 fish) and the other close to sediment (10 fish). Surface cages were plunged 30 cm beneath the water level, by a buoy-anchor system. Bottom cages were set at 15 cm from the sediment to prevent its direct contact with fish. Fish were visually examined and found to be in adequate conditions, when transferred to cages. During 3-day field exposure, fish were kept without any food supply. After the exposure and blood sampling, the ENA frequency and Hg_t levels in blood were determined. Hg_t concentrations in water (dissolved and SPM) and sediment were also measured.

2.4. Mercury analyses

2.4.1. Total mercury (Hg_t) in the water column and in sediment

Water samples were filtered with 0.45 µm Millipore filters. The filtrate was then acidified with HNO_3 (Merck, mercury-free) to pH < 2 and stored at 4 ºC until analysis. Suspended particulate material (SPM) collected in the filters was oven dried at 60 ºC until constant weight. Procedure blanks were always run with samples and its contribution corrected when necessary.

Total dissolved mercury concentrations were measured by Cold Vapour Atomic Fluorescence Spectrometry (CV-AFS; PSA model Merlin 10.023 equipped with a detector PSA model 10.003), using tin(II) chloride as reducing agent, after addition of 500 µL of a saturated solution of potassium persulfate to 50 mL of filtered water and irradiation by a UV lamp (1000 W) for 30 min. Following irradiation, the excess of oxidant was reduced with 37.5 µL of hydroxylamine solution 12% (w/v), prior to analysis (Mucci *et al.*, 1995). The system was calibrated every day with acidified (HNO_3, mercury-free) standard solutions prepared from a 1000 mg/L solution (BDH). The detection limit of CV-AFS technique was 0.5 ng/L.

Mercury in SPM was also determined by CV-AFS, after digestion of the filters in glass reactors with 50 mL of a solution 4 mol/L HNO_3, at 60 ºC for 4 h (Pereira *et al.*, 1995, 1998). Results presented for Hg_t concentrations in water column (total water), are always the sum of dissolved and SPM metal concentrations, expressed in µg/L, taking into account the mass of SPM and the volume of filtered water.

Sediment samples were oven-dried to constant weight at 60 ºC homogenized and sieved through a 1 mm sieve, prior to analysis. Samples were analysed by Atomic Absorption Spectrometry (AAS) with thermal decomposition of the sample using the equipment LECO AMA-254 (Advanced Mercury Analyser), with no pre-treatment of samples (Costley *et al.*, 2000). Accuracy was assessed with certified reference materials (CRMs) from the National Research Council of Canada (NRCC). The CRMs used were MESS-3 (0.091±0.009 mg Hg/Kg) and PACS-2 (3.04±0.20 mg Hg/Kg).

2.4.2. Total mercury (Hg_t) levels in blood

Blood were also analysed by AAS with thermal decomposition of the samples. As in sediment analysis, accuracy was assessed using certified reference materials, namely TORT-2 (0.27±0.06 mg Hg/Kg) and DORM-2 (4.64±0.26 mg Hg/Kg).

2.5. Scoring genotoxic damage

Genotoxicity was tested using the ENA assay, carried out in mature peripheral erythrocytes according to the procedures of Schmid (1976), Carrasco *et al.* (1990) and Smith (1990) as adapted by Pacheco and Santos (1996). Briefly, one blood smear per animal was fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. From each smear, 1000 erythrocytes were scored under 1000x magnification to determine the frequency of the following nuclear lesions categories: micronuclei (M),

lobed nuclei (L), dumbbell shaped or segmented nuclei (S) and kidney shaped nuclei (K). In addition, notched nuclei (N) were also scored as suggested by Ayllón and Garcia-Vasquez (2001). The results were expressed as the mean value (‰) of the sum (M+L+S+K+N) for all the lesions observed.

2.6. Immature erythrocytes (IE) frequency

IE were scored for each of the 1000 erythrocytes (mature+immature) per fish. Results were presented as a frequency resulting from the expression:

$$\text{IE frequency } (^0/_{00}) = \frac{IE}{(ME+IE)} \times 1000$$

(ME = Mature erythrocytes; IE = Immature erythrocytes)

The distinction between ME and IE was based on the criteria established by Hibiya (1982) and Smith (1990).

2.7. Statistical analysis

SigmaStat software (SPSS Inc.) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between fish groups, followed by Tukey test (Zar, 1996). Differences between means were considered significant when $P<0.05$. The relationship between ENA and IE frequencies and Hg_t concentration in blood was explored using linear regression analyses. The correlation coefficient (r) was calculated and its statistical significance (p) was determined from table of Critical Values for Correlation Coefficient, r in Zar (1996).

3. RESULTS

3.1. Wild fish survey

The hydrological parameters including water temperature, dissolved oxygen, salinity, pH, turbidity and depth, for each sampling season, are depicted in table I.

Table I

Hydrological characteristics determined seasonally on reference (REF) and contaminated (LAR) sites at Ria de Aveiro: water temperature (T), dissolved oxygen (DO), salinity, pH, turbidity and depth.

