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**CONTAMINAÇÃO DA BARRINHA DE ESMORIZ/LAGOA DE PARAMOS
POR METAIS PESADOS: EFEITOS BIOQUÍMICOS
E HISTOLÓGICOS EM *LIZA SALIENS***



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**Contaminação da Barrinha de Esmoriz/Lagoa de Paramos por
metais pesados: efeitos bioquímicos e histológicos em *Liza saliens***

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RESUMO

O presente trabalho centrou os seus objectivos na análise de alguns parâmetros químicos, bioquímicos, hematológicos e histológicos em tainha, *Liza saliens*, como medida do impacto da exposição crónica a metais pesados na Barrinha de Esmoriz/Lagoa de Paramos. Nesta laguna costeira a contaminação por metais pesados é uma preocupação porque são poluentes que apresentam ao mesmo tempo persistência, capacidade de bioacumulação e toxicidade.

A análise das concentrações totais de metais pesados (Cr, Cu, Pb e Zn), na água e nos sedimentos da Barrinha de Esmoriz/Lagoa de Paramos, mostrou que a ordem relativa de contaminação foi "Zn > Cu ~ Pb > Cr". Nos sedimentos as concentrações de metais foram muito superiores às da água e a média por estrato de profundidade não mostrou diferenças entre as estações amostradas, permitindo calcular a média de concentração de exposição para os peixes de 241 mg Zn·Kg⁻¹, 83 mg Cu·Kg⁻¹, 87 mg Pb Kg⁻¹ e 47 mg Cr·Kg⁻¹, sugerindo um padrão de deposição semelhante ao longo do tempo.

A quantificação dos metais, Cr, Cu, Pb e Zn, no fígado, na brânquia e no músculo de *Liza saliens* revelou existirem diferenças na bioacumulação de metais, entre diferentes tecidos. Os resultados mostram que o Cu e o Zn são os metais mais preocupantes neste ecossistema, atendendo aos valores de bioacumulação.

A relação dos factores de bioacumulação (BFAs) em relação aos sedimentos, do Cu na brânquia e no fígado com a idade dos peixes, revelou que o tempo de exposição afecta a capacidade homeostática de regulação deste metal, levando progressivamente à bioacumulação do Cu. Apesar da elevada concentração de Cu no meio, a concentração encontrada na brânquia é inferior à encontrada no fígado, sugerindo que as tainhas conseguem transferir o Cu da brânquia para o fígado que mostrou ser o principal órgão envolvido no metabolismo do Cu. Assim, os níveis de Cu acumulados pelo fígado de *L. saliens* mostram ser um bom indicador da contaminação deste metal na laguna.

A relação dos BFAs do Zn com a idade dos peixes, mostrou que o fígado parece regular a concentração do metal ao longo do tempo; na brânquia, embora ocorra alteração da regulação do metal, o aumento é repartido com o tecido muscular. Assim, a regulação do Zn parece envolver os três tecidos.

A brânquia, devido ao permanente contacto com os poluentes e à sua função que propicia rápida entrada de poluentes para a corrente sanguínea, mostrou estar histologicamente muito afectada. O destacamento do epitélio foi a alteração histológica mais grave pelo seu índice de extensão e severidade, seguida da

hiperplasia devido ao seu índice de extensão. Comparando com o fígado, bem como com tainhas recolhidas no mar, as brânquias de *L. saliens* aparecem com muitas lesões, mostrando uma prevalência maior do número de lesões e de cada lesão. Além disso, as alterações observadas foram comuns às que ocorrem devido à presença de Cu e Zn, referidas na bibliografia.

No fígado, a presença de extensas zonas de parênquima heterogéneo parece depender da maior acumulação de Cu e Zn no tecido. Apesar disso, as funções metabólicas do fígado aparentemente não se encontram comprometidas, nomeadamente os mecanismos envolvidos na destoxificação, visto que a prevalência do número de lesões e das diferentes lesões é moderada e o tecido não se encontra invadido por lesões pré neoplásicas, nem neoplásicas.

Vários indicadores bioquímicos e morfológicos sugerem uma situação geral de *stress* nas tainhas da laguna, como o aumento da glicemia e da proteinemia e o aumento do factor de condição que podem ser indicativos de efeitos tóxicos. O aumento do peso relativo do fígado pode estar relacionado com as lesões encontradas e com modificações metabólicas, incluindo a acumulação de gordura. Os valores de maior actividade da aspartato aminotransferase plasmática nos peixes da laguna também podem resultar de alterações no metabolismo proteico, eventualmente impostas pela exposição aos metais.

Apesar disso, os peixes mostraram possuir alguns mecanismos compensatórios na regulação iónica e no metabolismo da biotransformação, para fazer face à bioacumulação dos metais. Na brânquia, os mecanismos envolvem aumento do K^+ plasmático como consequência do destacamento do epitélio e o aumento do fósforo na compensação do equilíbrio osmótico e ácido-base. O aumento da actividade da superóxido dismutase na brânquia é indicador de uma resposta compensatória, face ao aumento do *stress* oxidativo que ocorre devido à acumulação de metais neste órgão. No fígado, estes mecanismos incluem a indução da actividade da catalase como resposta ao *stress* oxidativo devido à acumulação de Cu no tecido.

Os resultados de natureza química, bioquímica, hematológica e histológica, obtidos no presente estudo indicam que as condições ambientais na Barrinha de Esmoriz/Lagoa de Paramos são frágeis. A exposição crónica das tainhas a concentrações moderadas de Cu e Zn, afecta a resposta reguladora destes metais e envolve alterações metabólicas e estruturais, quer adaptativas com vista à desintoxicação, quer tóxicas face à acumulação de metais.

Não obstante as medidas de revitalização da Barrinha de Esmoriz/Lagoa de Paramos a contaminação em metais deve continuar a ser monitorizada, nomeadamente avaliando a recuperação da população piscícola.

ABSTRACT

This work focused on the analysis of some chemical, biochemical, haematological and histological parameters of mullet, *Liza saliens*, to assess the impact of chronic exposure to heavy metals in Esmoriz-Paramos coastal lagoon. In this lagoon heavy metals contamination is a concern since they are persistent, toxic and prone to bioaccumulate.

The first approach to this study was investigated heavy metal concentrations (Cr, Cu, Pb and Zn) in the water and sediments of the lagoon. These analyses showed the following relative order of contamination in "Zn > Cu ~ Pb > Cr" in both matrices, although metal concentrations in the sediments were higher than in water. Metals in sediments were determined in different sites and along depth, and significant differences were found between sites but not in depth, suggesting a similar pattern of sediment deposition over time, and allowing to calculate mean concentrations of fish exposure: 241 mg Zn·Kg⁻¹, 83 mg Cu·Kg⁻¹, 87 mg Pb Kg⁻¹ and 47 mg Cr·Kg⁻¹.

The same metals were also quantified in the living fish in order to evaluate their bioaccumulation. The results showed that Cu accumulated mostly in liver and Zn in gill, leading to conclude that these are the metals of concern in this lagoon.

BAFs of Cu in tissues were calculated relative to the sediments and a positive relation between Cu-BAF in liver and gill with fish age, revealed that time of exposure affects homeostatic regulation of copper. Despite the presence of high Cu concentration in the surroundings, Cu concentration in gill was much lower than in liver, suggesting that mullets are able to transfer Cu from gills to liver, the main organ of Cu metabolism. Therefore the levels of Cu in liver seem to be a good indicator of Cu contamination in this lagoon.

The relation between Zn-BAF in liver and gill with fish age revealed that liver keeps Zn under regulation. On the contrary, Zn increased in gill with time of exposure, but this increase was shared with the muscle, where it remained constant. Then, Zn metabolism involves the three tissues.

Gill histological alterations revealed extensive and severe epithelial lifting and extensive hyperplasia, and higher prevalence of lesions than mullets collected in the sea. In addition, gill damage observed was similar to that has been described in fish exposed to metals.

In liver, histological changes were less pronounced than in gill, but extensive areas of heterogeneous parenchyma were observed, which depend on Cu and Zn accumulation. Since the prevalence of lesions was mild and neither pre-neoplastic,

nor neoplastic alterations were observed, metabolic functions, such as metal detoxification seemed to be unaffected.

The measurement of several biochemical and morphological indicators suggest a general stress condition in the lagoon fish, such as hyperglycemia and proteinemia and increased condition factor. The higher aspartate aminotransferases can suggest an alteration in protein metabolism. The HSI was also increased in *L. saliens* which may be related with liver lesions and metabolic modifications, such as lipid accumulation.

Despite these observations, fish showed some compensatory mechanisms. In gill, this include a direct relation of epithelial lifting with an increase of K^+ in plasma and an increase of phosphorus concentration in an osmotic and acid-base compensatory mechanism. The increase of superoxide dismutase activity in gill and catalase in liver was seen as a response to oxidative stress caused by metals accumulated in these organs.

In conclusion, the chemical, biochemical, hematological and histological results of this work indicate that *Liza saliens* chronical exposure to moderate concentrations of Cu and Zn, affect the regulating mechanisms and involves structural and metabolic changes of adaptive nature to promote detoxification and even toxic.

At present, several actions have been implemented in Esmoriz/Paramos lagoon to improve water quality, nevertheless chemical monitorization and fish recovery assessmente is recommended.

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CAPÍTULO 1

INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

1.1. Metais

1.1.1. Considerações iniciais

A variedade de substâncias xenobióticas que usualmente são referidas como poluentes e que podem afectar os seres vivos é muito vasta, merecendo especial destaque os metais pesados. As concentrações de metais pesados nos solos e sedimentos não poluídos são geralmente baixas, dependendo essencialmente da sua pedogénese. No entanto, estudos sobre a distribuição de metais pesados em ecossistemas indicam que muitas áreas próximas de complexos urbanos e industriais, incluindo as de extracção de minério, apresentam concentrações elevadas destes elementos. Este aumento de metais ocorre devido a descargas de efluentes, à deposição em aterros e à emissão atmosférica, com a consequente deposição, contribuindo desta forma para o impacto negativo no ambiente. O uso de agro-químicos, designadamente de fertilizantes e pesticidas, contendo várias combinações de metais pesados, sendo alguns aplicados de forma intensiva, também contribui para a entrada antropogénica deste tipo de poluentes (Alloway, 1993; Novotny, 1995).

O efeito particularmente negativo dos metais pesados resulta da elevada persistência destes compostos no ambiente e da susceptibilidade de acumulação nos seres vivos, podendo atingir níveis tóxicos (Alloway, 1993). O ecossistema aquático é o que apresenta maior risco de poluição uma vez que as substâncias químicas, entre as quais os metais, podem ser eventualmente depositadas. Fenómenos de lixiviação, via efluentes ou via erosão, conduzem inevitavelmente ao transporte e à acumulação dos metais no meio aquático (Chapman et al., 2003), com a consequente distribuição nas fases que o integram, nomeadamente na água, nos sólidos em suspensão, nos sedimentos e biota. Os organismos aquáticos encontram-se progressivamente mais expostos a elevadas concentrações de metais e, consequentemente, numerosos estudos referem um aumento da sua concentração em peixes (Hornung e Ramelow, 1987; Romeo et al., 1999).

Contrariamente a outros poluentes, os metais embora sejam passíveis de uma certa regulação pelo organismo, não são irreversivelmente transformados ou metabolizados (Drexler et al., 2003). O aumento da sua presença no meio aquático, bem como a sua acção potencialmente tóxica, conduz a que os metais como poluentes sejam encarados com preocupação. É conhecido, a nível de

exposição aguda, que a presença de metais causa alterações morfológicas e bioquímicas nos peixes, podendo mesmo conduzir à morte. A título de exemplo, refira-se que o mecanismo da toxicidade aguda do Cu nos peixes é bem conhecido e envolve a acção directa em órgãos alvo (Handy, 2003). Contudo, a análise da informação actual sobre toxicidade crónica de metais nos peixes, permite concluir que os estudos que têm sido efectuados sobre os efeitos de vários anos de exposição ainda são escassos.

1.1.2. Biomarcadores

A monitorização de todos os contaminantes que representam uma potencial ameaça para o ambiente é difícil, quer sejam de natureza antropogénica, quer naturais. No entanto, a necessidade de avaliar o impacto da poluição na qualidade do ambiente, conduziu à análise de respostas bioquímicas que reflectem a potencialidade dos xenobióticos em prejudicar determinados processos fisiológicos (McCarthy e Shugart, 1990). Neste sentido, surgiram os marcadores de efeito biológico de contaminantes nos organismos, designados índices de *stress* no passado e que na actualidade são conhecidos como biomarcadores em diversos estudos de ecotoxicologia (McCarthy e Shugart, 1990; Livingstone, 1993; Timbrell, 1996).

Embora o conceito de biomarcador tenha evoluído, actualmente são considerados como sendo alterações traduzidas por uma resposta biológica, desde os níveis molecular, celular e fisiológico até comportamental, as quais podem estar relacionadas com a exposição de produtos químicos ou radiações libertadas no ambiente (NRC, 1989; Adams, 1990; Depledge et al., 1993; Peakall, 1994).

Dado que as alterações a nível molecular, bioquímico e fisiológico ocorrem numa fase precoce da exposição aos contaminantes, quando comparadas com as registadas ao nível da população ou comunidade os biomarcadores apresentam a possibilidade de funcionarem também como indicadores precoces de exposição (Au, 2004). O termo biomarcador foi definido pela "National Academy of Sciences" dos USA como sendo "uma variação induzida por xenobióticos em componentes celulares ou bioquímicos ou processos, estruturas, ou funções que são medidos num sistema biológico ou amostra" (Lam e Wu, 2003).

O objectivo do uso dos biomarcadores é o de relacionar a presença de substâncias tóxicas no meio com os efeitos no organismo, sendo que geralmente estes efeitos podem depender da toxicidade da substância, concentração e do grau de exposição. Apesar do uso de biomarcadores ser amplamente reconhecido,

existem algumas limitações à sua aplicabilidade que abarcam a complexidade e custo da metodologia, bem como os vários graus de especificidade. Muitos biomarcadores, talvez a maioria, são pouco específicos e traduzem uma resposta de *stress* do organismo devido à presença do contaminante (Lam e Wu, 2003). A aplicação de diferentes biomarcadores permitirá assim uma interpretação mais abrangente da resposta biológica do indivíduo, face à presença do xenobiótico. Por outro lado, a exposição crónica a contaminantes pode implicar alterações no indivíduo, com vista a desenvolver mecanismos de adaptação/compensatórios a distintos níveis e, neste caso, a resposta dos biomarcadores pode ser variável.

O fígado, considerando a sua diversidade funcional, possui um papel fisiológico de importância vital. Para além do seu papel na digestão, o fígado é um local de acumulação de lípidos, glicogénio e de vitaminas A e D, e encontra-se envolvido na secreção dos pigmentos biliares, bem como noutras actividades metabólicas. Por outro lado, é o principal órgão envolvido na acumulação, biotransformação e excreção de poluentes nos peixes (Hinton e Laurén, 1990; Triebkorn et al., 1997).

As brânquias são órgãos vitais responsáveis pelas trocas gasosas, osmoregulação e equilíbrio ácido-base, encontram-se envolvidas na excreção de compostos azotados e também desempenham um papel activo na excreção de poluentes (Hughes, 1984; Wood e Soivio, 1991; Evans et al., 1999). Dada a sua localização, podem estar continuamente expostas aos poluentes no meio aquático e, se por um lado são a primeira barreira entre o meio exterior e o meio interno, são também o local inicial onde eventualmente podem ser exercidos efeitos tóxicos (Playe et al., 1992; McDonald e Wood, 1993). Neste sentido a aplicação de biomarcadores de contaminação no fígado e na brânquia têm vindo a assumir uma importância crescente em estudos de ecotoxicologia de peixes (Hinton e Laurén, 1990).

1.1.3. Bioacumulação e toxicidade

Os estudos que envolvem bioacumulação de metais num sistema biológico potencialmente indicam a biodisponibilidade e a toxicidade destes contaminantes. A concentração de metais nos tecidos dum organismo, embora seja um indicador da exposição ao contaminante, não apresenta necessariamente uma relação directa com a concentração no meio, dado que a biodisponibilidade dos metais depende de diversos factores abióticos e bióticos. Por outro lado, embora a bioacumulação represente o balanço entre a entrada do contaminante e a sua depuração pelo organismo, envolve vários processos como a absorção, metabolismo, redistribuição,

compartimentação em tecidos específicos e excreção que vão influenciar a acumulação e a toxicidade.

Em termos gerais, quanto maior a bioacumulação de um poluente, maior é o risco de eventual toxicidade, embora nos peixes a acumulação de metais e a sua eventual toxicidade seja um processo complexo que depende de diversos factores que se referem de seguida.

1.1.3.1. Factores abióticos

A biodisponibilidade e a bioacumulação de metais no meio aquático podem ser controladas por diferentes processos. A biodisponibilidade tem sido definida de várias formas, embora a mais elucidativa considere a concentração de metais que é absorvida pelo organismo (Plette et al., 1999).

As principais vias de entrada de metais nos peixes são a ingestão e a absorção, em particular através das brânquias, atendendo a que pela derme o contributo é menor (Drexler et al., 2003). Os metais distribuem-se no meio aquático entre as fases aquosa e sólida e a especiação química pode condicionar a biodisponibilidade dos metais em ambas as fases. Alguns processos como a solubilidade, complexação, precipitação e adsorção que por sua vez dependem das propriedades do metal e das condições físico-químicas do meio, podem afectar a biodisponibilidade dos metais (Novotny, 1995; Plette et al., 1999; Chapman et al., 2003). Assim, num sistema natural a biodisponibilidade dos metais não é constante, pois vai depender da concentração total, bem como da dinâmica das condições do próprio sistema.

As condições físico-químicas do meio que podem influenciar a biodisponibilidade dos metais, incluem a concentração de oxigénio dissolvido, pH, temperatura, dureza da água, concentração de iões e a presença de agentes capazes de formar complexos com os metais, como é o caso da matéria orgânica (EPA, 1999; Plette et al., 1999; Chapman et al., 2003; Rajotte et al., 2003). De um modo geral, o ião metálico livre é a forma mais biodisponível (Drexler et al., 2003) e as condições que favorecem a complexação, adsorção e precipitação, tendem a diminuir a disponibilidade dos metais na fracção solúvel (Novotny, 1995). Contudo, o papel da dieta como veículo de entrada e de transferência de contaminantes pode assumir um papel relevante. Com o aumento da dureza da água, os metais tendem a precipitar (EPA, 1999) podendo a sua biodisponibilidade aumentar quando a via de entrada predominante é a ingestão de partículas.

1.1.3.2. Factores bióticos

Os factores biológicos também podem influenciar a bioacumulação dos metais, entre os quais se destacam a espécie, comportamento, fisiologia, morfologia, idade, tecido alvo e a capacidade reguladora do indivíduo.

O mecanismo de acumulação dos metais no peixe depende do tipo de dieta e do metabolismo, incluindo a assimilação, regulação e desintoxicação (Manon Vaal et al. 1997; Clearwater, 2002). O comportamento alimentar pode levar a um aumento da exposição ao contaminante, atendendo a que a ingestão de partículas em suspensão na água ou no sedimento pode representar uma fonte importante nos organismos detritívoros (Peplow e Edmonds, 2005). Alguns estudos demonstraram que diferentes espécies de peixes da mesma área geográfica, apresentavam diferentes concentrações de metais, consoante o tipo de dieta (Kalay et al., 1999; Canli e Atli, 2003; Marcovecchio, 2004).

Alguns metais, como o Cu e o Zn, desempenham funções essenciais em concentrações reduzidas. O Zn actua como componente metálico de diversas enzimas, sendo um cofactor regulador, funcional e estrutural. Alguns exemplos incluem a fosfatase alcalina, superóxido dismutase, e as ADN e ARN polimerases (Eisler, 1993; Kiekens, 1993; EPA, 2005). O Zn também é importante para o desenvolvimento normal das gónadas dos peixes (Kime, 1999; Kotze et al., 1999). O Cu é um elemento essencial para o metabolismo celular, pois é constituinte de algumas metaloenzimas, nomeadamente do complexo citocromo-oxidase, e assume um papel importante na síntese de hemoglobina (AEP, 1996).