Season	Site	Tide	Position	T (ºC)	DO (mg/l)	Salinity (‰)	pH	Turbidity (m)	Depth (m)
Winter	REF	Low	Surface	13.5	9.89	35	8.48	1.85	2.9
			Bottom	14.5	9.78	33	8.47		
		High	Surface	14.6	9.77	35	8.46	2.5	7.7
			Bottom	13.6	9.9	37	8.48		
	LAR	Low	Surface	13.5	8.79	15	7.48	0.4	2.05
			Bottom	13.5	10.5	14	7.62		
		High	Surface	13.8	8.62	21	7.75	0.8	1.8
			Bottom	13.8	8.61	23	7.76		
Spring	REF	Low	Surface	12	10.69	31	8.39	2	2.5
			Bottom	11.8	10.33	29	8.34		
		High	Surface	11.2	10.33	25	7.77	2.9	7
			Bottom	11	9.98	33	8.29		
	LAR	Low	Surface	11.9	8.67	16	7.76	0.5	2
			Bottom	11.8	8.56	18	7.83		
		High	Surface	12.3	9.88	24	8.28	0.8	2
			Bottom	11.9	9.93	27	8.32		
Summer	REF	Low	Surface	23.5	8.37	36	8.39	0.7	1.5
			Bottom	22.8	8.67	35	7.99		
		High	Surface	19.1	9.47	35	8.05	2.6	7
			Bottom	19.7	9.17	35	8.04		
	LAR	Low	Surface	27.7	5.37	35	7.052	0.7	1
			Bottom	27.4	6.12	35	7.93		
		High	Surface	23	6.7	36	8.05	1.2	2.8
			Bottom	24.1	6.86	25	7.75		
Autumn	REF	Low	Surface	18.1	7.52	35	7.42	0.9	1.2
			Bottom	18.3	6.65	35	8.04		
		High	Surface	16.8	7.07	35	7.88	7	7.5
			Bottom	16.9	6.99	35	7.32		
	LAR	Low	Surface	19.3	4.02	35	7.67	0.5	1
			Bottom	20.2	3.77	35	7.82		
		High	Surface	18.6	5.49	37	7.29	1.3	2.7
			Bottom	18.5	5.6	35	7.52		

3.1.1. Total mercury (Hg_t) levels in environment

Hg_t levels determined in the water column (dissolved mercury and mercury associated with suspended particulate matter) at two tide conditions (high and low tide) and in surface and bottom waters were always low in the two sampling stations (Table II).

Table II

Environmental total mercury (Hg_t) concentrations determined seasonally in water (sum of dissolved and particulate fraction) from reference (REF) and contaminated (LAR) sites at Ria de Aveiro.

Season	Site	Tide	Position	Hg_t in Water (µg/L)
Winter	REF	Low	Surface	0.6272
			Bottom	0.8245
		High	Surface	0.2580
			Bottom	mv
	LAR	Low	Surface	1.5917
			Bottom	3.3948
		High	Surface	1.4788
			Bottom	3.2966
Spring	REF	Low	Surface	1.9255
			Bottom	3.3845
		High	Surface	0.5131
			Bottom	1.1205
	LAR	Low	Surface	1.8848
			Bottom	1.4974
		High	Surface	0.9525
			Bottom	0.7297
Summer	REF	Low	Surface	0.3893
			Bottom	0.5906
		High	Surface	0.3469
			Bottom	0.2666
	LAR	Low	Surface	0.7365
			Bottom	0.5620
		High	Surface	0.3514
			Bottom	0.1774
Autumn	REF	Low	Surface	0.1666
			Bottom	0.3238
		High	Surface	0.2463
			Bottom	0.5150
	LAR	Low	Surface	0.8356
			Bottom	2.7331
		High	Surface	1.0655
			Bottom	0.3620

mv = missed value

Most of the times, the higher Hg_t concentrations occurred at deep layers of the water column during low tide conditions and mainly in autumn and winter surveys. This is probably due to the higher volumes of the water discharged from the upstream channels, enriched in mercury, into the Laranjo basin at this time of the year. In the reference site, higher concentrations were found in the bottom water sample, both in high and low tide

conditions; at this site no relevant differences were found in Hg_t concentrations in water column during the surveyed seasons.

As expected, higher Hg_t concentrations were found at LAR site near the anthropogenic source of the metal. The differences observed along the seasons are mainly due to the high hydrodynamic variability of the studied area. The higher concentrations found in the bottom can be related with remobilisation processes due to tidal dynamics.

3.1.2. Total mercury (Hg_t) levels in blood

The analysis of Hg_t in whole blood (Figure 2) revealed significant increases in fish captured at Laranjo (LAR) site in comparison with the reference site (REF) in all seasons, excluding spring. Moreover, the highest increment was observed in winter (approximately 10 times), whereas in summer and autumn the increment was 2.9 and 3.5 times, respectively.

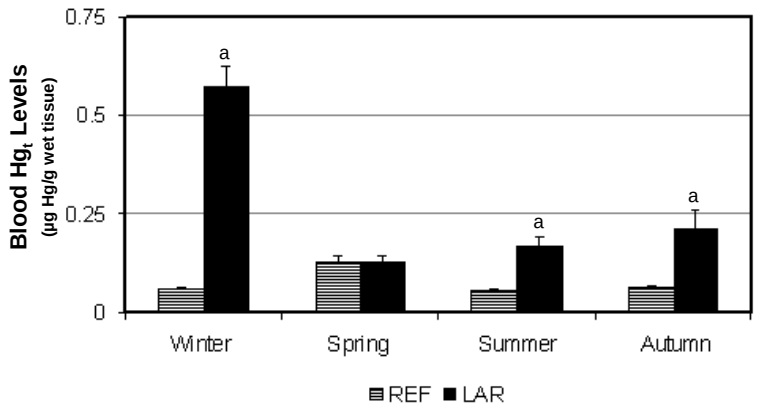

Figure 2 - Mean total mercury concentrations in the blood of indigenous *Liza aurata* collected seasonally from Ria de Aveiro. The letter (a) denotes statistically significant differences (p<0.05) between mercury-contaminated (LAR = Laranjo basin) and reference (REF) sites within each season. Error bars represent the standard error.

3.1.3. Erythrocytes Nuclear Abnormalities (ENA) frequency

The ENA frequency (Figure 3) demonstrated significant elevation at LAR site in summer and autumn compared with REF site, representing increments of 2.6 and 3.8 times, respectively. In spring, an ENA frequency increase was also perceptible, though no statistically significant.

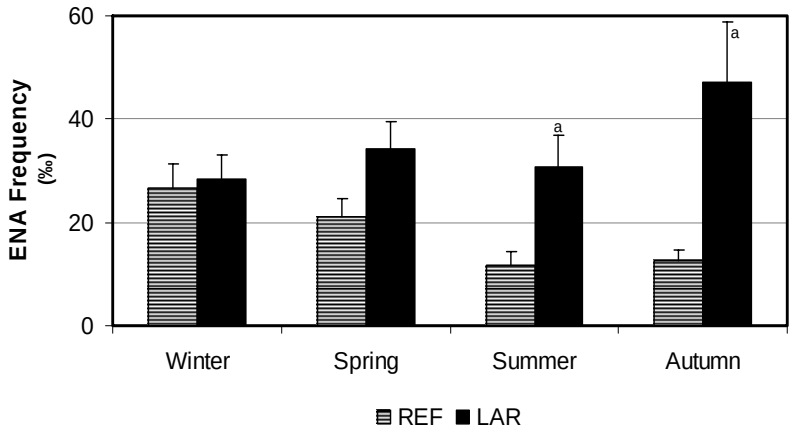

Figure 3 - Mean erythrocytic nuclear abnormalities (ENA) frequency in indigenous *Liza aurata* collected seasonally from Ria de Aveiro. The letter (a) denotes statistically significant differences ($p < 0.05$) between mercury-contaminated (LAR = Laranjo basin) and reference (REF) sites within each season. Error bars represent the standard error.

3.1.4. Immature Erythrocytes (IE) frequency

The analysis of IE frequency (Figure 4) revealed a significant decrease in fish captured at LAR in comparison with the REF site in all seasons, excluding autumn, exhibiting a 1.5, 2.5 and 1.9 times reduction, respectively for winter, spring and summer. In autumn, IE frequency at REF site showed a decrease, when compared with other seasons.

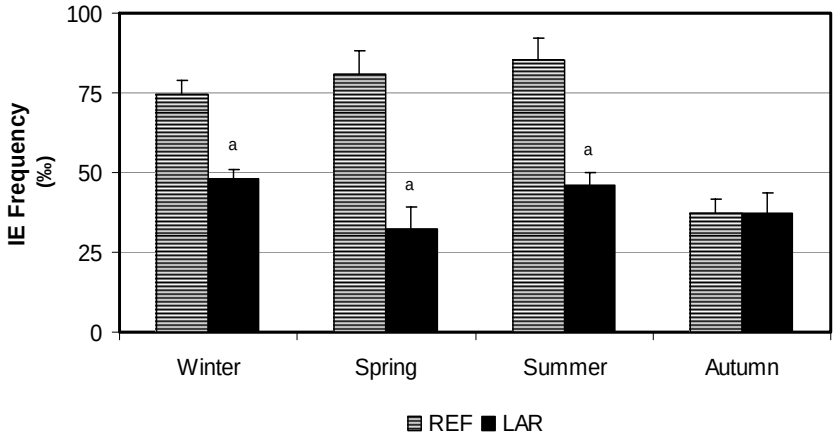

Figure 4 - Mean immature erythrocytes (IE) frequency in indigenous *Liza aurata* collected seasonally from Ria de Aveiro. The letter (a) denotes statistically significant differences ($p < 0.05$) between mercury-contaminated (LAR = Laranjo basin) and reference (REF) sites within each season. Error bars represent the standard error.

3.1.5. Correlations between biological parameters

The relationships between parameters were statistically tested analysing the data obtained in all sampling seasons. No correlations were found, i.e. blood Hg_t *vs*. ENA, blood Hg_t *vs.* IE and IE *vs*. ENA.

3.2. Caged fish experiment

The inter-sites comparisons were carried out separately for surface and bottom groups. The biological responses corresponding to surface group at LAR1 were not measured due to cage disappearance. Nevertheless, data concerning hydrological parameters and Hg_t environmental levels are presented.

The hydrological parameters previously mentioned were also measured for this component in all caging sites (REF, LAR1, LAR2 and LAR3) and presented in table III.