A bioacumulação dos metais essenciais, como o Cu e Zn, pode ser regulada pelo peixe, num intervalo de concentrações (Kiekens, 1993; Drexler et al., 2003; Rajotte et al., 2003). Todavia, estes metais podem apresentar efeitos tóxicos quando o equilíbrio homeostático é afectado e as concentrações nos tecidos são aumentadas (Eastwood e Couture, 2002). Pelo contrário, a bioacumulação dos metais não essenciais, como são os casos do Pb e Cr, reflecte a biodisponibilidade do meio, dado que não são activamente regulados (Chattopadhyay et al., 2002).

O tamanho do peixe é outro dos factores que pode também influenciar a bioacumulação de metais (Al-Yousuf et al., 2000; Canli e Atli, 2003). Em termos genéricos, os peixes mais jovens acumulam maior quantidade de metais, porque apresentam uma maior taxa de metabolismo e apresentam uma superfície corporal maior em relação ao volume, comparando com os mais velhos (Borga et al., 2004). A acumulação dos metais essenciais, como é o caso do Cu, também depende da fase de crescimento. Em situação de exposição crónica os peixes adultos

apresentam maior capacidade para regular a concentração do Cu, em comparação com os juvenis da mesma espécie (Handy, 2003).

Os níveis de metais que podem ser acumulados em cada tecido também são variáveis, com as funções fisiológicas do órgão. O músculo não é um tecido activo ao nível da acumulação e da regulação de metais quando comparado com o fígado e a brânquia (Romeo et al., 1999). Em consequência, alguns estudos demonstraram que existe uma correlação entre a exposição aos metais e a sua concentração na brânquia e fígado de peixes (Al-Yousuf et al., 2000; Olsvik et al., 2001; Chattopadhyay et al., 2002; Sekhar et al., 2003; Karadede et al., 2004).

A sazonalidade influencia a bioacumulação de metais através de alterações fisiológicas e comportamentais no peixe, bem como de alterações na biodisponibilidade dos metais, atendendo a que esta responde às alterações do meio. A reprodução, intimamente ligada a este aspecto, pode conduzir à mobilização de Zn, o qual é necessário para a maturação das gónadas, em particular, nas fêmeas (Kotze et al., 1999) e em alterações de comportamento, como migrações e hábitos alimentares.

Existem outros factores que afectam a bioacumulação dos metais, entre os quais se destacam os níveis e a duração de exposição. Vários estudos mostraram um aumento da bioacumulação como resposta a gradientes de concentração, em particular durante a exposição aguda. No entanto, durante a exposição crónica a relação da bioacumulação com a concentração de exposição pode ser diferente, em especial, para os metais essenciais, pois o organismo tem tempo para a regulação (Olsvik et al., 2001; Rajotte et al., 2003; Kraemer et al., 2005), designadamente na exposição crónica, a excreção do Cu pelo fígado é estimulada (Handy, 2003).

Do exposto, resulta que a bioacumulação de metais nos peixes nem sempre reflecte a concentração de exposição biodisponível, uma vez que as relações entre as concentrações nos tecidos e no meio podem variar com o metal (essenciais ou não), espécie (caso do comportamento alimentar) e tecido (fígado *versus* músculo).

Vários exemplos mostram que a toxicidade do contaminante está relacionada com a elevada concentração encontrada nos tecidos. De um modo geral, quanto maior a bioacumulação de metais, maior é o risco de toxicidade. No entanto não existe uma relação directa, pois a toxicidade depende também de diversos factores, nomeadamente do tipo de organismo, fase de crescimento, características do metal e do tempo de exposição, entre outros.

A sensibilidade à presença de metais depende da espécie e pode ser manifestada de diferente modo. Estudos laboratoriais com truta, salmão, tainha e

peixe-gato, mostraram diferentes efeitos negativos no seu crescimento e/ou sobrevivência quando expostos a concentrações de Cu na dieta (Clearwater, 2002).

A toxicidade aguda dos metais pode ser marcadamente influenciada pela idade (Eisler, 1993). Em geral, os peixes mais jovens são mais susceptíveis à toxicidade dos metais do que os adultos, dado que acumulam uma maior quantidade, e porque em fases mais precoces, os órgãos e o metabolismo, não se encontram completamente desenvolvidos.

Alguns estudos revelam que ocorre aclimação dos peixes, envolvendo processos fisiológicos que se traduzem num aumento da tolerância ao metal (Hollis, et al., 1999; Stubblefield et al., 1999; Alsop e Wood, 2000; Chapman et al., 2003; Drexler et al., 2003). As metalotioneínas são proteínas de baixo peso molecular que regulam os níveis de metais essenciais na célula, designadamente de Cu e Zn. A indução da síntese de metalotioneínas é um mecanismo comum, que possibilita aos peixes resistir ao aumento de metais (Olsvik et al., 2001; Chapman, et al., 2003), permitindo a sequestração de metais, em particular do Cd, além dos referidos (AETE, 1999; Drexler et al., 2003; Kraemer et al., 2005). O aumento da excreção no peixe através de diferentes órgãos, intestino (fezes), rim (urina), fígado (bólis), brânquias e pele, e a redução da absorção, são também mecanismos envolvidos na regulação e toxicidade dos metais (Bury et al., 2002; Clearwater, 2002; Grosell et al., 2002; Chapman et al., 2003).

Adicionalmente, a exposição crónica dos peixes aos metais pode conduzir ao desenvolvimento de outros mecanismos de adaptação que permitam minimizar os efeitos tóxicos. Alterações da estratégia reprodutiva ou da locomoção são exemplos adaptativos com vista a poupar energia (Handy, 2003).

A toxicidade dos metais pode ser afectada pelos parâmetros físico-químicos da água. O aumento da temperatura resulta na diminuição dos níveis de oxigénio na água, aumento do metabolismo e traduzem-se no aumento da toxicidade dos metais (Rajotte et al., 2003). Por exemplo, temperaturas elevadas e baixo teor de oxigénio dissolvido tendem a aumentar a toxicidade do Zn (EPA, 1999; Eisler, 1993).

O efeito tóxico também depende de outros factores, como a presença de outros compostos na água. A toxicidade do Cu nos peixes de água doce é reduzida com a presença de cálcio na água (Grosell et al., 2003), sendo a toxicidade do Zn diminuída com a presença de cálcio e magnésio (EPA, 1999). A interacção do Zn com outros metais existentes no meio também pode afectar a sua toxicidade. Em geral, a exposição a misturas de Zn e Cu em vários organismos aquáticos, incluindo os peixes, resulta em toxicidade para além do efeito aditivo (Eisler, 1993).

1.1.4. Parâmetros de condição

A determinação de alguns índices relacionados com o crescimento e a reprodução em peixes é útil na avaliação crónica da toxicidade dos metais. As alterações no tamanho do fígado devido a *stress* ambiental revestem-se de um particular interesse. Atendendo às funções metabólicas que este órgão desempenha, o índice hepatossomático (IHS) que relaciona o peso do fígado com o peso total do peixe, pode ser utilizado para avaliar indirectamente o tamanho deste órgão (Dethloff e Schmitt, 2000). Apesar de vários estudos relacionarem este índice com a exposição a contaminantes, a sua relação com os metais não é consensual. Na realidade, alguns peixes expostos a metais não mostraram variação nos valores de IHS (Martin e Black, 1996; Eastwood e Couture, 2002), enquanto outros mostraram uma diminuição (Norris et al., 2000).

O factor de condição de Fulton (*K*) relaciona o peso total do peixe com seu comprimento, sendo um indicador das reservas energéticas a longo tempo. Embora se trate de um parâmetro que não é específico, pode fornecer informação sobre o efeito de contaminantes crónicos e vários estudos relacionam este indicador com a exposição aos metais. Em geral, a presença crónica de metais conduz a uma diminuição dos valores de *K* (AETE, 1999; Laflamme et al., 2000; Norris et al, 2000; Eastwood e Couture, 2002; Rajotte et al., 2003).

Embora *K* e IHS possam variar com o ciclo reprodutivo e a presença de diversos contaminantes, a sua utilização em estudos de campo, pode constituir uma evidência de alterações ocorridas nos peixes em consequência da exposição a metais.

1.1.5. Histopatologia

As alterações que ocorrem nos órgãos alvo, como o fígado e a brânquia, revestem-se de particular interesse, dadas as funções fisiológicas que desempenham. A presença de lesões internas no fígado e na brânquia dos peixes tem sido usada como biomarcador da exposição aguda ou crónica a contaminantes, nomeadamente de metais (Hinton e Laurén, 1990; Munday e Nowak, 1997; Tricklebank, 2001; Au, 2004).

A etologia das alterações encontradas no fígado é conhecida com base no estudo dos mamíferos, cujo conhecimento é mais vasto. No entanto, o interesse por esta área de estudo nos peixes tem aumentado nos últimos tempos, representando os peixes um modelo para avaliar a saúde dos ecossistemas aquáticos e em toxicologia patológica (Law, 2003).

Entre as lesões que têm sido encontradas em estudos deste tipo, incluem-se as neoplasias malignas, como o carcinoma hepatocelular, e lesões toxicopáticas iniciais, como as não neoplásicas, designadamente o polimorfismo celular e nuclear (Mikaelian et al., 2002; Stentiford et al., 2003). O desenvolvimento de lesões pré-neoplásicas, apontadas como estádios iniciais da formação de neoplasias hepáticas, bem como de lesões neoplásicas, resulta de uma resposta a longo termo. Trata-se de uma resposta que é característica de exposição crónica a contaminantes e, deste modo, utilizadas como biomarcador histopatológico (Köhler et al., 2002; Stentiford et al., 2003; Au, 2004).

Embora as lesões histopatológicas no fígado de peixes não sejam, em geral, específicas de um determinado composto tóxico, alguns estudos relacionam a sua presença e incidência com o tempo de exposição e a concentração de poluentes, nomeadamente dos metais, tanto em laboratório (Schwaiger et al., 1997; Arellano et al., 1999; Paris-Palacios et al., 2000; Varanka et al., 2001; Olojo et al., 2005; Van Dyk et al., 2007) como em estudos de campo (Hinton et al., 1992; Teh et al., 1997; Wilhelm Filho et al., 2001; Mikaelian et al., 2002; Köhler et al., 2002; Stentiford et al., 2003).

O efeito dos metais na histologia da brânquia também tem sido estudado em diversas espécies de peixes. As brânquias, pela sua localização e ampla superfície, são sensíveis à presença de vários contaminantes, sofrendo alterações histopatológicas do epitélio, como por exemplo destacamento, hipertrofia, necrose, fusão de lamelas primárias e secundárias e edema dos capilares. Estas alterações branquiais ocorrem como resposta à exposição de poluentes e embora não sejam específicas de um determinado tóxico (Au, 2004), diferentes estudos em laboratório mostraram existir relação entre a manifestação de determinadas lesões e a presença de metais (Skidmore e Tovell, 1972; Schwaiger et al., 1997; Arellano et al. 1999; De Boeck et al. 2001; Mazon et al., 2002; Martinez, et al., 2004; Monteiro et al. 2005; Olojo et al., 2005).

Em síntese, diversos trabalhos relacionam a presença de metais com a presença de alterações histomorfológicas no fígado e na brânquia de peixes, considerando a histopatologia como um biomarcador de exposição.

No caso dos estudos de campo, apesar de diversos factores poderem condicionar as alterações histopatológicas, a análise da sua prevalência, severidade e extensão e a comparação com resultados obtidos em condições experimentais, poderão constituir indicadores de exposição crónica a contaminantes, nomeadamente metais.

1.1.6. Parâmetros do *stress* osmótico

A presença de metais no meio aquático, além de poder induzir alterações estruturais na brânquia, também pode afectar a capacidade respiratória e a regulação, quer iónica quer osmótica dos peixes (Wendelaar e Lock, 1992; Martinez et al. 2004). Nos peixes de água doce em geral, concentrações moderadas de metais influenciam a regulação iónica, enquanto concentrações elevadas podem alterar a capacidade respiratória, devido a alterações estruturais, podendo mesmo conduzir à morte (Playle, 1998).

A diminuição de sódio no plasma tem sido amplamente usada como um indicador de *stress* ambiental, devido à inibição da actividade da Na^+/K^+ -ATPase branquial (Mazon et al. 2002). Diversos estudos demonstraram existir uma diminuição do teor plasmático em sódio, como resposta à toxicidade aguda causada por metais, em peixes de água doce (Mazon et al., 2002; Martinez et al. 2004; Taylor et al., 2004; Monteiro et al., 2005). De igual modo, as concentrações plasmáticas de cloro, potássio e cálcio também têm sido utilizadas como indicadores da função osmótica. A título de exemplo, refira-se que o Cu pode induzir a inibição da captação activa de iões, através da inibição da Na^+/K^+ -ATPase (Li et al., 1998) e aumentar a permeabilidade do epitélio, conduzindo a um acréscimo do fluxo iónico (Lauren e McDonald, 1985).

As potenciais variações da capacidade de osmoregulação, traduzem-se numa diminuição do nível destes electrólitos (Mazon et al. 2002; Zsigmond et al. 2002). A acção tóxica do Zn pode traduzir-se na alteração da regulação do cálcio, podendo conduzir a uma hipocalcemia (Alsop e Wood, 2000).

Os metais com um potencial efeito negativo na regulação iónica podem ser agrupados nas seguintes categorias: os que afectam iões Na^+/Cl^- , como é o caso do Cu, e os que influenciam o Ca^{2+} , designadamente o Zn, de acordo com a afinidade para os substratos (Alsop e Wood, 2000; Chapman et al., 2003). Assim, podem ocorrer efeitos antagonistas, aditivos ou sinérgicos quando o peixe é exposto a diversos metais. Por outro lado, embora os mecanismos de resposta da capacidade osmoreguladora da brânquia, à toxicidade aguda induzida por metais, sejam identificáveis, o processo pode ser diferente na exposição crónica e merece ser estudado com maior detalhe.

1.1.7. Parâmetros do *stress* oxidativo

A presença de metais nos organismos vivos pode também originar a formação de espécies reactivas de oxigénio (EROs), também conhecidas por oxiradicaís (Ercal

et al., 2001; Durmaz et al., 2006; Lesser, 2006). As EROs podem ter influência ao nível da actividade de enzimas, da peroxidação lipídica, do ADN e, em casos extremos, conduzir à morte celular (Livingstone, 2001). As EROs podem ser desintoxicadas por acção de enzimas que participam nas fases I e II do mecanismo de biotransformação e por enzimas do *stress* oxidativo, nomeadamente a superóxido dismutase (SOD) e a catalase (CAT).

A SOD representa um grupo de metaloenzimas envolvidas na função antioxidante que catalisam a conversão do anião $O_2^{\cdot -}$ em H_2O_2 , enquanto a CAT cataliza a reacção do peróxido de oxigénio em água e oxigénio (Livingstone, 2001; Lushchak et al., 2001; Ozmen et al, 2004; Lesser, 2006). A glutathione S-transferase (GST) é uma enzima multifuncional da fase II que se encontra envolvida na conjugação dos xenobióticos com a glutathione, tendo como resultado um aumento da solubilidade dos xenobióticos, conduzindo a uma rápida excreção (Livingstone, 2003).

A alteração da actividade destas enzimas ocorre, particularmente no fígado, tendo sido usada na monitorização da exposição a contaminantes, constituindo um biomarcador de contaminação em numerosos estudos (Livingstone, 2001; Orbea et al, 2002; Livingstone, 2003; Regoli et al, 2004; Sen e Kirikbakan, 2004; Ferreira et al., 2005; Li et al., 2005). Os danos oxidativos, como o caso da peroxidação lipídica, também têm sido utilizados como potenciais biomarcadores em estudos de toxicologia ambiental (Sayeed et al., 2003; Bláha et al., 2004; Ozmen et al, 2004; Almroth et al., 2005; Durmaz et al., 2006).

Diversos estudos realizados em laboratório com peixes indicam que as actividades da SOD e da CAT são geralmente induzidas pelo *stress* oxidativo, devido à presença de metais (Paris-Palacios et al., 2000; Sanchez et al., 2005) ou outros poluentes (Palace et al., 1996; Figueiredo-Fernandes et al., 2006). Resultados similares têm sido obtidos em estudos de campo (Porte et al., 2002; Deviller et al., 2005; Ferreira, et al., 2005). Contudo, estes resultados não são consensuais. Na realidade, outros estudos realizados em peixes revelaram uma diminuição da actividade da SOD na presença de poluentes, tanto em laboratório (Peixoto et al., 2006) como em zonas poluídas (Wilhelm Filho et al., 2001).

De igual modo, os resultados sobre os efeitos dos poluentes na actividade da GST não são conclusivos, revelando existir indução em determinadas condições (Sen e Kirikbakan, 2004; Camargo e Martinez, 2006), inibição da actividade enzimática (Martinez-Lara et al., 1996; Wilhelm Filho et al., 2001), enquanto outros sugerem não existir alteração (Porte et al., 2002; Sanchez et al., 2005).

Em síntese, embora o *stress* oxidativo possa levar ao estímulo da actividade destas enzimas, esta resposta não é consensual, pelo que merece ser estudada.

1.1.8. Parâmetros hematológicos

Os indicadores hematológicos não são específicos na sua resposta a compostos químicos indutores de *stress*. No entanto, podem fornecer informação relevante em estudos de avaliação ambiental, uma vez que constituem uma indicação da fisiologia geral e do estado de saúde dos organismos (Wester et al., 1994). Nomeadamente, o aumento da actividade das aminotransferases, AST e ALT, no plasma é usado no diagnóstico de lesões causadas por poluentes, em diversos tecidos do peixe (Bucher e Hofer, 1990; De la Torre, et al., 2000; Yang e Chen, 2003). Na realidade, a actividade de enzimas plasmáticas possibilita uma avaliação do estado interno do peixe (Lusková et al., 2002), tendo vindo a ser aplicada na monitorização do efeito de diversos contaminantes (Oluah, 1999; Bernet et al. 2001; Begun, 2004).

As aminotransferases dos peixes, à semelhança de outros vertebrados, são enzimas intracelulares que estão, genericamente, envolvidas no catabolismo dos aminoácidos. Este grupo de enzimas cataliza a transferência de grupos amino durante a conversão de aminoácidos a alfa-cetoácidos.

A aspartato aminotransferase (AST), localizada nas células do fígado entre outros órgãos, catalisa a transaminação de aspartato e alfa-cetoglutarato em oxaloacetato e glutamato (Mosby, 1995; Ekrem, 2004).

Por seu lado, a alanina aminotransferase (ALT) tem como função catalizar a transaminação da alanina e alfa-cetoglutarato em piruvato e glutamato. Esta enzima encontra-se em diversos tecidos, em particular no fígado, sendo considerada hepato-específica, visto que na degeneração ou necrose hepática se observa um aumento significativo da sua actividade no plasma (Mosby, 1995).

A fosfatase alcalina (ALP) é um grupo de enzimas envolvido na separação dos grupos fosfato terminais dos estéres orgânicos, presente no intestino, nos rins e principalmente nos hepatócitos (Bernet et al., 2001).

A presença de metais pesados pode influenciar a actividade destas enzimas. A indução da actividade da AST e ALT, pela presença de Zn, Cu e Cd em condições controladas, foi demonstrada para *Clarias albopunctatus*, *Cyprinus carpio* e *Carassius auratus gibelio* (Oluah, 1999; Varanka et al., 2001; Zikic' et al., 2001), bem como em condições de exposição crónica a metais pesados na *Perca flavescens* (Levesque et al., 2002). A presença de outros poluentes também pode

influenciar a actividade destas enzimas. Peixes recolhidos em zonas com níveis de poluição diversos mostraram um aumento da actividade plasmática da ALP (Zsigmond et al., 2002) e da AST (Beyer, 1996; Van der Oost et al., 1998).

No entanto, a presença de poluentes nem sempre induz a actividade das transaminases e da ALP no plasma. Foi registado o aumento na actividade enzimática da AST e ALT em truta (*Salmo trutta*) sob exposição aguda a efluentes domésticos, enquanto que em condições de exposição crónica, a actividade destas enzimas voltavam aos valores considerados normais para a espécie (Bucher e Hofer, 1990). Bernet et al. (2001) observaram, por outro lado, uma diminuição da actividade da ALP em trutas (*Salmo trutta*) expostas a efluentes contaminados. Além disso, não foram encontradas diferenças na actividade da AST e ALT em enguias (*Anguilla anguilla*) recolhidas em locais com níveis de poluição consideráveis, em comparação com peixes de um local de referência (Van der Oost et al., 1996).