Table III

Hydrological characteristics of reference (REF) and contaminated (LAR1, LAR2, LAR3) sites on the caging experiment at Ria de Aveiro: water temperature (T), dissolved oxygen (DO), salinity, pH, turbidity and depth.

Site	Tide	Position	T (ºC)	DO (mg/l)	Salinity (‰)	pH	Turbidity (m)	Depth (m)
REF	Low	Surface	13.5	9.89	35	8.48	1.85	2.9
		Bottom	14.5	9.78	33	8.47		
	High	Surface	14.6	9.77	35	8.46	2.5	7.7
		Bottom	13.6	9.9	37	8.48		
LAR1	Low	Surface	13.8	8.64	9	7.42	0.35	2.7
		Bottom	13.8	8.9	17	7.69		
	High	Surface	13.8	8.83	23	7.78	0.85	1.8
		Bottom	13.5	9.66	25	7.95		
LAR2	Low	Surface	13.5	8.79	15	7.48	0.4	2.05
		Bottom	13.5	10.5	14	7.62		
	High	Surface	13.8	8.62	21	7.75	0.8	1.8
		Bottom	13.8	8.61	23	7.76		
LAR3	Low	Surface	13	10.3	2	7.06	0.25	2.15
		Bottom	13	10.13	6	7.29		
	High	Surface	14.5	8.5	18	7.58	0.5	2.7
		Bottom	14.2	8.43	20	7.69		

3.2.1. Total mercury (Hg_t) levels in the environment

Hg_t concentrations in water column (Table IV) at the reference site and at the three caging sites at Laranjo basin were always very low (less than 10 µg/L in most of the samples and only with one value higher than 40 µg/L). All the values are less than 1000 µg/L, the permitted value by law for mercury concentrations in water column of aquatic systems.

During high and low tide conditions, Hg_t concentrations in the water column are of the same order of magnitude. The higher Hg_t concentrations were found at the bottom almost in all studied stations located in the Laranjo basin. The differences observed between mercury concentrations in bottom and surface layers are attenuated as we move far away from the contamination source.

Hg_t concentrations in sediments (Table IV) revealed the pronounced human-induced environmental mercury gradient in the lagoon with the highest concentrations found near

the contamination source. This way, the results obtained for mercury concentrations in the sediments are mostly related with distance to the industrial source of the metal, with concentrations as high as 37 ng/mg measured in the most contaminated area, whereas in the reference site concentrations were very low (0.001 ng/mg).

Table IV

Environmental total mercury (Hg_t) concentrations in water (sum of dissolved and particulate fraction) and sediment of reference (REF) and contaminated (LAR1, LAR2, LAR3) sites on the caging experiment at Ria de Aveiro.

Site	Tide	Position	Hg_t in Water (µg/L)	Hg_t in Sediment (ng/mg)
REF	Low	Surface	0.6272	0.001
		Bottom	0.8245	
	High	Surface	0.258	
		Bottom	mv	
LAR1	Low	Surface	0.6833	3.0
		Bottom	0.7542	
	High	Surface	0.6876	
		Bottom	0.4056	
LAR2	Low	Surface	1.5917	7.1
		Bottom	3.3948	
	High	Surface	1.4788	
		Bottom	3.2966	
LAR3	Low	Surface	6.541	36.9
		Bottom	49.5915	
	High	Surface	2.585	
		Bottom	15.2026	

mv = missed value

3.2.2. Total mercury (Hg_t) levels in blood

Results concerning Hg_t levels in blood (Figure 5) revealed significant increases in comparison to REF site only among surface groups, namely at LAR2 and LAR3, corresponding to a 2 times increment in the last one. Though apparent, the increases detected for LAR2 (2 times) and LAR3 (2.3 times) bottom groups were not statistically significant. No differences were detected between surface and bottom groups in each site.

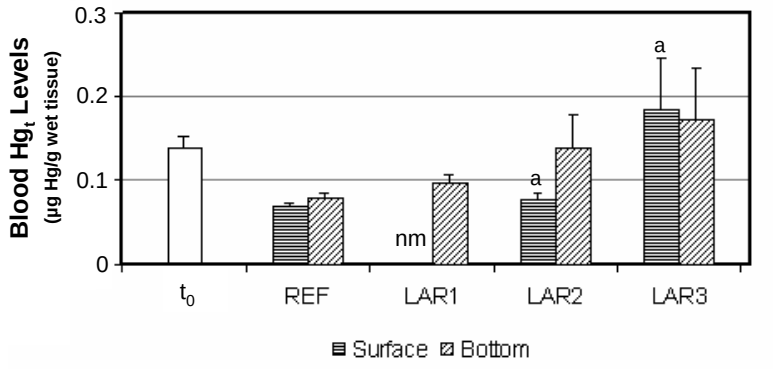

Figure 5 - Mean total mercury concentrations in the blood of *Liza aurata* caged during 3 days at different Ria de Aveiro locations, i.e. within a mercury-contaminated area (Laranjo basin - LAR1, LAR2 and LAR3) and a reference site (REF). t_0 represents the value at the beginning of the experiment. The letter (a) denotes statistically significant differences ($p< 0.05$) with respect to REF. Error bars represent the standard error. nm = not measured.

3.2.3. Erythrocytes Nuclear Abnormalities (ENA) frequency

Significant ENA frequency increases in comparison to REF site were found only at LAR3 for both surface and bottom groups, displaying approximately a 4 times increment (Figure 6). Moreover, both LAR3 groups were significantly higher than the corresponding LAR2 groups, whereas LAR3 bottom group was also different from LAR1 corresponding group. No differences were detected between surface and bottom groups in each site.

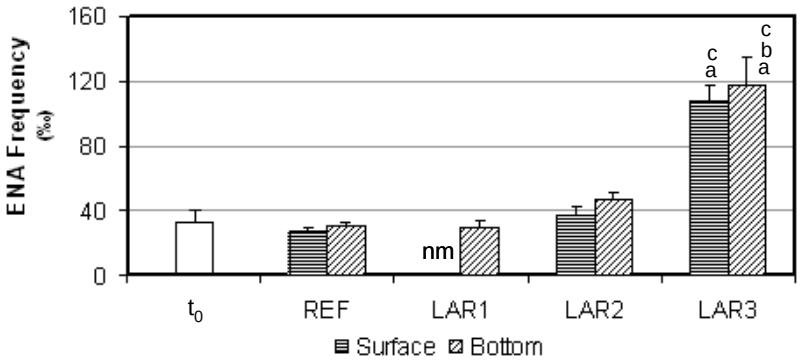

Figure 6 - Mean erythrocytic nuclear abnormalities (ENA) frequency in *Liza aurata* caged during 3 days at different Ria de Aveiro locations, i.e. within a mercury-contaminated area (Laranjo basin - LAR1, LAR2 and LAR3) and a reference site (REF). t_0 represents the value at the beginning of the experiment. Letters denote statistically significant differences ($p< 0.05$): (a) *vs* REF; (b) *vs* LAR1; (c) *vs* LAR2. Error bars represent the standard error. nm = not measured.

3.2.4. Correlations between biological parameters

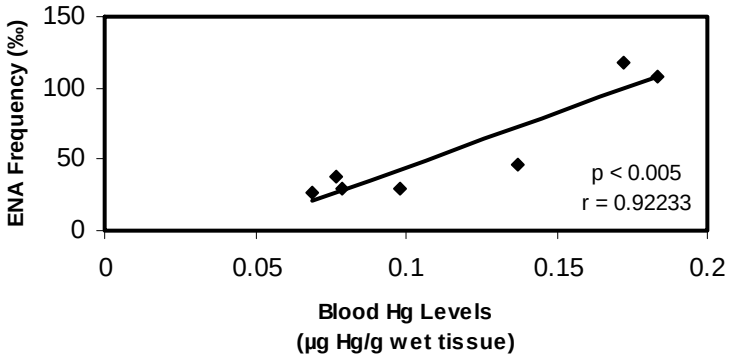

Figure 7 - Correlation between total mercury concentration in the blood and ENA frequency in caged *Liza aurata*. Statistical significance and correlation coefficient are represented by p and r, respectively.

The relationship between blood Hg_t and ENA frequency was statistically tested demonstrating a significant correlation when both surface and bottom groups were considered (Figure 7).

4. DISCUSSION

4.1. Wild fish survey

An ENA frequency increase was found in summer and autumn concomitantly with an elevation of Hg_t levels in blood. However, no significant correlation was found between these two biological parameters when all sampling seasons were considered. This absence of correlation is primary determined by the winter data, where Hg_t reached the maximum value at LAR, while the corresponding ENA frequency remained at the REF level, signaling a single deviation from an implicit correlation.

As previously demonstrated by Das and Nanda (1986) and Brunetti *et al*. (1988), the induction of nuclear lesions by genotoxic agents could be masked through a cytotoxic action, which killed the erythrocytes instead of forming non-lethal lesions. In addition, the appearance of nuclear lesions is determinately affected by a variety of factors such as erythropoiesis, cell maturation, and rate of mature or abnormal cells removal. A gradual decrease in frequency of MN with an increase in the exposure period, as well as at higher genotoxin concentrations was found by Das and Nanda (1986), and explained by an

inhibitory effect on cell division and subsequent hindrance in the passage of the affected cells into peripheral circulation (Nepomuceno *et al*., 1997). This effect may result either from inhibition of DNA synthesis (Williams and Metcalfe, 1992) or from direct inhibition of erythropoiesis (Dinnen *et al*., 1988). Moreover, circulating abnormal cells tend to be removed from the organism faster than undamaged ones (Das and Nanda, 1986), which was corroborated by the occurrence of splenic hemosiderosis concomitantly with ENA expression impairment (Pacheco and Santos, 2002). Considering the previous statements, as well as the suggestions of Minissi *et al*. (1996) and Pacheco and Santos (2002) about the importance to know the fish erythrocytes kinetics, in the current study it was assessed the frequency of immature erythrocytes (IE) in order to clarify the ENA results obtained principally in winter. The determination of IE frequency provides important information about haematological dynamics, but its isolated analysis does not allow a clear identification of the cause, since it reflects the balance between a variety of factors such as immature cells input *versus* splenic cells removal, and cell maturation rate. Nevertheless, current IE results support the idea that the ENA frequency in winter was conditioned by an altered haematological dynamics, which must be regarded as an integrated reflex of the different processes mentioned above. It may be assumed that the previous disruption prevented the ENA appearance in winter. In spring, the interference of a disrupted haematological dynamics cannot be excluded, despite the unaltered levels of Hg_t in blood. In that way, the IE decrease observed in spring rather than being determined by circulating mercury levels seems to be related with mercury accumulated in head kidney (haematopoietic organ) and/or in spleen (major blood cell removal organ).