Do exposto, pode-se sintetizar que a resposta aos poluentes varia com a espécie de peixe e com o tipo de poluente. Apesar disso, a actividade das enzimas plasmáticas poderá vir a ser considerado um biomarcador promissor para a presença de metais.

1.2. Barrinha de Esmoriz/Lagoa de Paramos

1.2.1. Caracterização geral

A Barrinha de Esmoriz/Lagoa de Paramos é uma laguna costeira situada no litoral Norte de Portugal, entre os concelhos de Ovar e Espinho que ocupa uma área de cerca de 396 ha. Esta laguna apresenta uma forma grosseiramente triangular, com cerca de 1500 m de comprimento, na direcção Norte-Sul, e cerca de 700 m de largura, na direcção Este-Oeste. Encontra-se afastada do mar cerca de 400 m, separada por dunas e pela faixa de areia. Na zona envolvente da Barrinha existem áreas de pinhal (Sul), bosques ripícolas/húmidos (Este e Sul), praia e dunas (Oeste), campos agrícolas (Norte, Leste e Sul), planície com vegetação rasteira/arbustiva (Norte) e construções (Norte, Este e Sul).

A frente litoral da Barrinha apresenta uma configuração suave, com uma inclinação da ordem dos 3%, da parte emersa da praia, e de 1.5%, nos fundos adjacentes à linha de costa (Almeida, 1998). A acumulação de água nesta laguna resulta essencialmente dos volumes drenados por duas ribeiras principais, Ribeira

de Paramos e Vala de Maceda. De um modo geral, trata-se de uma laguna com baixa profundidade, apresentando os seus fundos cobertos por sedimentos móveis.

Até ao início deste estudo (2004), a ligação da laguna com o mar era efectuada ocasionalmente por um canal de largura variável com cerca de 600 m de comprimento, de orientação Este-Oeste, com tendência a fechar por acção do transporte sólido litoral. A comunicação com o mar verificava-se na altura das marés-vivas, quando ocorria precipitação muito elevada ou, de forma artificial, através de dragagens. Quando a comunicação com o mar estava interrompida e a Barrinha estava cheia, a área do plano de água chegava aos 56 ha, correspondendo a um volume aproximado de 230.000 m³ de água (SIMRIA, 2002). Durante o Verão a Barrinha funcionava como um sistema isolado, sem comunicação com o mar, sendo o nível da água mantido pelos caudais dos seus afluentes, a cotas entre os 1.5-2.0 m, podendo pontualmente chegar aos 2.5 m (Almeida, 1998).

1.2.2. Caracterização ecológica

De forma geral, as lagunas costeiras constituem biótopos importantes para um número considerável de espécies animais e vegetais que podem ser classificadas como endémicas, raras ou ameaçadas. A Barrinha de Esmoriz/Lagoa de Paramos é a zona húmida mais significativa no litoral Norte, entre a Ria de Aveiro e o Estuário do Rio Minho (ICN, 2006).

Esta laguna apresentou onze habitats classificados como protegidos, incluídos no Anexo I da Directiva Habitats (Directiva 92/43/CEE, transposta pelo DL N^o 226/1997) que vão desde dunas fixas com vegetação herbácea, a florestas aluviais residuais. Este local foi considerado estratégico de estadia e/ou passagem para um grande número de aves e espécies dependentes deste ecossistema, que constam do anexo A1 do DL N^o 140/1999, referente a espécies de interesse comunitário, cuja conservação requer a designação de zonas de protecção especial. Esta laguna foi classificada como prioritária na Directiva Habitat e foi incluída na Lista Nacional de Sítios (Resolução do Conselho de Ministros N^o 76/2000).

Esta laguna costeira já foi o suporte de uma grande diversidade de espécies piscícolas, que pelas suas exigências em termos de qualidade ambiental foram desaparecendo, permanecendo as mais resistentes e de menor valor económico, como a tainha.

Actualmente, a Barrinha de Esmoriz/Lagoa de Paramos encontra-se integrada na Reserva Ecológica Nacional, classificada como biótopo CORINE (no âmbito da

Comissão da União Europeia) e integrada na rede Natura 2000 (SIMRIA, 2002; ICN, 2006).

1.2.3. Fontes de poluição

A Barrinha tem sido o destino final de inúmeras descargas de efluentes. As fontes de poluição na bacia de drenagem para este sistema lagunar foram até ao início deste trabalho sobretudo descargas não controladas de águas residuais domésticas e industriais e situações difusas referentes às actividades agrícola e agropecuária (SIMRIA, 2002; AMRIA, 2006).

A área da Bacia Hidrográfica que drena para a Barrinha de Esmoriz/Lagoa de Paramos apresenta uma forte ocupação humana e industrial, englobando parte dos concelhos de Espinho, Santa Maria da Feira e de Ovar. Por outro lado, as zonas agrícolas que constituem áreas bem representativas, localizam-se principalmente em zonas adjacentes às linhas de água. Embora nesta região as parcelas de terreno agrícola, em geral, não excedam os 5 ha são exploradas intensivamente, recorrendo aos fertilizantes e pesticidas (Almeida, 1998).

A zona Este da envolvente limítrofe da Barrinha também apresenta uma área habitacional, com diferentes actividades, como é o caso das oficinas de reparação de automóveis com efluentes residuais directos para a laguna.

Em termos de ocupação industrial, destacam-se diversas unidades pertencentes aos sectores de cortiça, curtumes, calçado, madeiras, papel, têxtil (cordoaria e tapeçarias), reparação automóvel, metalurgia e metalomecânica, indústria de produtos químicos (abrasivos, detergentes, tintas, vernizes) e indústrias agropecuárias, que se concentram predominantemente no Concelho de Santa Maria da Feira, no núcleo industrial da freguesia de Paramos e na zona industrial de Esmoriz (Almeida, 1998; SIMRIA, 2002). Os efluentes destas indústrias eram escoados pelas redes de drenagem de águas residuais urbanas, quando existentes e apresentavam carga poluente muito diversa, sendo frequente a presença de metais pesados (SIMRIA, 2002).

1.2.4. Rede hidrográfica

A Bacia Hidrográfica que drena para a Barrinha de Esmoriz/Lagoa de Paramos tem aproximadamente 78 Km² de área e 44 Km de perímetro (Almeida, 1998; SIMRIA, 2002). A rede hidrográfica é constituída por duas linhas de água principais, com direcção dominante Este-Oeste, a Ribeira de Paramos que desagua a Norte da laguna e a Vala de Maceda, que desagua a Sul da Barrinha. Estas linhas de água

apresentavam problemas de qualidade associados quer a descargas de efluentes domésticos e industriais, quer ao lançamento de resíduos diversos (SIMRIA, 2002; AMRIA, 2006).

Nas últimas décadas, tem-se verificado uma marcada diminuição, em largura e profundidade, do canal de comunicação com o mar e um acentuado assoreamento da Barrinha.

A diminuição do canal deveu-se principalmente ao arrastamento pelo vento de material das dunas e da praia e a consequente entrada na embocadura da Barrinha. A deposição continuada de sedimentos contaminados transportados pelas linhas de água, para além de contribuir para a diminuição da qualidade da água e sedimentos, tem sido o aspecto mais determinante para o assoreamento da Barrinha. O volume de transporte sólido drenado para a Barrinha de Esmoriz/Lagoa de Paramos estimou-se em 14 000 m³ por ano, contribuindo a Vala de Maceda maioritariamente, com cerca de 52%, e a Ribeira de Paramos com cerca de 39% (Almeida, 1998). Esta situação foi também agravada pela deficiente renovação de massas de água, em consequência das alterações no sistema natural de ligação desta laguna ao mar. Outras causas apontadas para o assoreamento da Barrinha foram o despejo de entulhos e são a tomada de terrenos adjacentes e o consequente aterro.

1.2.5. Estado da Barrinha

No início deste estudo, a Barrinha de Esmoriz/Lagoa de Paramos encontrava-se num estado avançado de degradação, devido a diversos factores. O assoreamento progressivo que levou à redução da área submersa (Figura 1.1), a taxa reduzida de renovação da água e os problemas de qualidade associados à ocupação humana, agrícola e industrial da Bacia Hidrográfica, bem como da zona envolvente, teve como consequência a diminuição da qualidade da água da Barrinha e dos seus efluentes, contribuindo para a alteração profunda que se verificou neste ecossistema.



Fig. 1.1 - Diminuição da profundidade média da laguna, com evidente redução da área inundada.

A vegetação da zona envolvente encontrava-se danificada e era visível a deposição de lixo variável, entulho e outros. A água da Barrinha na maioria das vezes transportava diversos detritos e apresentava um forte cheiro nauseante (Figura 1.2).



Fig. 1.2 - Exemplo de situação frequente na laguna, por forte presença de detritos e matéria em suspensão na água.

A ictiofauna encontrada era muito pouco diversificada, sendo dominada pela presença da tainha, concordante com a diminuição da qualidade da sua água e sedimentos.

Na actualidade, o estado desta laguna é diferente, denotando esforços para a sua revitalização. Para além de concluída a obra de gestão que controla a abertura da

Barrinha ao mar, foi efectuado o alargamento do Sistema Multimunicipal à Barrinha, rede que engloba colectores, ETARs e exutores submarinos (SIMRIA, 2006). Assim a qualidade da água poderá sofrer uma importante melhoria e a recuperação da laguna poderá ser uma realidade.

1.3. Biologia da tainha

1.3.1 Sistemática e distribuição geográfica

As tainhas pertencem à classe dos Actinopterygii, peixes caracterizados por apresentarem barbatanas suportadas por raios, esqueleto interno tipicamente calcificado e aberturas branquiais protegidas por um opérculo ósseo. Representam o grupo dominante dos vertebrados, apresentando milhares de espécies em todos os ambientes aquáticos (Wikipédia, 2006; ITIS, 2006)

A superordem Acanthopterygii, à qual pertencem estes peixes, representa um grupo monofilético que se caracteriza por várias estruturas e especializações funcionais, como o aparelho mandibular faríngeo e o mecanismo mandibular oral, englobando mais de metade de todas as famílias de peixes. A tainha é o nome vulgar de vários peixes da família *Mugilidae* que actualmente constituem o único membro da ordem dos Mugiliformes, da qual fazem parte várias dezenas de espécies e 17 géneros, entre os quais os géneros *Liza* e *Mugi*. Este último engloba a maior parte das espécies (Thomson, 1980; CSBD, 2006; Wikipédia, 2006).

Os mugilídeos encontram-se distribuídos por todo mundo, preferindo águas costeiras temperadas ou tropicais. De uma forma geral, distribuem-se preferencialmente junto à costa, na desembocadura de rios ou nos seus cursos baixos. A tainha é nativa da costa Ibérica e de abundância comum (Drake e Arias, 1991; Blasco et al., 1998). Actualmente, *Liza saliens* é a única espécie que abunda na Barrinha e, por este motivo, foi usada como modelo no presente estudo.

1.3.2. Características ecológicas

As tainhas apresentam uma elevada tolerância a uma vasta amplitude de parâmetros de qualidade da água, adaptando-se a condições aquáticas muito adversas. Com efeito, as tainhas são encontradas em habitats que outros géneros de peixes não toleram, sendo também muito resistentes à presença de substâncias tóxicas naturais ou de origem antropogénica.

São peixes eurialinos, estendendo-se o seu habitat desde a água salgada, salobra, existindo até algumas espécies que vivem em água doce (Minos et al., 1995). A *Liza saliens* tolera níveis de salinidade muito baixos, sendo usualmente encontrada em águas salobras. Segundo Koutrakis (2004) a ocorrência de diferentes mugilídeos em estuários e rios é influenciada pelos níveis de salinidade e no caso de *L. saliens* a maior percentagem de capturas, de acordo com este autor, ocorreu em zonas de baixa salinidade (0-3‰).

Em relação à temperatura, tratam-se de peixes euritêrmicos, preferindo no entanto águas quentes (Minos et al., 1995). No caso de *L. saliens*, tolera variações de 5 a 27 °C (CSBD, 2006).

As tainhas são peixes gregários, que se encontram principalmente junto à costa, mantendo-se nas camadas de água superficiais. A *L. saliens* prefere águas de profundidade acima dos 5-700 m (CSBD, 2006), entrando em lagoas e estuários de rios para se alimentarem. Os juvenis aparecem nos estuários cerca de 1 a 1,5 meses após a desova, dependendo da distância da zona de reprodução em relação à costa, visto que os ovos e os alevins são transportados por correntes marítimas (Koutrakis, 2004).

A maturação das gónadas e a reprodução tem lugar exclusivamente no mar (Fehri-Bedoui et al., 2002), nas zonas pelágicas, obrigando por isso a migrações sazonais. Geralmente, a maturação sexual ocorre a partir dos 3 anos, e é frequente que a maturação dos machos ocorra mais cedo do que a das fêmeas. Os machos de *L. saliens* atingem a maturidade sexual a partir dos 3 anos e as fêmeas após os 4 anos (CSBD, 2006). Algumas espécies de tainha, como *Mugil cephalus* têm tido assinalável êxito em cultivo. No entanto, a desova espontânea destas fêmeas não ocorre, visto não alcançarem a maturação sexual completa, sendo em alternativa induzida por hormonas, como a gonadotropina. Em contrapartida, os machos apresentam maturação sexual espontânea na estação normal de reprodução (Landeta, 1983).

A época da desova para cada espécie depende da latitude devido ao fotoperíodo e à temperatura (Landeta, 1983). No mar Cáspio e dependendo da temperatura, a reprodução de *L. saliens* ocorre de Junho a Setembro (CSBD, 2006). Os diferentes períodos de reprodução entre as espécies originam sucessivos picos de abundância de indivíduos ao longo do tempo nos sistemas estuarinos, com vantagens do ponto de vista alimentar. Em geral, coexistem não mais de 2 a 3 espécies de mugilídeos, conseguindo aproveitar os recursos alimentares disponíveis (Koutrakis, 2004).

As tainhas são omnívoras, alimentando-se de fitoplancton, zooplacton, organismos bentónicos e detritos. São peixes com uma grande versatilidade do ponto de vista alimentar, sendo frequente a sua presença junto a efluentes não tratados, como o que se pode usualmente observar no rio Douro e na Barrinha/Lagoa de Esmoriz.

Em termos económicos, a tainha é um peixe comestível pouco utilizado em Portugal e utilizado, particularmente, no Egipto, Espanha, França, Grécia, Israel, Itália, Irão e Tunísia, entre outros países, onde as capturas comerciais ascendem a várias toneladas (Fehri-Bedoui et al., 2002; CSBD, 2006).

1.4. Objectivos e metodologia geral

Os mecanismos envolvidos na toxicidade crónica de metais no meio aquático não se encontram completamente esclarecidos, já que a maioria da informação advém por um lado de um número restrito de espécies e, por outro, de estudos com pouca duração, quer no campo, quer no laboratório. No último caso, tem a agravante de não ser considerada a variabilidade inerente aos sistemas naturais.

O presente trabalho representa um contributo para o conhecimento dos mecanismos da acção sub-letal dos metais, particularmente, em peixes. Devido à presença abundante de tainha (*Liza saliens*) na Barrinha de Esmoriz/Lagoa de Paramos e pelas características apresentadas por esta, em particular, a contaminação por metais pesados e o isolamento da laguna, este mugilídeo foi usado para estudar o impacto da exposição crónica a metais.

No início deste trabalho, esta laguna caracterizava-se pela interrupção do canal de comunicação com o mar, sendo esta muito ocasional, o que dificultava a migração dos peixes. Em consequência, os peixes capturados apresentam fortes possibilidades de aí terem permanecido toda a sua vida, representando por isso o modelo ideal para estudos de exposição crónica. O peixe é considerado um bom modelo em estudos de toxicidade, visto que se encontra em contacto directo com os poluentes e, adicionalmente, muitos dos mecanismos bioquímicos envolvidos nos processos de toxicidade são comuns aos do Homem.

Como grupo controlo, utilizou-se um grupo de mugilídeos de alto mar, que foram capturados em simultâneo com os da laguna. Não obstante não serem peixes sedentários, supõem-se estarem sujeitos a menor concentração de metais ou de outros poluentes.

A avaliação da contaminação causada pelos metais pesados neste ecossistema envolveu, numa primeira fase deste estudo, a sua quantificação. Assim, no capítulo 2 são descritas as metodologias e os resultados da determinação de metais nos

sedimentos e na água, e estabeleceu-se a sua relação com a concentração encontrada em alguns tecidos de *Liza saliens*.

No capítulo 3 os factores de bioacumulação dos metais nos diferentes tecidos de *L. saliens*, nomeadamente brânquias, fígado e músculo, foram determinados, em função da contaminação nos sedimentos. A relação da bioacumulação dos metais nos tecidos com o tempo de exposição foi ainda estudada.

Os efeitos potencialmente tóxicos da exposição aos metais foram investigados, recorrendo a vários biomarcadores histopatológicos e bioquímicos, nos órgãos de entrada, como as brânquias e nos órgãos metabólicos, como o fígado, em *L. saliens*.

Assim, no capítulo 4 foram pesquisadas as alterações na histologia das células branquiais de *L. saliens* e avaliadas as suas prevalências. As alterações histopatológicas da brânquia, caracterizadas por grau de extensão e grau de severidade, foram relacionadas com a concentração de metais na brânquia. Foi ainda calculado um factor de impacto de cada lesão que permitiu avaliar o estado geral da população de peixes da laguna.

As alterações nas funções de regulação desempenhadas pela brânquia foram investigadas no capítulo 5. Os níveis de iões plasmáticos que interferem no metabolismo de regulação osmótica e iónica foram estudados e relacionados com as lesões e os metais encontrados na brânquia.

No capítulo 6 fez-se o estudo da actividade das enzimas envolvidas no *stress* oxidativo. A alteração da actividade destas enzimas foi pesquisada na brânquia e no fígado e os danos oxidativos foram avaliados no fígado.

As alterações na histologia do tecido hepático e a sua relação com os metais encontrados neste órgão, bem como a actividade das aminotransferases plasmáticas foram investigadas no capítulo 7.

Finalmente no capítulo 8 é apresentada uma síntese das principais conclusões dos efeitos da contaminação crónica de metais em *Liza saliens*, residente na Barrinha de Esmoriz/Lagoa de Paramos.

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CAPÍTULO 2

HEAVY METALS IN WATER, SEDIMENT AND TISSUES OF *LIZA SALIENS* FROM ESMORIZ-PARAMOS LAGOON, PORTUGAL

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2. HEAVY METALS IN WATER, SEDIMENT AND TISSUES OF LIZA SALIENS FROM ESMORIZ-PARAMOS LAGOON, PORTUGAL

2.1. Abstract

Esmoriz-Paramos lagoon is an ecosystem of great ecological importance that is located on the northwest coast of Portugal and has been degraded as a result of industrial and anthropogenic activities. To assess metal contamination in the lagoon its distribution among the different components of this habitat was evaluated. Concentrations of heavy metals (Cr, Cu, Pb and Zn) were measured in water, sediment and in tissues, liver and muscle of the dominant fish in the lagoon, the mullet, *Liza saliens*.

Metals in water were predominantly bound to particulate matter and their concentrations occasionally exceeded the limits of chronic reference values. Metal concentrations in sediments varied among sampled sites and their relative order of concentrations was $Zn > Cu \sim Pb > Cr$, the same pattern observed for metals in water. Metals in fish tissues showed higher concentrations in liver ($262 \text{ mg Cu}\cdot\text{Kg}^{-1}$ and $89 \text{ mg Zn}\cdot\text{Kg}^{-1}$) than in muscle ($< 3 \text{ mg Cu}\cdot\text{Kg}^{-1}$ and $26 \text{ mg Zn}\cdot\text{Kg}^{-1}$), while Pb and Cr were not detected. These results suggest that Cu and Zn are the metals of major concern in this lagoon. Mullet detritivorous feeding habits, metal bioaccumulation patterns and the high metal concentrations in the sediments relative to the water suggest that sediments can be the most important source of contamination in this ecosystem.

The positive relationship between Cu in liver and fish length found demonstrates that time of exposure is a crucial factor in bioaccumulation. Higher condition indices (*K* and HSI) in mullets from the lagoon compared to mullets from the sea, may suggest alterations in metabolism of the lagoon population.

In this lagoon, metals chronic exposure can contribute to considerable fish stress.