In summer, the IE frequency also signaled a disruption but, in this case, it was not sufficient to prevent a significant ENA increase. In this regard, the increased water temperature should be considered as an additive factor on ENA induction, as previously suggested for MN (Brunetti *et al*., 1992). Furthermore, it was demonstrated that water temperature might influence cell replication rates and DNA repair of poikilothermal organisms (Venier *et al*., 1997).

In autumn, IE data revealed that ENA expression was not conditioned by changes on haematological dynamics. Moreover, the observation that ENA frequency reached a maximum level in absence of IE frequency reduction represents a supplementary evidence of the interference of the haematological dynamics in ENA generation. In view of that, the absence of ENA increase, in this and future studies, should be carefully

interpreted since it can reflect a multi level impairment of ENA expression, rather than a fish ability to cope with DNA damaging agents or the nonexistence of genotoxic risk.

Analyzing the temporal variation of IE frequency, based on data recorded for REF site, it is evident a pronounced decrease on autumn, reinforcing a seasonal effect previously stated by Hibiya (1982).

4.2. Caged fish experiment

The ENA frequency increase observed in LAR3, for both surface and bottom groups, was expectable taking into account its closest location in relation to the mercury source. Moreover, a significant correlation was observed between ENA frequency and Hg_t levels in blood showing a clear cause-effect relation and corroborating the suitability of this genotoxic test. Under the present conditions, i.e. 3 days exposure and blood Hg_t levels up to 0.18 µg/g, the ENA expression was not limited by other physiological events able to mask this effect, namely those previously presented in wild fish survey. The current caging experiment demonstrated that 3 days exposure is sufficient to a significant mercury uptake. Moreover, as *L. aurata* feeding was almost completely limited, it can be stated that mercury was absorbed mostly through gills from the dissolved fraction and SPM, highlighting the importance of this uptake route.

Fish caged at LAR1 and LAR2 showed no ENA induction indicating the efficiency of defence mechanisms to cope with low to moderate Hg_t concentrations. These mechanisms can include antioxidants induction and/or activation of DNA repair processes.

The comparison between surface and bottom groups revealed no differences, indicating that the slight differences detected for dissolved and SPM Hg_t levels have no perceptible consequences on blood Hg_t concentrations.

4.3. General discussion

According to Sanchez-Galan *et al*. (1999), a theoretically ideal fish species to assess genotoxicity of pollutants should meet some criteria: (i) widespread in different ecosystems, cosmopolitan if possible; (ii) sensitive enough to detect genotoxicity of a wide range of pollutants at low doses; (iii) adequate for culture conditions in order to carry out laboratory experiments; (iv) large natural populations, to allow detraction of individuals without endangering their conservation. Taking into account the overall results of the current investigation, to what a bioindicator species concerns, *L. aurata* fulfils every

requirement. Juvenile specimens demonstrated to be an adequate life-cycle stage as it can better reflect recent exposures.

When natural fish populations are monitored for contaminant-induced changes, uncertainties arise from possible avoidance behaviour, the intermittent nature of the pollution or the duration of exposure (Kezic *et al.*, 1983). These factors can be under control using a caged fish strategy, improving the sensitivity of environment health assessment. On the other hand, Pacheco *et al.* (2005) stated that only field studies based on the capture of wild specimens could provide a definitive indication about the environment state and the ichthyic population's health. Thus, in the current study it was adopted a combined approach based on autochthonous fish survey and *in situ* caging experiment, which according to Pacheco *et al.* (2005) is an ideal strategy for biomonitoring purposes, providing an additional mechanistic interpretation of fish toxicological responses.

The biomarkers involved in the assessment of exposure to contaminants can comprise the quantification of chemicals (or their metabolites) in the organism's tissues or the biological responses induced by the exposure. The choice of the experimental strategy for the current study was based on the understanding that only the integration of both types of indicators can provide a solid environmental risk assessment. The overall results reinforced this option since it was demonstrated that misinterpretations could arise from the isolated analysis of either blood Hg_t levels or ENA frequency.

According to De Flora *et al.* (1994), the genotoxicity of organic and inorganic mercury compounds are qualitatively comparable, suggesting the involvement of a common genotoxic entity and thus, depending mainly on a differential bioavailability. Mercury organic forms are more likely to be responsible for the observed aneugenic and clastogenic action due to its lipofilicity. $MeHg^+$ in fish is originated by bacterial methylation of inorganic mercury, either in the environment or in bacteria associated with fish gills or gut (Boening, 2000). In relation to current caging experiment, methylation on gut should not be considered. Nevertheless, the involvement of mercury inorganic forms cannot be completely excluded.

The comparison between wild (winter data) and caged fish reveals higher level of Hg_t in blood in the former probably related to a longer exposure to Laranjo basin contaminated environment and/or the involvement of both uptake routes (food and water).