Keywords: Esmoriz-Paramos - heavy metals - sediment - water - fish

2.2. Introduction

The pollution of the aquatic environment with heavy metals has become a worldwide problem during recent years, because they are indestructible and most of them have toxic effects on organisms (MacFarlane & Burchet, 2000). Among environmental pollutants, metals are of particular concern, due to their potential toxic effect and ability to bioaccumulate in aquatic ecosystems (Miller, Sweet, Adams, Omann, Passino-Reader, & Meter, 2002; Censi, Spoto, Saiano, Sprovieri, Mazzola, Nardone, Geronimo, Punturo, & Ottonello, 2006). The final fate of metals is typically the sediments via sedimentation, adsorption to particulate matter, precipitation and/or co-precipitation (Chapman, Wang, Janssen, & Kamunde, 2003).

In aquatic ecosystems heavy metals are accumulated in sediments, where they may reach concentrations several orders of magnitude greater than in the overlying water (Bryan & Langston, 1992). Sediment-associated metals pose a direct risk to detritus and deposit-feeding organisms, and may also represent long-term sources of contamination to higher trophic levels (Eimers, Evans, & Welbourn, 2001; Marín-Guirao, Cesar, Marín, Lloret & Vita, 2005). Due to the ecological importance and the persistence of pollutants in the aquatic ecosystems, sediments are more appropriate to be monitored in environmental evaluations and understand their potential toxic impacts (Von Gunten, Sturm, & Moser; Kwon & Lee, 1997; Kwon & Lee, 2001; Ghrefat, & Yusuf, 2006; Cesar, Choueri, Riba, Morales-Caselles, Pereira, Santos, Abessa, & DelValls, 2007).

Trace metals can be accumulated by fish, both through the food chain and water. The potential metal effects on fish can be assessed examining their accumulation in target tissues (Rajotte, Pyle, & Couture, 2003; Mendil & Uluözölü, 2007). Heavy metals like copper and zinc are essential for fish metabolism, while others such as lead have no function in biological systems. However, all may exert harmful effects on fish, depending, among others, upon concentration.

Several studies have demonstrated that high concentrations of metals can affect wild fish species (Romeo, Siau, Sidoumou, & Gnassia-Barelli, 1999). The use of condition factor (K) and hepato-somatic index (HSI) can provide insight into longer-term responses of fish to stressors (Dethloff and Schmitt, 2000). Condition factor is a commonly measured variable that can be used as a general index of fish health. Although it may not be specific, several studies have related it with metal contamination (Laflamme, Couillard, Campbell, & Hontela, 2000; Eastwood &

Couture, 2002). The hepatic-somatic index reflects the status of liver and its variation may be associated to contaminant exposure (Dethloff & Schmitt, 2000).

Coastal lagoons are considered a priority habitat, according to the European Community Habitats Directive. The Esmoriz-Paramos lagoon is located on the Northwest coast of Portugal, it is included in the National Ecological Reserve and is classified as CORINE biotope. The quality of this ecosystem has been degraded due to agriculture and industrial activities, including cork, leather, wood, textile, paper, painting and metallurgical industries (SIMRIA, 2002). Over the past decades, population in this region has increased and it is estimated to grow from the actual 60 000 to 237 000 inhabitants in 2030 (Almeida, 1998; SIMRIA, 2002). However, despite these many inputs, the amounts and spatial distribution of metals in water, sediments and biota have been poorly documented.

The dominant fish in the lagoon is *Liza saliens*, whose family has been considered sentinel organisms for pollution impact research (Chen & Chen, 1999; Marcovecchio, 2004; Ferreira, Moradas-Ferreira, & Reis-Henriques, 2005). Mullet feeding behaviour, filter and detritus-mud feeder, exposed them to sediments and water contamination (Minos, Katselis, Kaspiris, & Ondrias, 1995).

The aim of this study was to assess metal contamination in this ecosystem, by measuring Cr, Cu, Pb and Zn in water, sediments and in tissues of fish *Liza saliens* from the Esmoriz-Paramos lagoon. Condition indices (*K* and HSI) of mullet from the lagoon were compared with mullet caught at the sea.

2.3. Material and Methods

Study area

The Esmoriz-Paramos lagoon, 1500 m in length (N-S), 700 m wide (W-E), 2.5 m maximum depth and a catchment area of 78 Km², is located on the Northwest coast of Portugal. It receives two main water inflows (Figure 2.1): one from the North (Paramos) and another from the South (Maceda), and occasional effluents from local housing and small industry. The lagoon communication with the sea is established through a non-permanent channel with small dimensions, particularly in the shoreline. Major inputs of contaminants into the lagoon are mostly industrial or municipal, mainly untreated sewage from its tributaries and runoff from contaminated soils and surfaces (Almeida, 1998; SIMRIA, 2002).

Sample collection

Surface water samples were collected, in three sites: channel (P), Paramos (Pa) and Maceda (M), between November 2003 and September 2004, using polyethylene bottles (Figure 2.1), according to standard procedures (Forrest, 2000). Heavy metals were analysed in the dissolved and bound to particulate matter fractions.

Sediment cores (30 cm depth) were also collected, between February and March 2003, using polymethyl methacrylate corers (7 cm diameter x 60 cm height), according to general standard procedures. The sampling sites were chosen to cover the lagoon area, including the main tributaries (E1 and E5) and occasional water inflows (E6 and E9). Triplicate cores were taken from each site and vertically fractionated in 2 cm sections, from 0-10 cm, and 4 cm sections, from 10-30 cm. The triplicate fractions of same depth were pooled, homogenized and stored at -20°C until metal analyses. Appropriate decontamination procedures were employed throughout.

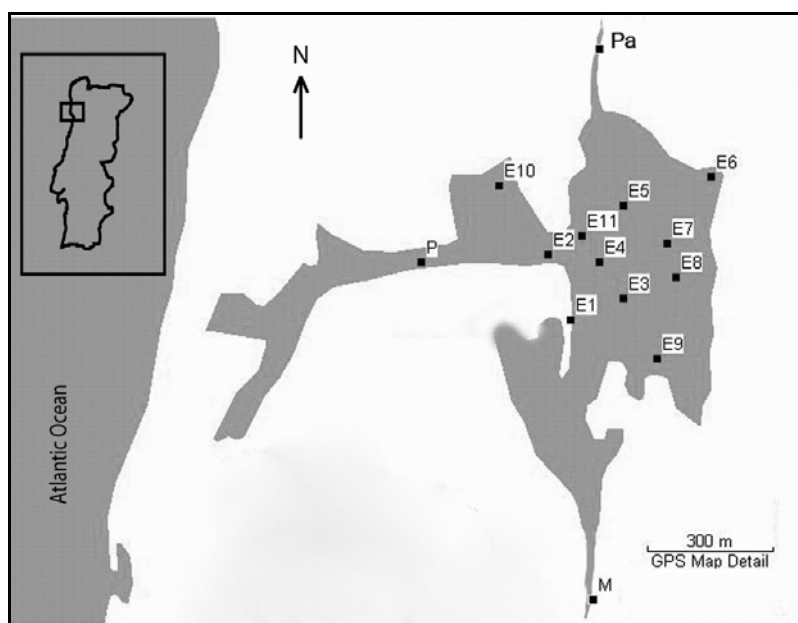


Fig 2.1 - Location of study area and sampling map.

Organic matter and grain size analyses were done for all sites, in 3 core sections, 0-10, 10-20 and 20-30 cm. Organic matter was determined by gravimetric analysis using 5 g of dried sediment incinerated at 375 °C for 16 h. Grain size determination was carried out in a Retsch GmbH, AS 200 sieve, using 100 g of dried sediments and 2, 1, 0.5, 0.25, 0.125, 0.063 and < 0.063 mm sieves at 1.6 mm vibration height for 10 minutes. Results are expressed as percentage.

Fish, from the lagoon and from the sea, 14 Km northwards from the lagoon, were captured by gill net in April 2004. Fish handling was in accordance with institutional guidelines for animal welfare. They were anaesthetised and their total length and weight recorded to calculate condition factor ($K = \text{body weight (g)} / (\text{fish length (cm)})^3 \times 100$). Livers were removed and weighed to obtain hepato-somatic index ($\text{HSI} = \text{liver weight (g)} / \text{body weight (g)} \times 100$).

The liver and dorsal muscle of fish from the lagoon were dissected and stored in plastic bags at $-20\text{ }^{\circ}\text{C}$, until metals analysis.

Analytical methods

Total metal concentrations were measured using a Philips PU9100X flame atomic absorption spectrophotometer (FAAS). Sediment samples were oven-dried, homogenized and digested according to HMSO (1986) and the results expressed in $\text{mg}\cdot\text{Kg}^{-1}$ dry weight.

Water samples were filtered through $0.45\ \mu\text{m}$ membrane filters (Schleicher & Schuell), using a volume of 0.25-1 L. The dissolved metal fraction was quantified by direct analysis of the filtered water, after acidification with HNO_3 (0.2%). Quantification of metals bound to particulate matter was done digesting filters as described for the sediments. The results are expressed in $\text{mg}\cdot\text{L}^{-1}$.

Liver and muscle tissues were freeze-dried and digested according to Ferreira, Cortesão, Castro, & Vale (1990) and metal concentrations are expressed in $\text{mg}\cdot\text{kg}^{-1}$ dry weight. The analyses were done in duplicates and the chemicals used were of analytical reagent grade. Blank assays were done with Milli-Q50 water and blank filters. Certified reference materials (National Research Council of Canada) were as follows: PACS-2 for sediment, DOLT-3 for liver, and DORM-2 for muscle (Table 2.1).

Data analyses

Statistical analyses were carried out using SPSS statistical program. Kolmogorov-Smirnov was used to test normal variable distribution and two-way ANOVA was used to compare differences in sediment metal concentrations along depth and among sites. Multiple comparisons between sites were made using Tamhane test, since homogeneity of variance could not be assumed. Differences in metal concentrations in water were tested with Mann-Whitney. Differences between tissue metal concentrations were compared with t test, as well as differences between condition indices of the two fish populations. Relationships between sediment parameters (metal concentrations, organic matter and grain size) were evaluated with Spearman's correlations, while Pearson's correlations were applied

to the relationships between tissue metals content and fish length and weight. The significance was set at 0.05.

Table 2.1 – Certified metal concentrations (mg.Kg⁻¹ dry weight) in reference material, results of analyses (N=10), and [recoveries obtained].

REFERENCE material		<i>Cr</i> λ (357.9 nm)	<i>Cu</i> λ (324.8 nm)	<i>Pb</i> λ (217nm)	<i>Zn</i> λ (213.9 nm)
PACS-2	CERTIFIED VALUE	90.7 ± 4.6	310 ± 12	183 ± 8	364 ± 23
	ANALYZED VALUES	49.48 ± 4.21 [51-60%]	306 ± 4 [97-101%]	179 ± 10 [93-104%]	362 ± 18 [95-106%]
DOLT-3	CERTIFIED VALUE	-	31.20 ± 1.00	0.319 ± 0.045	86.60 ± 2.40
	ANALYZED VALUES		29.13 ± 0.59 [93-96%]	a	86.41 ± 1.62 [97-101%]
DORM-2	CERTIFIED VALUE	34.7 ± 5.5	2.34 ± 0.16	0.065 ± 0.007	25.60 ± 2.30
	ANALYZED VALUES	⁽¹⁾ 19.9 ± 1.1 [54-61%]	⁽¹⁾ 2.45 ± 0.19 [93-109%]	a	27.34 ± 1.84 [95-112%]

(1) N=6; a: below the quantification limit; Values were rounded of the same decimal than the certified value

2.4. Results

Table 2.2 shows metal concentrations in water, as dissolved and bound to particulate matter. Metals in water were predominantly bound to particulate matter.

Table 2.2 – Mean concentrations of metals in the Esmoriz-Paramos lagoon water: dissolved and bound to particulate matter fractions.

Sampling sites	Months	Metal concentration (mg.L ⁻¹)					
		Zn		Cu		Pb	
		Bound	Dissolved	Bound	Dissolved	Bound	Dissolved
M	Nov	n.d.	0.267	0.006	n.d.	n.d.	n.d.
	Jan	n.d.	n.d.	0.006	n.d.	n.d.	n.d.
	Mar	0.006	n.d.	0.003	n.d.	0.010	n.d.
	Set	< 0.063	< 0.063	< 0.03	< 0.03	< 0.038	< 0.038
P	Nov	0.112	0.699	0.015	n.m.	n.d.	n.d.
	Jan	0.019	n.d.	0.006	n.d.	n.d.	n.d.
	Mar	0.012	n.d.	0.005	n.d.	0.011	n.d.
	Set	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Pa	Nov	0.134	0.519	0.031	n.d.	0.024	n.d.
	Jan	n.d.	n.d.	0.014	n.d.	n.d.	n.d.
	Mar	0.034	n.d.	0.025	n.d.	0.026	n.d.
	Set	< 0.063	< 0.063	< 0.03	< 0.03	< 0.038	< 0.038
Mean ± sd		0.264 ± 0.341		0.012 ± 0.010		0.018 ± 0.008	

n.m.: not measured; n.d.: not detected, detection limits for dissolved fraction: 0.038 mg Zn.L⁻¹, 0.005 mg Cu.L⁻¹, 0.004 mg Pb.L⁻¹ and 0.012 mg Cr.L⁻¹. Detection limits for bound fraction as in sediments.

However, Zn occurred occasionally in both fractions and Cr was not detected. Zn was significantly higher than Cu. No significant differences in water metal concentrations among sites sampled were found.

Metal concentrations in sediments are presented in Table 2.3. The ranges of mean concentrations were as follows: 15-545 mg Zn·kg⁻¹, 6-232 mg Cu·kg⁻¹, 2-255 mg Cr·kg⁻¹, and 7-299 mg Pb·kg⁻¹. The distribution of heavy metals in sediments varied significantly among sampling sites. The lowest mean metals concentrations were measured at E1, except for Cr that was lowest in E2. The highest metal concentrations in sediments were found in E8 and E9, and the highest concentrations of Pb were found in E6.

Results also revealed that organic matter varied significantly among site, showing higher percentages at E3, E6, E7, E8 and E9, but not with depth. A positive correlation between organic matter and metal concentrations in sediments was found for Cu, Pb and Zn within 0-20 cm and for Cr within 20-30 cm depth.

Table 2.3 – Metal concentrations in sediments from the Esmoriz-Paramos lagoon: minimum (min), maximum (max) and mean ± sd (mg.kg⁻¹ d.w.).

sites	Cr		Cu		Pb		Zn	
	min-max	mean	min-max	mean	min-max	mean	min-max	mean
E1	26-57	41 ± 9	14-41	22 ± 8	15-67	32 ± 16	44-134	80 ± 26
E2	2-47	24 ± 16	13-118	74 ± 37	<7-66	40 ± 19	50-427	221 ± 129
E3	25-156	73 ± 38	62-154	107 ± 28	35-102	75 ± 20	163-485	339 ± 104
E4	n.d.-72	37 ± 22	<6-108	48 ± 39	<7-114	59 ± 41	33-240	129 ± 79
E5	25-58	42 ± 9	42-102	65 ± 21	43-99	70 ± 16	141-298	222 ± 51
E6	29-86	48 ± 17	55-163	117 ± 41	95-299	153 ± 70	96-384	265 ± 108
E7	21-52	31 ± 11	77-139	106 ± 22	90-144	115 ± 18	196-486	337 ± 92
E8	21-63	32 ± 14	85-184	119 ± 34	75-184	130 ± 38	247-544	374 ± 100
E9	37-255	103 ± 75	125-232	170 ± 37	71-150	117 ± 24	331-545	457 ± 73
E10	n.d.-59	41 ± 19	<6-126	47 ± 48	18-278	126 ± 105	15-216	82 ± 75
E11	19-56	40 ± 11	15-64	40 ± 15	17-54	40 ± 12	73-188	148 ± 35

n.d. not detected detection limits: 0.048 mg Zn.Kg⁻¹, 0.013 mg Cu.Kg⁻¹, 0.019 mg Pb.Kg⁻¹ and 0.037 mg Cr.Kg⁻¹.

Sediment grain size analysis was characterised mostly by coarse sand (>0.25 mm; 63%), followed by fine sand (0.25-0.063 mm; 19%) and fine particles including clay plus silt (<0.063 mm; 18%). No significant relationship between metals and grain size was found, although a large percentage of fine particles in E8, E3 and E7 sites (30%, 32% and 34%, respectively) were observed.

Averaging metal concentrations of all sites, the depth profile did not show any significant differences. The calculation of mean metal concentrations was then 241 mg Zn·Kg⁻¹, 83 mg Cu·Kg⁻¹ (Fernandes, Fontainhas-Fernandes, Peixoto, & Salgado, 2006), 87 mg Pb ·Kg⁻¹ and 47 mg Cr·Kg⁻¹. The resultant order of metal concentrations in sediments was Zn > Cu ~ Pb > Cr.

Metal content in fish tissues are shown in table 2.4. Cu and Zn were significantly higher in liver, ranging 51-547 mg Cu·kg⁻¹ and 26-190 mg Zn ·kg⁻¹, than in muscle, where Zn ranged 13-64 mg·kg⁻¹ and Cu was always below 2.64 mg·Kg⁻¹ (Fernandes, Fontainhas-Fernandes, Peixoto, & Salgado, 2007) and Pb and Cr, were not detected in none of the tissues.

Table 2.4 - Metals distribution in muscle and liver of the *Liza saliens* from lagoon (mg.Kg⁻¹ d.w. ± standard deviation). N=35

Tissues	Cr	Cu	Pb	Zn
Liver	« a	262.08 ± 140.71	« a	88.64 ± 32.00
Muscle	« a	< 2.64	«a	25.71 ± 10.08

a: below the analytical detection limits (the same as in table 2.3).

Table 2.5 compares condition indices between mullets caught at Esmoriz-Paramos lagoon and at sea, within the same size class. Condition factor (*K*) was significantly higher in fish from the lagoon than in fish from the sea (range 0.72-1.10 g.cm⁻³ and 0.31-0.99 g.cm⁻³, respectively). Fish from the lagoon also showed higher HSI than fish from the sea (range 1.76-4.38 % and 0.36-2.21 %, respectively).

Table 2.5 – Mean values and standard deviations of length (L), weight (W), condition factor (*K*) and hepatosomatic index (HSI) of mullet from the lagoon and sea.

	Lagoon N = 30	Sea N = 32
L (cm)	28.63 ± 2.21	28.05 ± 3.20
W (g)	230.08 ± 62.05	173.43 ± 81.79
K (g.cm⁻³)	0.95 ± 0.08 **	0.75 ± 0.14
HSI (%)	2.56 ± 0.65 **	1.26 ± 0.41

**p<0.01

A positive relationship between Cu-liver content and lagoon fish length was also found (Figure 2.2).

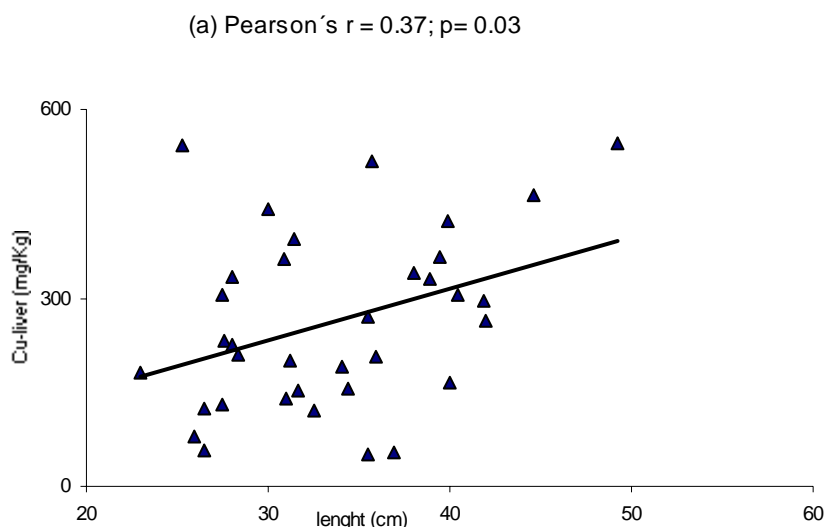


Fig 2.2 - Relationships between Cu-liver and length in fish from the Esmoriz-Paramos lagoon.

2.5. Discussion

The present study showed that metal concentrations in the water were predominantly bound to particulate matter. However, an exception was registered for Zn in November, when the dissolved fraction reached $0.7 \text{ mg}\cdot\text{L}^{-1}$. Zn was the metal showing the highest total concentration in water, while Cr was not detected at all. Generally, mean metals concentrations in the water were within or occasionally exceeded the limit of chronic reference values, suggested by USEPA (1999): $0.11 \text{ mg Zn}\cdot\text{L}^{-1}$, $0.012 \text{ mg Cu}\cdot\text{L}^{-1}$ and $0.0032 \text{ mg Pb}\cdot\text{L}^{-1}$.