The ENA frequency showed to be not predictable on the basis of a concentration dependent pattern, mainly due to masking processes affecting its expression under high Hg_t blood concentrations. For that reason, the suitability of ENA assay may be limited in situations of intense metals contamination, which is in agreement with a previous statement of Pacheco and Santos (2002). Comparing with MN, ENA confirmed to be a more sensitive assay, as no MN induction was detected either in wild or caged fish (maximum MN frequency < 3 ‰).

In order to gain a better understanding of the mercury dynamics, a considerable number of studies have been carried out on the distribution and retention of this metal in fish tissues (muscle, liver, gonad, brain, and gill). In this context, present findings demonstrated that blood, as an important route of mercury distribution, could reflect current body burdens, as previously stated by Cizdziel *et al*. (2003).

5. CONCLUSIONS

The current study demonstrated the mercury genotoxicity through clastogenic and/or aneugenic actions in both wild and caged fish. It was confirmed the usefulness of ENA assay on the assessment of aquatic genotoxicants, though some limitations were evident in the presence of high mercury concentrations. The mercury uptake from the water showed to be sufficient *de per se* to significantly increase Hg_t levels in blood and the consequent genetic damage.

The present findings confirm the obvious need for this kind of integrated approach, comprising the assessment of mercury accumulation and genetic end-points, for achieving more reliable and functionally relevant results. *Liza aurata* showed potential as a metal biosentinel because it is commonly found in both unpolluted and metal-contaminated environments, easy to catch and responsive in terms of the assessed parameters. Finally, the results reflect a serious environmental risk to native ichthyofauna at Laranjo basin due to mercury contamination.

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DISCUSSÃO GERAL

A identificação de alterações bioquímicas precoces, e porventura reversíveis em peixes (biomarcadores de efeitos), poderá fornecer importantes indicações acerca da exposição e absorção de contaminantes e, consequentemente, acerca do estado dos ecossistemas aquáticos. Essa informação pode ainda ser consolidada pela avaliação de alterações citogenéticas que traduzem danos a um nível celular ou subcelular decorrentes da exposição. Por outro lado, biomarcadores de exposição (ou de dose interna) podem ser usados para confirmar e avaliar a exposição a um contaminante em particular, estabelecendo uma ligação entre a exposição externa e a quantificação da exposição interna, resultante da absorção desse contaminante. Este tipo de parâmetros poderá reflectir a quantidade da substância ligada ao local-alvo ou biodisponível para interagir com o material biológico, sendo particularmente informativo relativamente a compostos com elevadas taxas de bioacumulação, como é o caso do mercúrio.

Nesta perspectiva, o presente trabalho teve como objectivo geral, o estudo integrado de respostas bioquímicas/metabólicas e citogenéticas, assim como da acumulação de mercúrio em tecidos alvo, pretendendo contribuir para um melhor entendimento da toxicocinética e toxicodinâmica deste metal em peixes.

A análise comparativa dos níveis de mercúrio total (Hg_t) acumulados nos três tecidos avaliados em *Liza aurata* revelou, em termos gerais, e no caso dos peixes selvagens a seguinte ordenação: fígado > músculo > sangue. Relativamente a exposições *in situ*, esta hierarquização foi alterada para: fígado > sangue > músculo. Daqui resulta a eleição do fígado como órgão preferencial de bioacumulação de mercúrio, apresentando níveis 6 (selvagens) e 8 vezes (engaiolados) superiores quando comparados com os outros tecidos, o que confirma dados anteriormente obtidos em peixes (Abreu *et al.*, 2000; Cizdziel *et al.*, 2003). Este órgão mostrou ser capaz de reflectir, através dos níveis de mercúrio acumulados, quer uma exposição de curta duração (3 dias de exposição *in situ*), quer uma exposição mais prolongada, como previsivelmente terá acontecido com os animais selvagens capturados. Adicionalmente, a concentração de Hg_t no fígado parece reflectir a duração da exposição, uma vez que os teores encontrados em peixes selvagens foram, em média, o dobro dos encontrados em peixes engaiolados.

A acumulação de mercúrio no sangue tem sido apresentada como um parâmetro que, dada a dinâmica característica deste tecido, reflecte fundamentalmente exposições recentes (Cizdziel *et al.*, 2003), perdendo alguma capacidade informativa relativamente a exposições que não se prolonguem até ao momento da amostragem. Em contraste, os níveis de mercúrio no músculo parecem reflectir, fundamentalmente, exposições de longo

prazo que não terão obrigatoriamente que ser recentes, e traduzem uma exaustão da capacidade de desintoxicação e/ou retenção e acumulação em órgãos, como o fígado. Desta forma se explica quer a diferença entre os teores encontrados no músculo de animais engaiolados e de animais selvagens, quer a inversão na hierarquização dos níveis acumulados no sangue e músculo. Importa aqui salientar ainda que, tal como referido nos capítulos I e II, as diferenças observadas entre animais selvagens e engaiolados traduzem, para além de hipotéticas diferenças na duração da exposição, diferenças nas vias de absorção, que foram restringidas, devido à forma de exposição dos últimos.