Metal concentrations in sediments varied among sites. The highest metal concentrations were found in sites with reduced water inflow, near occasional effluents of domestic and small industry origin. The low water exchange associated to high organic matter composition in these areas, probably favours metal deposition. It is well known that metals (Cu, Zn and Pb) have high affinity to humic substances present in organic matter (Sekhar, Chary, Kamala, Raj, & Rao, 2003). The present study shows a positive relationship between organic matter and the metal content in sediments.

On contrary, the mean metal concentrations of all sites did not vary along depth, which reflects a similar pattern of deposition over time. Adsorption of heavy

metal ions onto organic particulate debris has been demonstrated (Chattopadhyay, Chatterjee, & Mukhopadhyay, 2002). Metal accumulation in sediments results mainly from this deposition and, to a lesser extent, from diffusion of dissolved metals in the water (Chapman, Wang, Janssen, & Kamunde, 2003). Total suspended solids (TSS) load varied with particles input, water chemistry and flow regime. The high TSS measured, 76 mg.L⁻¹; n= 3x12 (Fernandes; personal communication) and the slow water flow into the lagoon, enhances particle settling and metal accumulation in sediments. The relative order of concentrations in the sediments was Zn > Cu ~ Pb > Cr, similar to that in water.

Trace metal contamination in sediment can affect the water quality and the bioaccumulation of metals in aquatic organisms, resulting in potential long-term implications on human health and ecosystem. This study revealed that that *L. saliens* displayed high concentrations of Cu and Zn in liver, and Zn in muscle than related species from other polluted ecosystems (Blasco, Rubio, Forja, Gómez-Parra, & Establier, 1998; Yilmaz, 2003; Joyeux, Filho, & Jesus, 2004; Karadede, Oymak, & Ünlü, 2004). As reported by Mansour & Sidky (2002, 2003), feeding behaviour make *Mugil* sp. more vulnerable to sediment-pollution, than other fish species, such as *Solea* sp. and *Tilapia* sp. Mullet detritivorous feeding habits and the high concentrations of metals in lagoon sediments, relatively to the water, suggest that sediments can be the most important source of contamination in this ecosystem.

The relationship between metals concentrations in tissues and sediments found in the present study reveals that direct up-take was not the unique process involved in bioaccumulation. This was particularly evidenced for Pb and Cr since they were not detected in tissues, regardless sediment high concentrations. The bioaccumulation of metals depends on the total metal content of exposure, chemical composition and several environmental and biological conditions. Speciation of metals is expected to influence metal bioavailability and thereby metal content in biota (Plette, Nederlof, Temminghoff, & Van Riemsdijk, 1999; Sekhar, Chary, Kamala, Raj, & Rao, 2003). On the other hand, metal biological characteristics, such as bioactivity, play an important role in the homeostatic regulation.

Environmental concentrations of non-essential toxic metals, such as Pb and Cr, can determine their accumulation (Chattopadhyay, Chatterjee, & Mukhopadhyay, 2002). However, the physico-chemical conditions of the lagoon must have regulated Cr and Pb low bioavailability. On the contrary the accumulation of bioactive metals, such as Cu and Zn, are under homeostatic control and increased metal content in fish tissues may be an indicator of increased metal availability,

overload of homeostatic control processes and therefore potential metal stress (Eastwood & Couture, 2002). The high content of Cu-liver in *L. saliens* may be attributed to their bioavailability and loss of liver homeostatic capacity. The hepatic Zn reflects its bioavailability, and it is apparently under regulation, as previously demonstrated (Fernandes, Fontáinhas-Fernandes, Peixoto, & Salgado, 2007). The referred previous study also revealed that time of Cu-exposure is other crucial factor involved in liver bioaccumulation in this ecosystem (Fernandes, Fontáinhas-Fernandes, Peixoto, & Salgado, 2007). We found a similar relationship between Cu-liver content and fish length.

Alterations in fish condition indices (*K* and HSI) are sometimes indicative of toxicant effects (Mayer, Versteeg, McKee, Folmar, Graney, McCume, & Rattner, 1992). Condition indices were higher in fish collected in the lagoon than in fish caught in the sea. Several studies have shown lower *K* in the presence of metal contamination (Laflamme, Couillard, Campbell, & Hontela, 2000; Eastwood & Couture, 2002; Rajotte, Pyle, & Couture, 2003). Previous studies suggested that *K* was not significantly different in fish exposed to pollutants compared to the controls (Van der Oost, Lopes, Komen, Satumalay, van den Bos, Heida, & Vermeulen, 1998; Gadagbui & Goksoyr, 1996), while others, reported that an increase of *K* is sometimes indicative of toxicant effects (Figueiredo-Fernandes, Fontáinhas-Fernandes, Peixoto, Rocha, & Reis-Henriques, 2006).

In the present study we found an increase of HSI that can be due to metabolic modifications induced by chronic exposure to heavy metals. Stephensen, Svavarsson, Sturve, Ericson, Adolfson-Erici, & Förlin (2000) consider that the higher HSI found in the sculpin (*Myoxocephalus scorpius*) caught in polluted areas can be indicative of increased activity of xenobiotic biotransformation enzymes. Results from different studies with fish exposed to different pollutants, like PCB, OCP and PCDD also showed a significant increase of HSI (Newsted & Giesy, 1993; Arnold, Pluta, & Braunbeck, 1995; Gadagbui & Goksoyr, 1996). In contrast, some studies revealed that HSI of fish exposed to metals are not significantly different from the controls (Martin & Black, 1996; Eastwood & Couture, 2002). Norris, Camp, Maldonado, & Woodling (2000) found significant sex and site differences in HSI, with large livers observed in fish living at the uncontaminated site.

In conclusion, metal contamination in water and sediments from Esmoriz-Paramos lagoon followed the order Zn > Cu ~ Pb > Cr. Metals in the water were within, or occasionally exceeded, reference values, whereas in the sediments they were high and probably constituted the main source of bioaccumulation in fish tissues. Concentrations of metals found in fish liver were high and followed an

inverse order relative to water and sediments, Cu > Zn, showing that bioaccumulation of metals in mullets was driven by biotic as well as abiotic factors.

The results of this study supplied valuable information on the metals levels in water, sediment, and *Liza saliens* from the Esmoriz-Paramos lagoon, and also show that this lagoon is an area of environmental concern.

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CAPÍTULO 3

BIOACCUMULATION OF HEAVY METALS IN *LIZA SALIENS* FROM THE ESMORIZ- PARAMOS COASTAL LAGOON, PORTUGAL

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3. BIOACCUMULATION OF HEAVY METALS IN *LIZA SALIENS* FROM THE ESMORIZ-PARAMOS COSTAL LAGOON, PORTUGAL

3.1. Abstract

Heavy metal (Cu and Zn) concentrations in liver, gill and muscle of leaping grey mullet *Liza saliens*, from the Portuguese Esmoriz-Paramos coastal lagoon were measured to evaluate their bioaccumulation as a function of sediment contamination. The highest metal concentrations were observed in the liver (254 mg Cu.kg⁻¹) and gill (114 mg Zn.kg⁻¹). Bioaccumulation factors (BAFs) were found to follow the order: Cu-liver > Cu-gill > Cu-muscle and Zn- gill > Zn-liver > Zn-muscle. The highest BAFs were observed in the organs mainly implicated in metal metabolism and a significant positive relationship was found between BAFs and fish age. These results suggest the loss of homeostatic capacity of *L. saliens* under chronic metal exposure leading to bioaccumulation. Furthermore, Cu-liver and Zn-gill accumulation can be good environmental indicators of metal stress in *L. saliens*.

Keywords: Bioaccumulation - chronic exposure - heavy metals - *Liza saliens*

3.2. Introduction

Metals can be taken up by fish from water, food, sediments and suspended particulate material (Hardersen and Wratten, 1998). However, the presence of a given metal at high concentrations in water or sediments does not involve direct toxicological risk to fish, especially in the absence of significant bioaccumulation. It is known that bioaccumulation is to a large extent mediated by abiotic and biotic factors that influence metal uptake (Rajotte et al., 2003).

Due to the deleterious effects of metals on aquatic ecosystems, it is necessary to monitor their bioaccumulation in key species, because this will give an indication of the temporal and spatial extent of the process, as well as an assessment of the potential impact on organism health (Kotze et al., 1999).

The Esmoriz-Paramos coastal lagoon represents an ecosystem of great physical as well as ecological significance. However, as a result of industrial, agricultural and anthropogenic activities, this aquatic ecosystem has been progressively degrading over the past decades (Almeida, 1998; SIMRIA (Saneamento Integrado dos Municípios da Ria), 2002). The heavy metal

contamination has been an important factor in the decline of water and sediments quality and may adversely affect fish health.

In the present work the leaping grey mullet, *Liza saliens*, was studied because it is the most dominant species in the lagoon. It is a filter feeder and also feeds on detritus mud, and is therefore exposed to contaminated sediment. In turn, metals bound to the sediments might pose a threat to aquatic biota either through leaching into the aqueous phase or by direct contact with the organisms (D'Itri, 1990).

The aim of the present study was to assess metal concentrations of Cu and Zn in sediments and their bioaccumulation in various fish tissues, such as muscle, liver and gill. In order to evaluate the effect of chronic fish exposure to metals, the relationships between bioaccumulation factors (BAFs) and fish age were determined.

3.3. Material and Methods

The Esmoriz-Paramos Lagoon is located on the Northwest coast of Portugal and receives two main water inflows (Fig. 3.1). The communication of the lagoon with the sea is established through a non-permanent channel, and the system receives mostly untreated effluents via its tributaries. The particle deposition rate has been relatively high during the last 25 years and the wet area of the lagoon has decreased (Almeida, 1998; SIMRIA, 2002): the annual solid transport from land, estimated to be as much as 14,000 m³ (Almeida, 1998), caused a ca. 20% reduction of the immersed area between 1970 and 1995 (SIMRIA, 2002).

Sampling stations were set within the wet area of the lagoon (Fig. 3.1). Eleven sediment cores (30 cm long) were collected in February-March 2003, whereas 34 *L. saliens* specimens were captured during April 2004, in the post-spawning period in accordance with institutional guidelines for animal welfare. Fishes were anesthetized and liver, gill and dorsal muscle samples were taken and stored at -20 °C until analyses. The age was determined by reading the annual ring structure of scales.

Sediments were extruded and sectioned in 2-4 cm thick slices, which were dried for 24 h at 105 °C and digested with hydrochloric-nitric acid mixture according to HMSO (1986). Analysis of sediments included organic matter determination and granulometry for each station and at two depths (0-10 cm and 10-20 cm). Organic matter determination was done by gravimetric analysis after incineration of 5 g of dried sediment at 375°C for 16 h. Grain size determination was done using 100 g of sieved dried sediments (2, 1, 0.5, 0.25, 0.125, 0.063 and

< 0.063 mm) at 1.6 mm vibration height for 10 minutes (Retsch GmbH, AS 200). Results from duplicate samples are expressed in percentages.

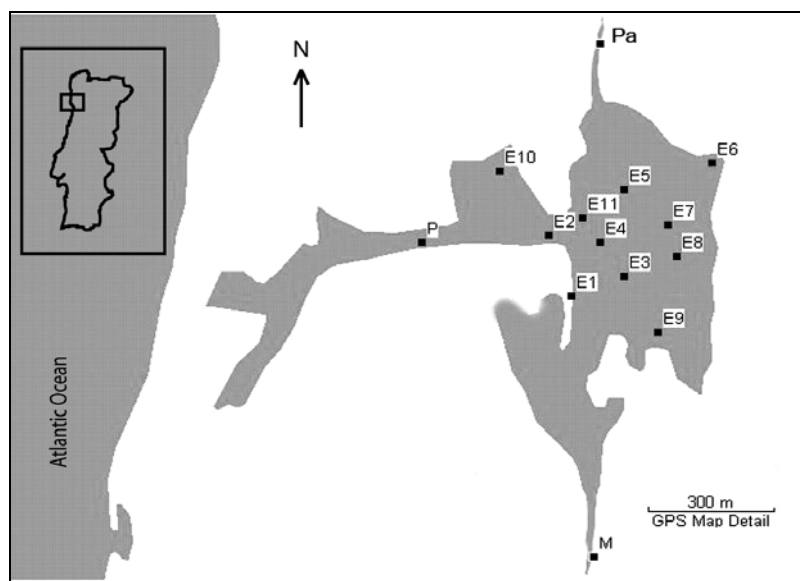


Fig. 3.1 – Map showing the Esmoriz/Paramos lagoon sampling locations. Water inflows: north, Ribeira de Paramos (Pa); south, Vala de Maceda (M). Channel (P).

Fish tissues were digested based on the method of Ferreira et al. (1990). Metal concentrations in sediments, liver and muscle were measured by flame atomic absorption spectrometry (Philips PU9100X). The gill soft tissue was also acid-digested by nitric acid in a ratio of W (g): V (ml) = 1:20 and analysed in a graphite furnace atomic absorption spectrometer (UNICAMP 939 AA - GF90).

Certified reference materials, i.e PACS-2 (sediment from National Research Council of Canada-NRC), DOLT-3 (dogfish liver from NRC) and DORM-2 (dogfish muscle from NRC) were analysed to check analytical accuracy and precision. Few discrepancies were observed between the metal concentrations of analysed and certified reference materials (Table 3.1). The standard recoveries obtained were: 97-101% and 95-106%, respectively, for Cu and Zn in PACS-2; 93-96% and 97-101%, respectively for Cu and Zn in DOLT-3; and 93-109%, and 95-112%, respectively for Cu and Zn in DORM-2. The precision of the methods for the sediment and tissue analysis, for both metals, varied between 1-5% and 2-8%, respectively.

All reagents were analytical grade and blank determinations were done using the same procedure with Milli-Q50 water. Results were expressed in $\text{mg}\cdot\text{Kg}^{-1}$ dry weight.

Table 3.1 - Certified metal concentrations in reference material and results of analyses (N=10), in mg.Kg⁻¹ dry weight (mean ± sd).

REFERENCE		<i>Cu</i>	<i>Zn</i>
PACS-2	CERTIFIED VALUE	310 ± 12	364 ± 23
	ANALYZED VALUE	306 ± 4	362 ± 18
DOLT-3	CERTIFIED VALUE	31.20 ± 1.00	86.60 ± 2.40
	ANALYZED VALUE	29.13 ± 0.59	86.41 ± 1.62
DORM-2	CERTIFIED VALUE	2.34 ± 0.16	25.60 ± 2.30
	ANALYZED VALUE	2.45 ± 0.19 ^a	27.34 ± 1.84

Note: Values were rounded to the same decimal place as certified value.

^a N=6

Bioaccumulation factors (BAFs) were calculated according to the formula $BAF = \frac{\text{metal concentration (mg}\cdot\text{Kg}^{-1} \text{ dry wt.) in tissue}}{\text{metal concentration (mg}\cdot\text{Kg}^{-1} \text{ dry wt.) in sediment}} \times 100\%$, according to Barron (1995).

Statistical analyses were carried out using the SPSS statistical package program. The Kolmogorov-Smirnov method was used to test normal variable distribution and two-way ANOVA was used to compare differences in metal concentrations in the sediments along depth and stations. Multiple comparisons between stations were made using the Tamhane test, since homogeneity of variance could not be assumed. Metal tissues concentrations were also compared by one-way ANOVA. BAFs in the different tissues were tested by Pearson rank correlation and linear regression analysis was applied to BAFs and fish age. Partial correlations analyses were used to determine associations between Zn-BAFs. Nonparametric correlations were applied for organic matter and grain size. The significance level (α) was set at 0.05.

3.4. Results

Total metal concentrations in the sediments along depth are shown in Table 3.2. No significant differences were found along depth, although they varied among stations, ranging from 4-232 mg Cu·Kg⁻¹ and 15-545 mg Zn·Kg⁻¹. Stations E6, E8 and E9 showed significantly higher levels of copper whereas E3, E7, E8, and E9 were contaminated by zinc.

Table 3.2 - Sediments metal concentrations (mg metal·Kg⁻¹ d. w, mean ± (sd), N=2) from 11 stations along depth.

Depth (cm)	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	Mean
Cu												
0-2	41 (0.9)	33 (0.9)	80 (1.6)	83 (2.1)	86 (2.1)	130 (2.1)	77 (1.5)	93 (0.0)	125 (9.3)	126 (2.8)	31 (0.5)	82
2-4	30 (0.4)	13 (1.8)	62 (1.6)	108 (3.1)	45 (2.1)	127 (2.1)	87 (1.5)	85 (1.5)	137 (3.7)	98 (2.8)	35 (6.4)	75
4-6	24 (0.4)	28 (3.3)	86 (1.6)	91 (4.1)	50 (3.7)	148 (10.6)	113 (1.5)	105 (0.0)	161 (0.0)	102 (8.3)	51 (0.0)	87
6-8	25 (0.4)	104 (9.8)	112 (3.1)	81 (5.2)	77 (3.7)	152 (4.2)	79 (1.5)	135 (0.0)	154 (1.9)	68 (0.9)	64 (1.4)	96
8-10	20 (1.3)	69 (4.9)	93 (1.6)	41 (4.1)	82 (0.5)	157 (2.1)	93 (0.0)	86 (3.0)	157 (1.9)	40 (2.8)	29 (2.3)	79
10-14	18 (0.4)	118 (0.0)	104 (1.6)	37 (4.1)	102 (0.5)	104 (4.2)	105 (6.0)	101 (0.0)	230 (1.9)	21 (0.0)	40 (0.0)	89
14-18	18 (0.0)	104 (6.5)	136 (0.0)	16 (0.0)	55 (3.7)	67 (2.1)	124 (0.0)	112 (6.0)	232 (7.4)	< 4	60 (0.9)	84
18-22	16 (0.4)	79 (9.8)	154 (0.0)	9 (0.9)	70 (3.7)	68 (0.0)	139 (3.0)	184 (0.0)	182 (3.7)	< 4	44 (0.5)	86
22-26	14 (0.9)	81 (9.8)	125 (3.1)	< 6	45 (2.1)	55 (6.4)	132 (1.5)	166 (4.5)	182 (14.9)	< 4	29 (1.4)	76
26-30	15 (1.3)	107 (6.5)	121 (3.1)	< 6	42 (1.1)	163 (2.1)	115 (1.5)	123 (10.5)	139 (3.7)	< 4	15 (0.9)	77
Mean	22	74	107	48	65	117	106	119	170	47	40	
Zn												
0-2	134 (2.5)	91 (0.2)	282 (19.6)	200 (0.7)	283 (8.2)	330 (11.2)	196 (14.0)	280 (3.3)	404 (13.6)	216 (0.0)	126 (4.3)	231
2-4	97 3.0)	50 (4.0)	163 (8.7)	187 (2.0)	141 (6.8)	330 (4.3)	232 (5.8)	246 (13.2)	384 (6.1)	100 (3.9)	122 (13.5)	187
4-6	84 (1.6)	61 (1.5)	264 (5.1)	240 (11.5)	191 (4.8)	361 (24.2)	356 (13.2)	302 (0.8)	501 (7.6)	174 (10.7)	179 (12.2)	247
6-8	95 (3.2)	427 (1.0)	279 (0.0)	208 (4.7)	250 (9.5)	364 (5.2)	255 (9.9)	381 (5.8)	485 (1.5)	151 (9.0)	183 (7.9)	280
8-10	74 (1.6)	328 (18.9)	264 (9.4)	116 (3.4)	227 (0.7)	384 (9.5)	298 (25.5)	320 (17.3)	473 (11.3)	67 (4.3)	145 (4.6)	245
10-14	84 (5.3)	196 (10.9)	423 (12.3)	157 (1.4)	298 (0.7)	164 (7.8)	359 (0.8)	318 (7.4)	545 (1.5)	32 (0.3)	161 (9.5)	249
14-18	72 (0.0)	323 (19.9)	485 (5.8)	64 (7.4)	213 (2.0)	96 (0.9)	412 (5.8)	391 (14.0)	331 (8.3)	21 (2.6)	188 (4.0)	236
18-22	60 (3.9)	175 (5.0)	387 (4.4)	51 (3.0)	259 (7.5)	109 (1.7)	486 (1.6)	516 (14.0)	535 (12.9)	18 (1.6)	153 (0.7)	250
22-26	53 (6.4)	237 (24.9)	379 (2.2)	33 (3.7)	176 (18.3)	241 (20.0)	426 (25.5)	544 (9.9)	515 (17.4)	15 (1.3)	146 (25.3)	251
26-30	44 (6.6)	318 (9.9)	467 (5.1)	36 (3.8)	177 (9.5)	270 (13.8)	349 (24.7)	439 (26.3)	393 (6.1)	22 (0.3)	73 (0.6)	235
Mean	80	221	339	129	222	265	337	374	457	82	148	

Based on both lagoon area and solid transport rate, the sediment deposition was estimated to be at most 2 cm per year. Since fish mobility is random around all the stations and no significant differences were found between mean concentrations of each metal along depth (Table 3.2), the average sediment concentration was taken into account for metal exposure: 241 mg Zn·Kg⁻¹ and 83 mg Cu·Kg⁻¹.

Concentrations of Cu and Zn in the liver, gill and muscle were significantly different ($P < 0.05$) from each other (Fig. 3.2). Liver contained Cu levels significantly higher ($P < 0.001$) than the gill and muscle, 30- and 96-fold, respectively.

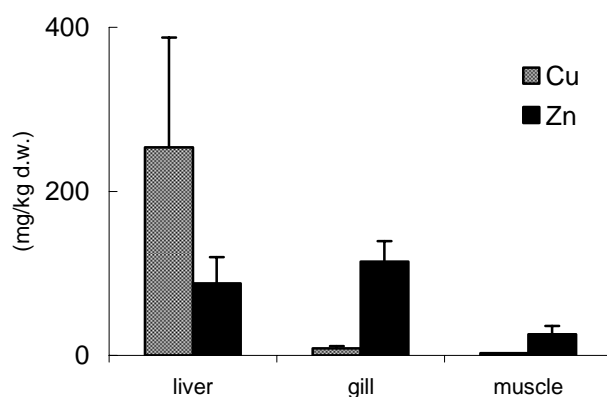


Fig. 3.2 – Mean tissue concentrations of Cu and Zn in *Liza saliens* from Esmoriz/Paramos lagoon.

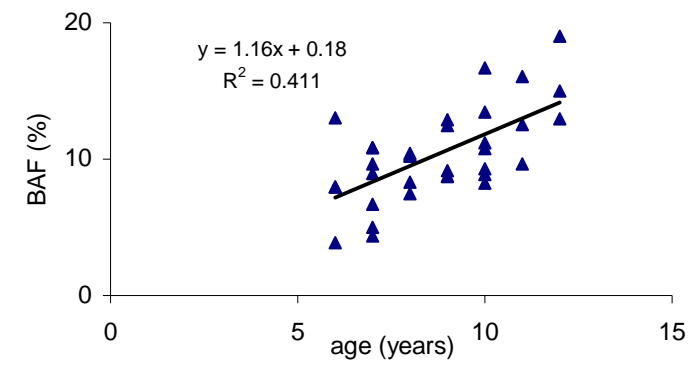
The mean concentration of Cu in the liver was $253.68 \text{ mg}\cdot\text{Kg}^{-1}$ with a range of $51\text{-}544 \text{ mg}\cdot\text{Kg}^{-1}$, while in the muscle it was always below $2.64 \text{ mg}\cdot\text{Kg}^{-1}$. The level of Zn was significantly higher in the gill than in liver and muscle tissues ($P < 0.001$). The mean concentration of Zn in the gill was $114.41 \text{ mg}\cdot\text{Kg}^{-1}$, 4.4- and 1.3-fold the content in the muscle and liver, respectively.

Table 3.3 shows the comparison of BAFs. Similarly to the concentration values of Fig. 3.2, the variability is high, and much more accentuated for Cu. Actually, the highest and lowest BAFs are a characteristic of copper: 305% and $< 3\%$ in the liver and muscle, respectively. Zinc displayed the highest BAF (47%) in the gill, whereas the lowest value (11%) was found in the muscle. Thus, the BAFs for these metals were found to follow the order: Cu-liver $>$ Cu-gill $>$ Cu-muscle and Zn-gill $>$ Zn-liver $>$ Zn-muscle.

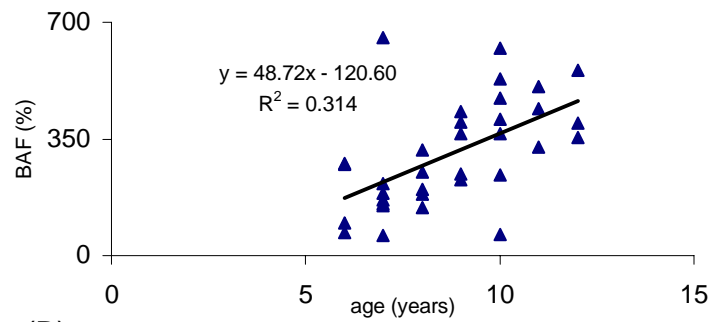
Table 3.3 - Metal concentration of sediment exposure and the bioaccumulation factors in fish tissues (mean \pm sd).

	Zn	Cu
Sediment exposure concentration (mg.Kg-1 d.w.)	241 \pm 145	83 \pm 52
BAF (%)		
Pooled livers	36.37 \pm 13.26	304.98 \pm 160.66
Pooled muscles	10.72 \pm 4.23	$<$ 3.17
Pooled gills	47.45 \pm 10.27	10.34 \pm 3.36

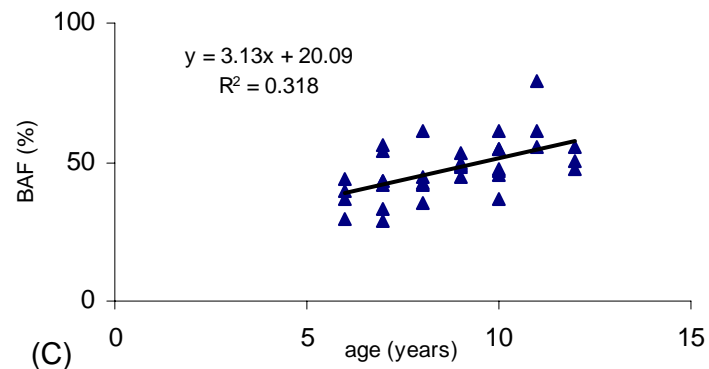
Fish age varied between 6 and 12 years. A clear dependence upon age was found for Cu-BAF in liver and gill, as well as for Zn-BAF in the gill (Fig 3.3).



(A)



(B)



(C)

Fig. 3.3 – Relationships between fish age and BAFs: (A) Cu-BAFs in gill, (B) Cu-BAFs in liver, and (C) Zn-BAFs in gill.

3.5. Discussion

The present study shows that metals concentrations in the sediments tend to vary among stations, and some stations (E3, E6, E7, E8, and E9) displayed particularly high levels. This variation may be due to the differences in the sources of metal pollution and physical-chemical conditions favouring sediment contamination. The correlations between the percentage of organic matter and Cu and Zn in the sediments showed that they are directly related. Spearman's test gave particularly good correlations for Cu, at depths 0-10 and 10-22 cm ($r = 0.873$, $p < 0.01$; $r = 0.933$, $p < 0.01$) and for Zn at the same depths ($r = 0.673$, $p = 0.023$; $r = 0.767$; $p = 0.016$). Grain size analyses, in the 0-10 and 10-20 cm of depth, showed a wide range of grain sizes, from sand to clay, yet no evidence of significant relationships with metal content was found.

The 30 cm of sediment sampled include the entire range of sediments that the oldest fish found in the lagoon (12 years) has been exposed to. Rather surprisingly, no significant differences were found between mean concentrations of metals along depth, despite the variations mentioned above. Therefore, average concentrations of $241 \text{ mg Zn}\cdot\text{Kg}^{-1}$ and $83 \text{ mg Cu}\cdot\text{Kg}^{-1}$, assuming random fish mobility around stations, can be used to assess the fish-sediment accumulation factors.

Bioaccumulation is species-dependent and therefore feeding habits and life style can be strongly related to the sediment exposure (Chen and Chen, 1999). On the other hand, bioavailability of metals can be influenced by inorganic and organic factors that control metal speciation and thereby bioaccumulation (Sekhar et al., 2003). The uptake of sediment-associated contaminants by fish may occur by respiratory and dietary routes, whereas the dermal route is usually a minimal contributor of exposure, due to the often effective barrier provided by the external epithelium. Our data show that mean concentrations of metals in the gill, liver and muscle are very variable. Previous studies also indicated that different fish species from the same area contained different metal levels in their tissues (Kalay et al., 1999; Canli and Atli, 2003; Marcovecchio, 2004). The metal accumulation in different fish organs depends on their physiological role, behaviour and feeding habits, as well as regulatory ability, as reported by Chattopadhyay et al. (2002) and Clearwater (2002). Other factors, such as sex and size, may also influence metal bioaccumulation (Al-Yousuf et al., 2000; Canli and Atli, 2003). The concentrations of essential metals, such as Cu and Zn in organisms, tend to be highly regulated compared to nonessential. Fish can use different strategies of metal homeostasis to achieve a steady-state balance. The mechanisms of reducing metal accumulation and toxicity include uptake inhibition, increased elimination and detoxification, and

storage. This study showed that the highest BAF for Cu was in liver and for Zn was in the gill. The lowest BAFs, for both metals, were found in muscle. Similar findings were obtained in the muscle of *Liza macrolepis* and *Oreochromis mossambicus* (Chen and Chen, 1999; Kotze et al., 1999).

Gill is the first organ to be exposed to resuspended sediment particles, so they can be significant sites of interaction with metal ions. On the other hand, the liver has a key role in basic metabolism (Moon et al., 1985) and is the major site of accumulation, biotransformation and excretion of contaminants in fish (Triebkorn et al., 1994, 1997). It is well known that large amount of metallothionein induction, caused by contamination, occurs in liver tissues of fish (Olsvik et al., 2001). In contrast, the muscle tissues are not considered an active site for metal accumulation (Romeo et al., 1999).

Liver and gill are known to be target organs for Cu in fish (Arellano et al., 1999). Results of the present study showed a positive correlation between the two BAFs (Cu-liver and Cu-gill) and the highest BAF, found in liver, revealed that this organ is involved in the metabolism of copper. On other hand, the Cu-BAF in liver increased with age, showing that time of exposure affects Cu bioaccumulation in *L. saliens*. Prolonged exposure may result in impairment of the normal detoxification response for copper, leading to liver bioaccumulation.

Although an increase in Cu-BAF in gill with age was observed, the ratio between Cu-BAFs in liver and gill (31- fold) shows that gill is not the primary organ involved in copper metabolism. As mentioned, gill can uptake heavy metals at higher levels due to their distinct role in metal exposure. They are involved in Zn regulation, either reducing influx or increasing efflux rates, reaching a steady state (Kraemer et al., 2005). The highest Zn-BAF found in gill, and the metal increase over time can indicate loss of homeostatic capacity by this organ.

The role of liver in zinc metabolism should also be considered, but zinc is apparently well regulated in this organ, since no increase of BAF with age was found. No relationships were found between Zn-BAFs in muscle and age or Zn-BAFs in gill and muscle. However, an increase of Zn-BAF in muscle and gill depending on fish age ($r = 0.365$; $p = 0.036$) was evident. This suggests that the metabolism of zinc is related with muscle tissue. The relationship found between chronic exposure and metal bioaccumulation in aquatic biota, shows that usually Zn content is well regulated (Marcovechio and Moreno, 1993; McGeer et al., 2003). However, the lowest BAF found in muscle does not exclude Zn toxic effects in fish. Marcovechio (2004) recorded mean values of zinc in muscle of $48.8 \text{ mg}\cdot\text{Kg}^{-1}$ (wet weight) in *Mugil liza*, and Yilmaz (2003) found $51.13 \text{ mg}\cdot\text{Kg}^{-1}$ (wet weight) in *Mugil cephalus*,

both from contaminated sites. The present study found values twice as high (99.38 mg Zn·Kg⁻¹) that clearly indicate a metal-contaminated environment, eventually leading to toxic effects.

3.6. Conclusions

The relatively high content of metals found in liver and gill tissues may be due to the metal concentrations in this ecosystem and time of exposure, which is a function of fish age. The significant positive relationship observed between fish age and bioaccumulation of copper in the metabolic organs (liver and gill) suggests a loss of homeostatic ability. Zn metabolism seems to be dependent on the three tissues. Zinc is homeostatically maintained within a range of concentrations in liver, whereas its increase in the gill seems to be partitioned, within a range of concentrations, with the muscle. In summary, the results of this study reveal that Cu-liver and Zn-gill accumulation in this species can be used as environmental indicators of metal stress.

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CAPÍTULO 4

HISTOPATHOLOGICAL GILLS CHANGES IN WILD LEAPING GREY MULLET (*LIZA SALIENS*) FROM THE ESMORIZ-PARAMOS COASTAL LAGOON, PORTUGAL

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4. HISTOPATHOLOGICAL GILLS CHANGES IN WILD LEAPING GREY MULLET (*LIZA SALIENS*) FROM THE ESMORIZ-PARAMOS COASTAL LAGOON, PORTUGAL

4.1 Abstract

The histopathological changes are among the most recognized responses to environmental stressors, namely heavy metals. *Liza saliens* were sampled, in the Portuguese Esmoriz-Paramos coastal lagoon to assess their gill histopathological response to Cu and Zn contaminated sediments. A lesion prevalence index, severity and extension scores of each lesion, as well as an assessment value (severity x extension), were determined to evaluate the effect of environmental heavy metal exposure.

The main histopathological changes observed were aneurisms, hyperplasia, lifting and vasodilation. A high prevalence of lesions (65 to 85%) was found, in addition a high number of simultaneous lesions. Vasodilation and hyperplasia were the lesions that showed higher prevalence indexes. The lifting, followed by hyperplasia, were the lesions with highest assessment value. The prevalence of the number of lesions found in *L. saliens* collected from Esmoriz-Paramos lagoon was high when compared with mullet caught in the sea. A previous work showed that fish collected in the lagoon showed elevated Cu and Zn levels in their gills, in a positive correlation with age. However, no positive correlation was observed between assessment value and gill metal levels.

Keywords: Histopathological changes – gill – metals - *Liza saliens*

4.2. Introduction

The increase of domestic, industrial and agricultural activities has resulted in an increasing number of freshwater systems being impacted by the contaminants present in wastewater release. The heavy metal contamination of the aquatic ecosystems is a severe problem because these pollutants persist in the environment and are potentially harmful to most organisms.

Heavy metals, like copper and zinc, are essential for the normal metabolism of fish. However they are present at high concentrations in freshwaters, generally as a result of industrial pollution (Alsop and Wood, 2000; Arellano et al., 1999). High levels of heavy metals in the aquatic environment induce fish gill structure damage,

affecting physiological functions such as respiratory gas exchange, acid-base balance, water and ion exchange (Wendelaar Bonga and Lock, 1992).

Histopathological studies, in laboratory and in field experiments, have proved to be a sensitive tool to detect direct toxic effects of chemical compounds within target organs of fish (Schwaiger et al., 1997; Au, 2004). The gills of freshwater fish are the largest fraction of the total body surface area, which is in direct contact with the water (Hughes, 1984). The complexity and constant contact with the surrounding water make the gill the first target to waterborne pollutants (Perry and Laurent, 1993). In fact, pollutants enter the organism through the gills and exert their primary toxic effects on the branchial epithelium (Playe et al., 1992). Thus, changes in fish gills are among the most commonly recognized responses to environmental stressors (Mallat, 1985; Au, 2004) and are an indicative of physical and chemical stress. Although they are not diagnostic of particular chemicals or mode of action, the prevalence, severity and extension of the lesions might be an accurate indicator of the toxicant levels.

The Esmoriz-Paramos is a coastal lagoon on the Northwest coast of Portugal that plays a crucial role on the ecology of the ecosystem. However, as a result of industrial, agricultural and other anthropogenic activities, this aquatic ecosystem has been degraded over the last decades. The heavy metal contamination has been an important factor to the decline of water and sediment quality and may adversely affect fish health (Fernandes et al., 2007).

In the present work the leaping grey mullet *Liza saliens* was studied because it is the dominant species in the Esmoriz-Paramos lagoon. It is a filter feeder and also a detritus-mud feeder, and is therefore exposed to contaminated sediments. In fact, the sediment compartment is the final receptor of the insoluble or weakly water soluble pollutants acting both as a deposit and as a source of pollutants (Fracácio et al., 2003).

Data from a previous study showed that the metal concentrations in the sediments collected in different stations of the Esmoriz-Paramos lagoon did not varied significantly with depth and its mean metal concentrations were 234 mg Zn·kg⁻¹ d.w. and 84 mg Cu·kg⁻¹ d.w. (Fernandes et al., 2007). The range of metal concentrations in the water was 0.006-0.811 ppm for Zn and 0.003-0.031 ppm for Cu and was high when compared with reference guidelines (Fernandes et al., 2007 accepted).

The purpose of the present study was to evaluate the gill histopathological changes in *Liza saliens* caught in the Portuguese Esmoriz-Paramos coastal lagoon,

and to determine the prevalence of the number of lesions and the prevalence of the severity and extension of each lesion.

4.3. Material and Methods

Study Area and Fish Sampling

The Esmoriz-Paramos coastal lagoon is located on the Northwest coast of Portugal and is about 1500 m long (N-S), 700 m wide (W-E) and presents a maximum depth of 2.5 m. It receives mostly untreated effluents via its tributaries: one from the north, Ribeira de Paramos and other from the south, Vala de Maceda. The lagoon communication with the sea is established occasionally when its channel extends through the sand dunes to the ocean. The particle deposition rate has been relatively high during the last 20 years and the wet area of the lagoon has decreased (SIMRIA, 2002).

Liza saliens from their natural populations were captured during April 2004 in Esmoriz-Paramos lagoon, using a gill net. Mullet from the sea, 14 Km northwards from the lagoon, were also caught during April 2004. Fish was anesthetized and gill samples were randomly taken for histopathological examination and metal analyses. Fish age was determined by reading the annual ring structure of scales removed from opercular region (Muir and Den Haas, 2003).

For metals quantification the gill soft tissue was lyophilized, and digested overnight with nitric acid (supra pure grade) at 60 °C. The digested samples were analysed in a graphite furnace atomic absorption spectrometer (UNICAMP 939 AA-GF90). Blank determinations were done using the same procedure with Milli-Q50 water. The results were expressed in mg·kg⁻¹ dry weight. The analytical accuracy and precision was checked using certified reference materials, i.e DOLT-3 and DORM-2 (National Research Council of Canada).

Light Microscopy

For light microscopy, the gill tissue previously fixed in buffered formalin (10%) fluid for 48 h, was dehydrated in graded ethanol concentrations, and embedded in paraffin wax. Sagittal sections (5 µm of thickness) were stained with hematoxylin-eosin (HE). Changes observed in gill tissue were analyzed in a light microscope Nykon and photographed using an Olympus digital camera (Camedia 5050).

Analysis of the Gill Lesions

A score system was used to rank the severity and extension of the gill lesions. The severity of the lesions was scored, according to Schwaiger et al. (1997), as

follows: 0 = no pathological alterations, 1 = focal mild pathological alterations, 2 = moderate pathological alterations, 3 = severe pathological alterations. The extension of lamellae affected was scored as follows: 0 = 0%, 1 ≤ 10%, 2 = 11 to 49%, 3 = 50 to 69%, 4 ≥ 70%.

For each fish and lesion, the product severity x extension was calculated to establish an assessment value per gill filament varying through 1-12. Assessment values ≥ 6 were assumed to be of high impact. To reduce bias, the codes for individuals were broken only after the final histopathological examination. The number of lesions, the scores of severity and extension, and the large assessment values (6-12) were recorded and their prevalence index calculated. The prevalence was registered as the percentage of fishes that evidenced the lesion.

Statistical Analysis

The relationships between the assessment value for each fish lesion and gill metal levels, and fish age were tested using Pearson's correlation test, as well as the relationship between gill metal levels and fish age. The level of significance was set at $p < 0.05$.

4.4. Results

Fish age varied between 6 and 13 years old. A mucous layer was frequently observed on the gills surface. Gill epithelium was typical of other teleost fish. Filamentar epithelium was constituted by several cell types, namely chloride cells, pavement and mucous cells. The filament is vertically intersected by the lamella vascular axis, which in its apical region is covered by pavement cells. The lamellar vascular axis is supported by endothelial modified cells, the pillar cells.