A amostragem de Inverno de peixes selvagens, proporcionou, contudo, um perfil diferente das demais estações do ano no que respeita aos teores de Hg_t: fígado > sangue > músculo. Esta diferença pode ser igualmente explicada à luz dos princípios anteriormente referidos, uma vez que este momento de amostragem correspondeu a uma época de fortes correntes resultantes da elevada pluviosidade, a qual originou a ressuspensão dos sedimentos (confirmada pelo elevada turbidez medida) e um consequente aumento da biodisponibilidade de mercúrio circunscrito no tempo.

O alargamento do número de tecidos analisados poderia permitir um melhor entendimento dos processos de absorção (via água *versus* via alimento), assim como da toxicodinâmica do metal. Nesta perspectiva, a inclusão de guelras, rim e intestino em futuros estudos seria particularmente relevante.

A interpretação dos níveis de Hg_t no músculo de *L. aurata* em termos de risco para a saúde humana demonstrou valores abaixo do limite máximo permitido pela UE (Official Journal of the European Communities, 1994). Contudo, e de acordo com o que foi já implementado em países como o Canadá (Health and Welfare Canada, 1979), seria recomendável o estabelecimento de níveis que contemplem a taxa de consumo médio de peixe pela população em causa. Esta questão assume uma maior relevância no caso presente pelo facto da população portuguesa estar no topo da lista de países da UE relativamente a esse aspecto. Desta forma, o reconhecimento dos níveis observados no presente estudo como seguros, deverá ser encarado com prudência.

Os dados do presente trabalho não sinalizam evidências de stresse oxidativo na população de *L. aurata* que habita o Largo do Laranjo (Ria de Aveiro, Portugal). Todavia, a indução de defesas antioxidantes (ex. glutationa total - GSH_t – tendo em consideração peixes selvagens e engaiolados, assim como a actividade da catalase em peixes engaiolados) denotou uma produção acrescida de espécies reactivas de oxigénio (ROS, do

inglês “reactive oxygen species”) associada à contaminação da área de estudo, o que não permite excluir em absoluto o risco de stresse oxidativo.

A genotoxicidade do mercúrio foi demonstrada no sangue sem, contudo, ter sido possível clarificar o(s) mecanismo(s) envolvido(s) nessa acção genotóxica. Nesse sentido, seria recomendável em futuros estudos avaliar simultaneamente em diferentes tecidos (ex. sangue, guelras, rim e fígado) o dano genético, parâmetros de defesa antioxidante e níveis de ROS. Esta abordagem permitiria esclarecer quais as formas mercuriais responsáveis pelo efeito genotóxico, uma vez que há indicações de que associação entre ROS e genotoxicidade surge fundamentalmente para as forma inorgânicas (De Flora *et al.*, 1994), assim como um melhor conhecimento da toxicocinética do mercúrio.

Algumas limitações pontuais na interpretação dos resultados deverão ser entendidas como inerentes ao trabalho de campo e suas especificidades. Não obstante essa dificuldade, a estratégia adoptada no presente trabalho demonstrou, globalmente, a sua validade na avaliação da qualidade de ambientes aquáticos costeiros, tendo as respostas biológicas registadas permitido identificar uma situação de contaminação por mercúrio (Largo do Laranjo) através da comparação com o local de referência (S. Jacinto).

Um dos objectivos centrais da presente dissertação era a obtenção e integração de informação relativa a níveis de Hg_t nos tecidos e a respostas bioquímicas e citogenéticas em *L. aurata* após exposição ambiental a este metal. Neste contexto, foi possível estabelecer uma correlação apenas entre os níveis de Hg_t no sangue e frequência de anomalias nucleares eritrocíticas (ANE) em animais engaiolados. O facto de se ter encontrado uma correlação entre parâmetros analisados no sangue, em animais engaiolados, vem dar apoio à ideia anteriormente apresentada relativamente à capacidade de cada tecido reflectir exposições de diferente duração.

A dificuldade em reconhecer uma associação generalizada das respostas metabólicas medidas no fígado e os respectivos níveis de Hg_t em animais selvagens vem enfatizar a necessidade de, tal como referido no capítulo II, estudar a indução de metalotioninas neste órgão, dado o seu importante papel no sequestro deste metal (Schlenk *et al.*, 1995), participando assim de forma determinante na defesa do organismo contra os efeitos tóxicos do mercúrio.

Num outro contexto, a proximidade do peixe em relação ao sedimento, investigada na exposição *in situ*, demonstrou a sua importância ao nível das respostas hepáticas (concentração de Hg_t e GSH_t), confirmando o sedimento como o principal compartimento do ambiente no que respeita à determinação da biodisponibilidade do mercúrio. Torna-se,

assim, possível reforçar a nomeação do fígado como órgão alvo de grande valor informativo na detecção de exposição de peixes a mercúrio.

Os dados obtidos neste estudo confirmam uma relação clara entre a presença de mercúrio no ecossistema e o risco existente para as ictiopopulações. Deste modo, conclui-se que a estratégia utilizada (engaiolados/selvagens) se revelou bastante promissora, tendo permitido colmatar algumas lacunas pré-existentes em estudos com uma só componente. Adicionalmente, a integração de dois níveis de resposta, representados pela concentração de Hg_t nos tecidos e por respostas bioquímicas/metabólicas e citogenéticas, permitiu obter um conhecimento com carácter mais sólido e informativo, quando comparado com o estudo destes níveis isoladamente ou com a determinação dos níveis ambientais do contaminante.

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