Gill epithelium of exposed fish showed different histopathological effects. These main lesions were lamellar capillary aneurisms, epithelial hyperplasia, occasionally resulting in lamellar fusion, epithelial lifting and vasodilation (Fig 4.1). These lesions were observed at varying degrees of extension and severity.

Table 4.1 shows the prevalence of the number of gill lesions and the prevalence of each lesion. All the fishes presented at least one histological alteration in the gill. However, the prevalence of 3 or 4 different lesions (76%) was found to be higher than the prevalence of 1 or 2 lesions (24%). All the lesions showed high prevalence, being the vasodilation the one with the highest value recorded, whereas aneurism showed the lowest. The prevalence of the number of

lesions found in *L. saliens* collected from Esmoriz-Paramos lagoon was high when compared with mullet caught in the sea.

Table 4.1 - Prevalence of the number and kind of lesions.

<i>Parameters</i>		<i>Prevalence (%)</i>	
		Reference (N=10)	Esmoriz-Paramos lagoon (N=34)
Number of lesions	1 - 2	71	24
	3 - 4	29	76
Lesion	Aneurism	0	65
	Hyperplasia	14	82
	Lifting	43	74
	Vasodilation	53	85

The number of lesions was estimated as the number of different gill lesions present simultaneously in each fish; The prevalences indicates the percentage of fish presenting 1-2, 3-4 number of gill lesions, or a specific gill lesion (aneurism, hyperplasia, lifting or vasodilation).

The prevalence relative to severity and extension scores for each lesion and the large assessment value are presented in Table 4.2. There is a predominance of lesions with low severity and extension (score 1), except lifting which showed 32 and 24% of the fishes affected, respectively, in a very extensive and severe way.

Table 4.2 - Prevalence (%) of the severity and extension scores for each lesion.

			Extension				Severity			Assessment value
			4	3	2	1	1	2	3	6 to 12
Lesions	Reference (N=10)	Vasodilation	0	0	14	86	57	43	0	2
		Aneurism	0	0	0	0	0	0	0	0
		Hyperplasia	0	0	43	57	100	0	0	1
		Lifting	0	0	57	43	71	29	0	2
	Lagoon (N=34)	Vasodilation	0	7	24	69	48	38	14	10
		Aneurism	0	0	14	86	50	36	14	5
		Hyperplasia	14	14	25	47	50	43	7	21
		Lifting	32	12	20	36	44	32	24	40

Assessment value was obtained by multiplying the severity and extension from each lesion. High impact is assumed to be assessment value ≥ 6 .

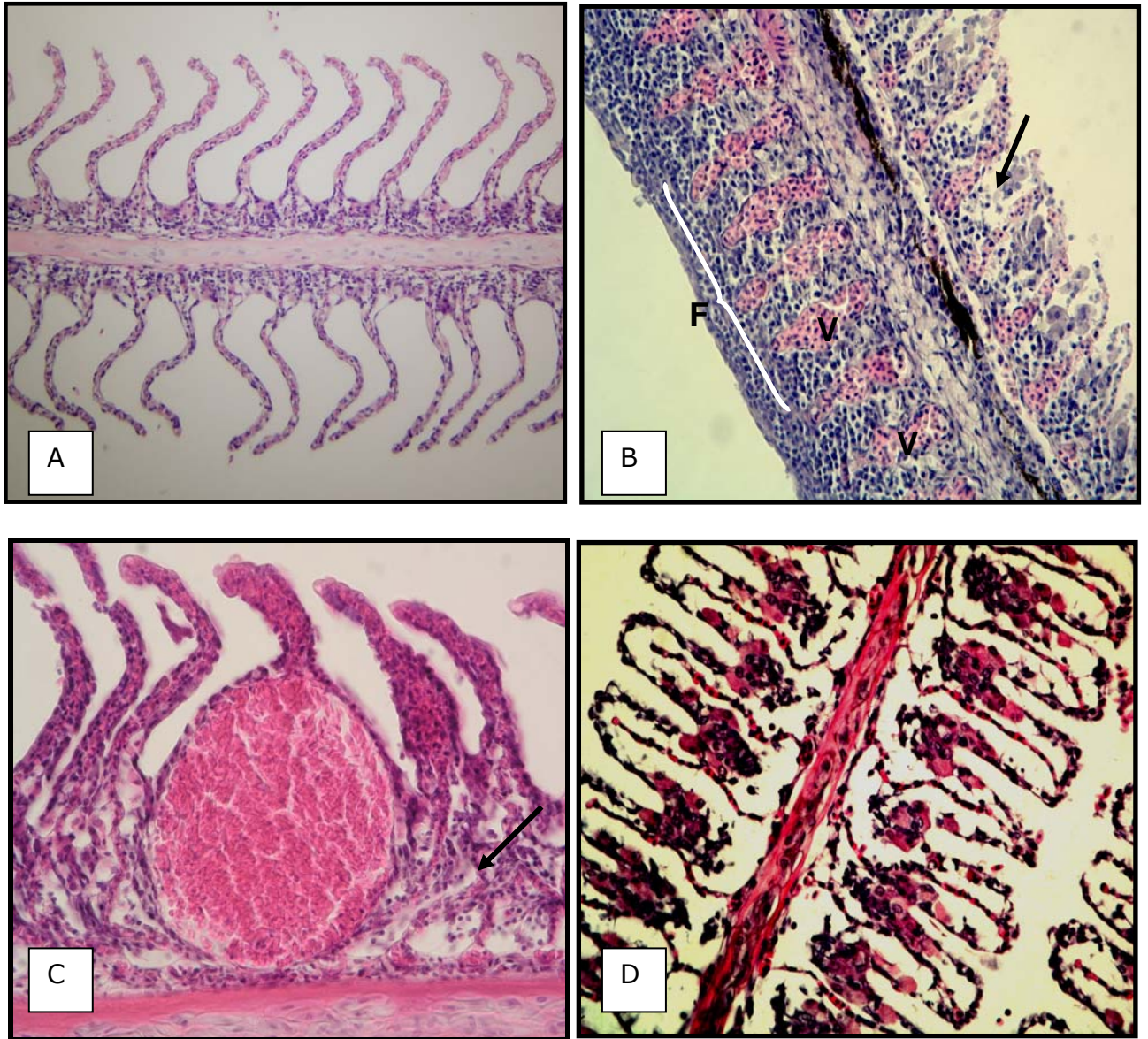


Fig. 4.1 – **A**, Gill filament of mullet collected from the sea; Gill histopathology of mullet (*Liza saliens*) from the Esmoriz-Paramos lagoon: **B**, High severity of filamentary epithelium hyperplasia that induced completely lamellar fusion (F). High severity vasodilation (V) in the lamellar vascular axis and necrosis with rupture of covering filamentary epithelium (arrow); **C**, Aneurism with high grade of severity that extends through the entire lamellar vascular axis and lifting of lamellar epithelium (arrow); **D**, High severity degree of lifting of filamentary and lamellar epithelium. In filament, the lifting might be due to degeneration of epithelial cells and edema. The lifting observed in lamellae is probably due to the high levels of edema. (Magnification, 200x).

The analysis of metal concentrations in the gills showed that the zinc levels were significantly higher than copper. The minimum and maximum concentrations were 69 to 191 mg Zn·kg⁻¹ and 3 to 16 mg Cu·kg⁻¹, respectively. Table 4.3 shows the relationships between the histological assessment values for each gill lesion, fish age and metals levels and the relationships between gill metal levels and fish age. The concentrations of Cu and Zn in gills were positively correlated with fish age (p<0.01). Lifting was the single lesion showing significant negative correlations with both metals and with fish age.

Table 4.3 – Relationship between histological assessment values for each gill lesion, fish age and metal levels, and relationship between metal levels and fish age.

	<i>AVA</i>	<i>AVH</i>	<i>AVL</i>	<i>AVV</i>	<i>Age</i>
Age	-0.064	0.250	-0.395 *	-0.155	
Cu-gill	0,016	0.334	-0.517 **	-0.087	0.577 **
Zn-gill	0.116	0.259	-0.374 *	0.033	0.450 **

AVA - assessment value of aneurism; AVH - assessment value of hyperplasia; AVL - assessment value of lifting; AVV - assessment value of vasodilation; fish age was determined by reading the annual ring structure of scales; p < 0.05; ** p < 0.01;

4.5. Discussion

Histological alterations in marine organisms have been identified as useful biomarkers of environmental contamination (Hinton and Laurén, 1990, Hinton et al., 1992; Munday and Nowak, 1997; Tricklebank, 2001). The histopathological changes observed in *Liza saliens*, collected from Esmoriz-Paramos lagoon, were previously identified in other teleosts exposed to copper or zinc (Arellano et al., 1999; Chen and Lin, 2001; De Boeck et al. 2001). However, these alterations are not specifically induced by these metals. In fact, other pollutants may cause the observed changes, such as endosulfan (Nowak, 1992), arsenic (Hwang and Tsai, 1993), nickel (Pane et al., 2004) or cadmium (Reid and McDonald, 1988).

The results of the present study reveal that all the analysed fish from Esmoriz-Paramos lagoon showed some degree of gill injury and high percentage of fishes had more than two types of lesions simultaneously. The prevalence of the number of lesions found in *L. saliens* collected from lagoon was high when compared with fish caught in the sea, which is considered an unimpacted area.

However, considering the extension and the severity, not all the lesions had high impact. The severity and extension of aneurism and vasodilation was generally low, resulting in a few number of fishes with high impact assessment. On the other hand, fish presenting lesions with high impact assessment were the ones with more

extense lesions. In fact, about 44% of the fish showing lifting and 28% presenting hyperplasia had a severe extension. As a result, lifting, followed by hyperplasia, were the lesions with the highest number of fish showing elevated assessment value.

Lifting constitutes a typical defence mechanism that increases the diffusion distance between blood and the waterborne pollutants, and was probably induced by the incidence of severe edema (Arellano et al., 1999; Pane et al., 2004; Schwaiger et al., 2004). Hyperplasia of filamentar epithelium, in turns, reduces the superficial area exposed to the pollutants. Previous experimental studies have demonstrated that lifting and hyperplasia of the epithelium are frequent lesions observed in gill epithelium of fish exposed to Cu (Arellano et al., 1999). The exposure of rainbow trout (*O. mykiss*) to Zn can also lead to epithelial lifting and lamellar aneurism (Skidmore and Tovell, 1972). However, epithelial lifting and hyperplasia of undifferentiated epithelial cells are non-specific alterations that can be caused by other factors, namely ammonia or protozoan ectoparasites (Schwaiger et al., 1997; Martinez et al., 2004).

The aneurisms are gill lesions that may result from extended vasodilation with rupture of pillar cells, and are less often associated with heavy metals (Mallatt, 1985). However, experimental studies reveal that exposure to Cd (Thophon et al. 2003), Pb (Martinez et al., 2004) and Cu (Mazon et al., 2002) induced this lesion in the gill epithelium. On the other hand, field studies also referred the presence of aneurisms (van den Heuvel et al. 2000; Handy et al., 2002).

It is generally accepted that heavy metal uptake occurs mainly from water, food and sediment. The leaping grey mullet lives in close contact to the sediments and its feeding strategy make them particularly vulnerable to sediment associated pollution that may accumulate in the target organs such as liver, kidney and gills. Gills are metabolic active tissues that accumulate heavy metals at higher levels as has been observed in experimental and field studies (Allen, 1995; Karadede and Unlu, 2000; Yilmaz, 2003). The age, size and feeding habitats of fish, besides their retention time in polluted waters, affect the heavy metal accumulation (Schumacher et al., 1992; Al-Yousuf et al., 2000; Canli and Atli, 2003). Corroborating these results, the present study showed a positive relationship between Cu and Zn concentrations in gills and fish age. However the present study revealed no significant relationship between Cu and Zn gill content and the different kind of lesions, except for lifting that showed a negative correlation.

In conclusion, this study supply new insights about histopathological gill changes that are indicative of the poor environmental conditions of the studied are, mainly

showed by the epithelial lifting and hyperplasia assessment values and by a high number of simultaneous lesions and a high prevalence for each lesion. However, it is important to determine additional information about other contaminants present in the Esmoriz-Paramos lagoon in order to explain the observed lesions.

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CAPÍTULO 5

CHANGES IN PLASMA ELECTROLYTES AND GILL HISTOPATHOLOGY IN WILD *LIZA SALIENS* FROM THE ESMORIZZ-PARAMOS COSTAL LAGOON, PORTUGAL

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5. CHANGES IN PLASMA ELECTROLYTES AND GILL HISTOPATHOLOGY IN WIL *LIZA SALIENS* FROM THE ESMORIZ-PARAMOS COSTAL LAGOON, PORTUGAL

5.1. Introduction

The Esmoriz-Paramos is a lagoon of great ecological significance located on the Northwest coast of Portugal. The quality of water and sediment within this ecosystem has been gradually degraded due to the discharges of mostly untreated industrial waste and domestic sewage. Contaminants include heavy metals that can be taken up by fish from water, food, sediments, and suspended particulate material. Fish inhabiting polluted water bodies tend to accumulate many chemicals in high concentrations, even when the environmental contamination levels are low (Colombo et al. 1995). The leaping grey mullet (*Liza saliens*) when in contact with xenobiotics in the water and, when feeding, in the sediments is one of a few dominant species living in this environment.

Previous studies have analyzed heavy metal concentrations in the water and sediments of this lagoon, and evaluated their bioaccumulation in *L. saliens*. The seasonal range of metal concentrations in surface water was 0.003-0.031 mg Cu·L⁻¹, 0.006-0.811 mg Zn·L⁻¹ and 0.01-0.026 mgPb·L⁻¹, all mainly found in particulate matter. The main metals found in sediments were Cu, Zn and Pb, respectively 83, 241 and 87 mg·Kg⁻¹d.w. The Cu and Zn concentrations in *L. saliens* liver were 262 and 89 mg·Kg⁻¹d.w and below the detection limit for Pb (0.073 ppm), whereas these concentrations in the gill were 9 mg Cu·Kg⁻¹ d.w.; 114 mg Zn·Kg⁻¹ d.w. and 0.6 mg Pb·Kg⁻¹ d.w. (Fernandes et al. 2007). Also in this study, were noted significant age-related increases of metal concentration in tissue for Cu in liver and gill and for Zn only in gill.

The gill is particularly sensitive to physical and chemical changes in the aquatic environment and it is the main target organ in fish for toxic waterborne heavy metals (McDonald and Wood 1993). Effects of metals on ionoregulatory gill functions have been well demonstrated, including regulation of plasma electrolytes (Mazon et al. 2002; Grosell et al. 2003; Martinez et al. 2004). The histological effects of metals on fish gill have also been studied in several fish species (Arellano et al. 1999; De Boeck et al. 2001). The mechanisms of acute Cu toxicity include the osmoregulatory disturbances involving Na⁺, Cl⁻ and K⁺ uptake by the gill (Mazon et al. 2002; Grosell et al. 2003). Although the mechanisms of heavy metals toxicity are well known in acute exposure, the process may differ in chronic exposure (Handy 2003).

The objectives of this study were to evaluate the plasma electrolyte concentrations of wild *Liza saliens* from the Esmoriz-Paramos lagoon and to assess the osmoregulatory responses and branchial histopathological changes related to chronic heavy metal exposure in this habitat.

5.2. Material and Methods

Mugilidae specimens were captured from the Esmoriz-Paramos lagoon and from the sea, in April 2004 in post-spawning period. The water quality parameters of the lagoon were monitored monthly during 2003-2004: temperature 10-23 °C, pH 6.9 -7.7 and salinity 0.17-2.60 ppt. Ten fish from the lagoon, with total length of 27-45 cm, and 15 fish from the sea, with total length of 22-47 cm, were randomly collected and quickly euthanized in the field by a sharp blow to the head. Blood was drawn from the caudal vessels with heparinized syringes. The second right gill arch was randomly collected for histopathology according to the usual methodology (Pane et al. 2004) and immediately fixed in buffered formalin (10%) for 48 h. The remaining gill tissue and liver samples were frozen at -20 °C until metal analyses. Fish age (7-12 years old) was determined by reading the annual ring structure of scales.

Plasma was obtained by centrifugation (5 min, 10.000 g, 4°C) and electrolyte measurements were carried out for Na⁺ (mEq Na/L), Cl⁻ (mEq Cl/L), K⁺ (mEq K/L), Ca²⁺ (mg Ca/L) and inorganic phosphorus (mg P/dL), using an automatic dry chemistry system analyzer (Clinical Diagnostics, VITROS 950). Na⁺, Cl⁻ and K⁺ were measured by direct ion-selective electrodes using SRM 919^a and 956 from National Institute for Standards and Technology. According to National Committee for Clinical Laboratory Standards, colorimetric methods were employed to measure inorganic phosphorus and calcium by the ammonium molybdate reaction and arsenazo (III) dye formation, respectively.

Fish tissues were acid-digested based on the method of Ferreira et al. (1990). Metal concentrations in liver were measured by flame atomic absorption spectrometry (Philips PU9100X). The gill metal concentrations were analyzed in a graphite furnace atomic absorption spectrometer (UNICAMP 939 AA - GF90). Certified reference materials, i.e DOLT-3 (dogfish liver from NRC) were analyzed to check analytical accuracy and precision. Results were expressed in mg·Kg⁻¹ dry weight (Fernandes et al. 2007).

The gill tissue was prepared for analysis for light microscopy, being dehydrated in graded ethanol concentrations, and embedded in paraffin wax.

Sagittal sections (5 μm of thickness) were prepared and stained with hematoxylin-eosin (HE). Changes observed in gill tissue were analyzed using a Nykon E 200 microscope at magnification of 200x and photographed using an Olympus digital camera (Camedia 5050).

To evaluate gill histopathological changes, 5 filaments and at least 100 lamellae per filament for each fish were evaluated using a scoring system to rank severity and extent. Severity was scored as follows: 0 = no pathological alterations, 1 = focal mild pathological alterations, 2 = moderate pathological alterations, 3 = severe pathological alterations (Schwaiger et al. 1997). A mean severity value for each histopathological change was calculated per filament to normalize the results. The extent of histopathological change was quantified, counting the number of lamellae showing alterations in each filament. The extent of lamellae affected was scored as follows: 0 = 0%, 1 \leq 10%, 2 = 11 to 49%, 3 = 50 to 69%, 4 \geq 70%. The mean extent value of the 5 filaments was then calculated for each histopathological change. Previous histopathological results, using a larger sample of fish from the lagoon, have shown high prevalence of lesions with high extent (Fernandes et al. 2007 accepted).

The relationships between plasma electrolyte concentrations, metal levels, and histopathological evaluation were tested with nonparametric correlations (Kendall's $r - p$), and differences between metal concentrations in tissues were tested with Mann-Whitney. A 5% significance level was applied throughout. Data are presented as mean values (standard deviation).

5.3. Results and Discussion

Table 5.1 shows the average plasma ion levels in the two fish populations. Plasma concentrations of P and K^+ , in fish from the lagoon were significantly higher when compared with plasma from sea ($p < 0.01$ and $p = 0.035$, respectively).

Gill epithelium of fish from the lagoon showed histopathological changes. The main lesions were epithelial hyperplasia, occasionally resulting in lamellar fusion, epithelial lifting, vasodilation and lamellar capillary aneurisms (Fig. 5.1). At a minimum, all the fish analyzed showed hyperplasia of filamentar and lamellar epithelium and vasodilation of the vascular axis. Lifting of lamellar epithelium and vascular aneurisms were found in 50% and 80%, respectively of the analyzed fish.

Table 5.1 - Plasma electrolytes concentrations in fish collected from the lagoon and from the sea. Mean (sd) and [range]

Parameters	Lagoon fish (N=10)	Sea fish (N=15)
Ca (mg/L)	7.06 (0.98) [5.30-8.20]	6.47 (0.60) [5.50-7.40]
Cl (mEq/L)	129.66 (7.55) [119-148]	134.23 (6.94) [122-146]
K (mEq/L)	5.31 (1.41) [2.90-6.90]	4.26 (1.15) [2.30-5.90]
Na (mEq/L)	152.28 (9.11) [136-168]	161.27 (12.29) [143-188]
P (mg/dL)	15.88 (2.08) [12.30-18.50]	12.05 (3.39) [5.80-17.50]

These gill changes were observed at varying degrees of extent and severity. For fish exhibiting aneurism, hyperplasia and vasodilation severity ranged from 1-3, whereas severity ranged from 1-2 for epithelial lifting. The mean severity of hyperplasia was 2 and for the rest of the changes was 1. The extent of hyperplasia and lifting ranged from 1-4 with a mean of 2. The extent of vasodilation, ranged from 1-2, and extent was 1 for aneurism; both of these lesions had an extent mean of 1.

L. saliens from the lagoon exhibited highest Cu content in liver, ranging from 53 to 464 mg·kg⁻¹ d.w. (mean: 283 mg·kg⁻¹ d.w.), than in gill, where ranging from 7 to 16 mg·kg⁻¹ d.w. (mean: 10 mg·kg⁻¹ d.w.). Zn concentration was higher in gill 136 mg·kg⁻¹ d.w. (range 107-191) than in the liver where 109 mg·kg⁻¹ d.w. (range 63-190) were obtained.

The plasma electrolyte levels showed no significant correlation with Cu-gill or Zn in tissues. However, K⁺ was positively correlated with liver Cu content (Kendall's $r = 0.556$, $p = 0.037$), and also with gill lifting (Kendall's $r = 1$, $p = 0.042$, for both severity and extent).

Phosphorus levels showed a positive correlation with severity of hyperplasia (Kendall's $r = 0.568$, $p = 0.041$). Cu-liver content was positively correlated with fish length (Kendall's $r = 0.556$, $p = 0.025$).

The gill Cu and Zn levels of *L. saliens* found in this work are similar to those observed in laboratory exposures that produced several histopathological changes, such as lifting and hyperplasia of the epithelium (Skidmore and Tovell 1972; Arellano et al. 1999).

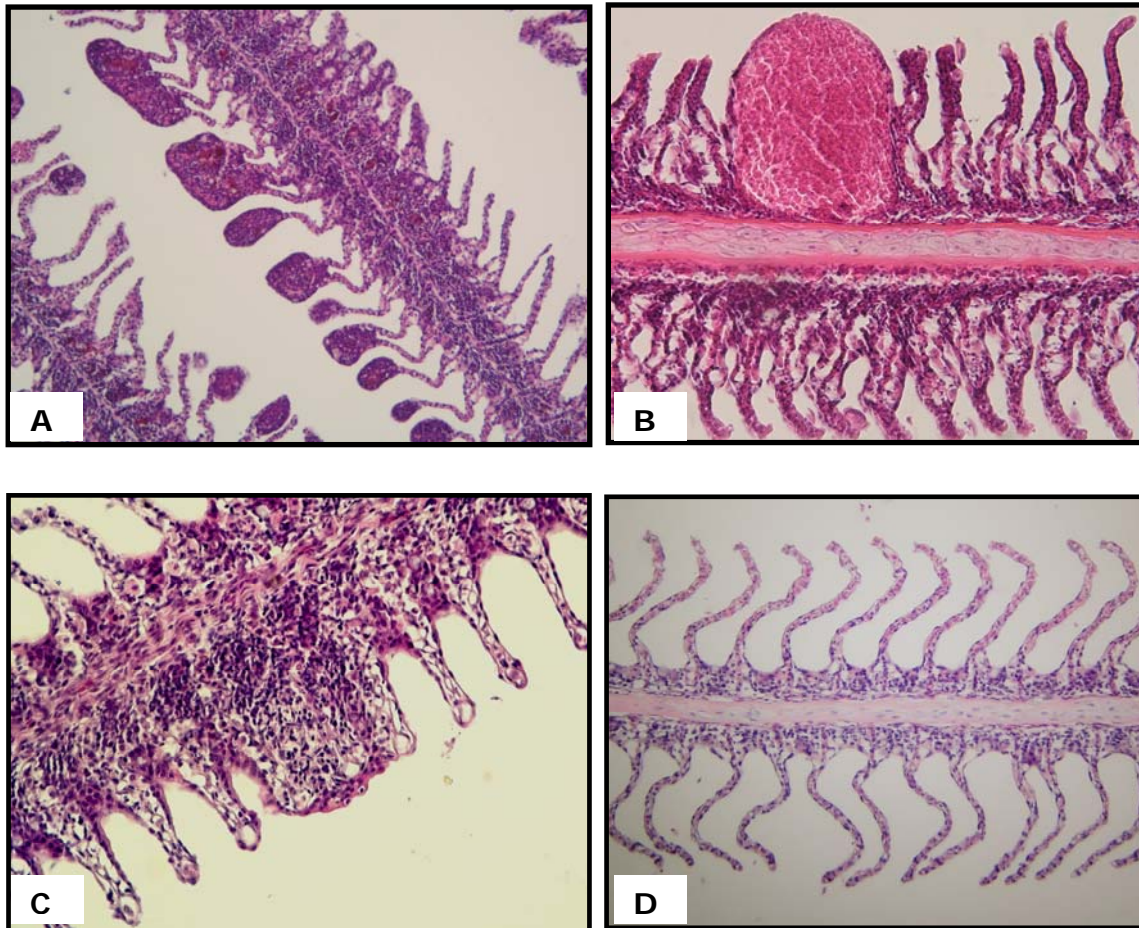


Fig. 5.1 - Gill histopathology of *Liza saliens* from the Esmoriz-Paramos lagoon. **A**, High severity of lamellar epithelium hyperplasia with fusion of adjacent lamellae (200x); **B**, Aneurism, with high grade of severity that extends through the entire lamellar vascular axis and lifting of lamellar epithelium (200x); **C**, Filamentar epithelium hyperplasia with focal lamellar fusion (200x); **D**, Gill filament of fish collected from the sea with no histopathological changes (200x).

Branchial Zn and Cu accumulations result from the chronic metal exposure (Taylor et al. 2004). In fact, the highest Zn concentration was found in gill and an increase kept within an interval was, over time, observed (Fernandes et al. 2007). The mean gill to liver-Cu ratio was 1:28, and the range of hepatic Cu content found in this study ($53\text{-}464\text{ mg}\cdot\text{kg}^{-1}$) suggests an above-normal accumulation (Paris-Palacios et al. 2000). Furthermore, the increased Cu levels noted with increased fish length, indicates some loss of liver homeostatic capacity, as also noted in previous studies (Fernandes et al. 2007).

Besides metal accumulation and associated gill histopathological changes, the stress caused by chronic metal exposure, can also affect the plasma K^+ levels in *L. saliens*. In this system, Cu-liver was a better environmental indicator of Cu stress, rather than Cu-gill (Fernandes et al., 2007). Additionally, plasma K^+ levels in fish

from the lagoon were higher than this electrolyte measured in fish from the sea exposed to low concentrations of contaminants. Thus, the positive relationship between Cu-liver and K^+ concentrations can indirectly indicate that chronic exposure affects this plasma electrolyte. Plasma K^+ levels may increase in fish from the lagoon due to osmotic adjustment, when compensating for a decline of other serum components, or resulting from disruption of K^+ regulatory ability (Marcaldo-Allen et al. 2004). Plasma K^+ alterations noted in this study may also have been caused by the disruption of cell membrane integrity due to gill lesions. In fact, a positive relationship was established between epithelial lifting (severity and extent) and K^+ levels. A comparison of plasma K^+ levels between fish exhibiting epithelial lifting (6.40 ± 0.36 mEq/L) and fish without this gill change (4.44 ± 1.31 mEq/L) further reinforces this concept. Other investigations have also described an increase of K^+ levels with Cu acute toxicity (Mazon et al. 2002).

The mechanisms of acute Cu toxicity also include osmoregulatory disturbance of Na^+ uptake by the gill (Grosell et al. 2003; Taylor et al. 2004), related to inhibition of branchial Na^+/K^+ -ATPase (Mazon et al. 2002). In contrast, the present results showed that plasma Na^+ levels in fish from the lagoon were not affected. No relationship between Na^+ levels and gill changes or metals were established, nor differences between Na^+ levels from the two populations indicate any effects on the Na^+/K^+ -ATPase function.

Plasma Ca^{2+} and Cl^- concentrations are parameters used to characterize the general osmoregulatory condition of the fish. Both of these plasma electrolytes generally decrease in cyprinids following exposure to different stressors (Jeney et al. 2002; Mazon et al. 2002). No trends in Ca^{2+} or Cl^- electrolytes in fish from the lagoon were found. The same electrolytes had similar levels in the two populations, which suggest that *L. saliens* was not sensitive enough or even adaptation has taken place. Tsuzuki et al. (2001) reported that moderate salinities (3-5 ppt) can reduce the stress response and ionic unbalances, because less osmotic work is needed to maintain stable ion levels. Indeed, in this lagoon the annual salinity range was 0.17-2.6 ppt, which may have contributed to a mild ionic stress response.

The increase in phosphorus concentration found in *L. saliens*, from the lagoon when compared with fish from the sea, could be a compensatory mechanism to maintain the number of osmotically active particles in the plasma (Mercaldo-Allen et al. 2004) and/or, as an important buffer involved in acid-base balance. In addition, the relationship between phosphorus levels and severity of hyperplasia

may be explained by decreased cell integrity resulting in phosphorus release from the cell.

Our results show potential evidence of anthropogenic effects on blood chemistry parameters of *L. saliens* collected from Esmoriz-Paramos lagoon. The chronic heavy metal toxicity may involve a series of adjustments or adaptations that contribute to the leaping grey mullets long-term survival. The effect of chronic Cu and Zn exposure in adult *L. saliens* in this lagoon included high gill and liver metal content, histological damage to specific target organs (e.g the gills) and indirectly to ionoregulatory disturbances. The increase of plasma K⁺ and phosphorus could be a consequence of changes in gill permeability and cell integrity, associated with the gill histopathological alterations. In conclusion, this study provides information about the nature of chronic adverse and adaptive effects on aquatic biota undergoing exposure to metals in Esmoriz-Paramos lagoon.

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CAPÍTULO 6

OXIDATIVE STRESS RESPONSE IN GILL AND LIVER OF *LIZA SALIENS*, FROM THE ESMORIZ-PARAMOS COASTAL LAGOON, PORTUGAL

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[Submitted to *ARCHIVES ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY*]

6. OXIDATIVE STRESS RESPONSE IN GILL AND LIVER OF *LIZA SALIENS*, FROM THE ESMORIZ-PARAMOS COASTAL LAGOON, PORTUGAL

6.1. Abstract

Tissue-specific responses against the oxidative stress and lipid peroxidation were analyzed in wild adult mullet (*Liza saliens*) caught in the portuguese costal lagoon Esmoriz-Paramos. Parameters measured were catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) activities in liver and gill tissues, and lipid peroxidation. The enzyme activities were related with gill histopathological alterations, as well as with heavy metals (Cu and Zn) concentrations in these tissues.

Gill epithelium of *L. saliens* showed histological alterations, such as epithelial hyperplasia that results in lamellar fusion, epithelial lifting, vasodilatation and lamellar aneurisms, which prevalences range 62 to 92%. The high Cu content was found in liver ($379 \text{ mg}\cdot\text{kg}^{-1}$), while the high Zn content was observed in gill ($119 \text{ mg}\cdot\text{kg}^{-1}$).

SOD and CAT activities showed differences between gill and liver. The high activities found were SOD in gill (10.1 U/mg prot.) and CAT in liver (39.2 mmol/min/mg prot.). In gill, CAT activity was negatively related with both Cu levels and gill lifting, while a positive relationship was found between SOD activity and fish age. A positive relationship was found, between hepatic CAT activity and Cu-liver that suggests a high metabolic level is related with Cu oxidative stress. The decrease of gill CAT activity can be due to osmotic stress caused by damaged gill epithelium. CAT activity in liver can be an appropriate biomarker of oxidative stress in Esmoriz-Paramos lagoon.

Keywords: Oxidative stress - antioxidant enzyme - liver - gil - *Liza saliens* - heavy metals

6.2. Introduction

The effects of pollutants in fish can be properly evaluated if bioaccumulation is complemented with other biomarkers. Contaminants usually appear in the environment as very complex mixtures that can cause interactive effects, thus biomarkers offer an integrated measurement of these effects (Orbea *et al.* 2002; Ferreira *et al.* 2005). Among pollutants that could be accumulated in fish, heavy

metals are of great interest because they could trigger oxidative stress in fish (Bláha *et al.* 2004; Deviller *et al.* 2005), by reactive oxygen species (ROS) generation (Durmaz *et al.* 2006; Lesser 2006).

Several studies revealed that exposure to contaminants in aquatic ecosystems can enhance intracellular formation of ROS, which are able to origin oxidative damage to biological systems (Livingstone 2003; Ferreira *et al.* 2005). ROS can be detoxified by an enzymatic defence system, that includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Halliwell and Gutteridge, 1989). SOD is the enzyme that catalyzes the dismutation of the superoxide anion to O₂ and H₂O₂ and CAT reacts with H₂O₂ to form water and molecular oxygen (Livingstone 2001; Lushchak *et al.* 2001; Ozmen *et al.* 2004). Glutathione-S-transferase form a family of multifunctional phase II biotransformation enzymes, present in cytosol of most cells, that catalyzes the conjugation of glutathione to a variety of compounds (Livingstone 2003).

Recent data indicate that changes in the levels of antioxidant enzyme activities can be used as contaminant biomarkers in different aquatic organisms (Orbea *et al.* 2002; Livingstone 2003; Regoli *et al.* 2004). Lipid peroxidation is one of the main manifestations of oxidative damage induced by various compounds, including metals (Ercal *et al.* 2001; Livingstone 2003; Lesser 2006), and it has also been used as biomarker (Sayeed *et al.* 2003; Bláha *et al.* 2004; Ozmen *et al.* 2004; Almroth *et al.* 2005; Durmaz *et al.* 2006).

The Esmoriz-Paramos is a coastal lagoon on the Northwest coast of Portugal, which receives untreated industrial and domestic sewage that promotes a decline of water and sediments quality and a decrease of biodiversity. Heavy metals have been an important source of contamination in this ecosystem that can be up-taken by fish from water, sediments, and suspended particulate material (Hardersen and Wratten 1998).

The vertebrate model most used in ecotoxicological studies is fish, which allows the evaluation of antioxidant responses in tissues and the hepatic oxidative damage caused by metal contamination (Orbea *et al.* 2002). The liver is the main detoxification organ (Olsvik *et al.* 2001). The gill is the osmoregulatory surface tissue and it is the primary site of uptake of waterborne pollutants (McDonald and Wood, 1993). The leaping grey mullet (*Liza saliens*) is the dominant specie in the lagoon that contacting with water and sediments pollutants, through its detritus feeding behaviour (Fernandes *et al.* 2007). Previous studies have shown Cu and Zn bioaccumulation in liver and in gill of *L. saliens* from the lagoon, and that the metal

level increases for Cu-liver, Cu-gill and for Zn-gill with fish age (Fernandes *et al.* 2007).

The aim of the present study is to determine the antioxidant enzymes activities in liver and gill in *L. saliens* from the Esmoriz-Paramos coastal lagoon. Lipid peroxidation was also measured in liver to assess oxidative damage and fish age was determined to assess time of exposure. Additionally, the enzyme activities were related with gill alterations and heavy metals (Cu and Zn) content.

6.3. Material and Methods

Study Area

The Esmoriz-Paramos lagoon, 1500 m in length (N-S), 700 m wide (W-E), 2.5 m maximum depth and a catchment area of 78 Km², is located on the Northwest coast of Portugal (Almeida 1998). It receives two main water inflows: one from the north and another from the south, and occasional effluents from local housing and small industry. The lagoon communication with the sea is established through a non-permanent channel with small dimensions, particularly in the shoreline. Major inputs of contaminants into the lagoon are mostly industrial or municipal, untreated sewage from its tributaries and runoff from contaminated soils and surfaces (SIMRIA 2002).

Fish Sampling

Liza saliens from their natural populations were collected during April 2004 in Esmoriz-Paramos lagoon, using a gill net. Mullet from the sea, 14 Km northwards from the lagoon were also caught during April 2004 and CAT liver and gill activities were measured and compared with equivalent activities from lagoon mullets. Fish was anaesthetised and gill and liver samples were frozen in liquid nitrogen and stored at -80 °C, until biochemical assays. Gill samples were also randomly taken for histopathological examination and gill and liver samples for metal analyses. The fish age was determined by reading the annual ring structure of scales removed from the opercular region (Muir and Den Haas 2003).

Biochemical Analysis

Livers were homogenised in ice-cold sodium phosphate buffer 50 mM, Na₂EDTA 0.1 mM, pH 7.8. Gills were homogenised in ice-cold imidazol buffer 50 mM, sucrose 150 mM, 10 mM Na₂EDTA, pH 7.3 (homogenizer T 1500 Ystral GmH). Mitochondrial fractions were obtained after centrifugation at 15 000 X g for 20 min. at 4°C.

SOD (EC 1.15.1.1) activity was determined by an indirect method involving the inhibition of cytochrome c reduction at 550 nm (Mc Cord and Fridovich 1969). The concentration of the reactives was: buffer 50 mM, pH 7.8, hypoxanthine 50 μ M, xanthine oxidase 1.98 mU/mL and cytochrome c 10 μ M (Ferreira *et al.* 2005). Enzyme activity was expressed in units per mg of protein (U/mg prot.), where 1 unit corresponds to 50% inhibition of the xantine oxidase reaction.

GST (EC. 2.5.1.18) activity was determined according to Habig *et al.* (1974) adapted to microplate by Frasco and Guilhermino (2002). Concentrations of the reactives were: glutathione (GSH) 10 mM in buffer 0.1 M, pH 6.5, and 1-chloro-2,4-dinitrobenzene (CDNB) 60 mM in ethanol prepared just before the assay. The reaction mixture was in a proportion of 4.95 mL (buffer): 0.9 mL (GSH solution): 0.15 mL (CDNB solution) (Ferreira *et al.* 2005). GST activity was measured every 20 s at 340 nm, during the first 5 min., and calculated using the period of linear change in absorbance. Enzyme activity was expressed in nanomoles per minute per mg of protein (nmol/min/mg prot.).

CAT (EC 1.11.1.6) activity was determined by measuring the consumption of H₂O₂ monitored spectrophotometrically at 240 nm, according to Aebi (1974). The reaction volume was 1 mL and contained 67.5 mM potassium phosphate buffer, pH 7.5, and 12.5 mM H₂O₂ (Ferreira *et al.* 2005). The CAT activity was expressed in millimoles of decomposed hydrogen peroxide per minute per mg of protein (mmol/min/mg prot.).

Oxidative Damage

The peroxidative damage of lipids that occurs with free radicals generation, and that results in malondialdehyde (MDA) production was assessed by thiobarbituric acid method (TBARS) adapted to microplate (Ferreira *et al.* 2005). The absorbance was measured at 532 nm and the concentration of MDA was expressed as nanomoles MDA per g of liver (nmol MDA/g liver). Total protein was measured by Lowry method adapted to microplate (Ferreira *et al.* 2005).

Tissue Metal Content

For metals quantification gill and liver samples, stored in plastic bags at -20 °C, were lyophilized, and analyzed according to Fernandes *et al.* 2007. Certified reference materials, i.e DOLT-3 (dogfish liver from National Research Council of Canada) were analyzed to check analytical accuracy and precision. The results were expressed in mg·kg⁻¹ dry weight.

Light Microscopy

For light microscopy, the gill tissue previously fixed in buffered formalin (10%) fluid for 48 h, was dehydrated in graded ethanol concentrations, and embedded in paraffin wax. Sagittal sections (5 µm of thickness) were stained with hematoxylin-eosin (HE). Changes observed in gill tissue were analyzed in a light microscope Nykon.

A score system was used to rank the severity and extension of the gill lesions. The severity of the lesions was scored as follows: 0 = no pathological alterations, 1 = focal mild pathological alterations, 2 = moderate pathological alterations, 3 = severe pathological alterations. The extension of lamellae affected was scored as follows: 0 = 0%, 1 ≤ 10%, 2 = 11 to 49%, 3 = 50 to 69%, 4 ≥ 70%. For each fish and lesion, the product severity x extension was calculated to establish an assessment value per gill filament varying between 1 and 12.

Statistical Analysis

Data are presented as mean ± standard deviation. Statistical calculations were performed with SPSS software. Differences between tissues metal content, enzymatic activities and assessment values of gill lesions were tested using the Mann-Whitney U-Test. The relationships between the different quantified parameters were tested with Spearman´s correlations. A 5% significance level was employed throughout.

6.4. Results

The age from fish collected from the lagoon ranged between 7 and 13 years old and length ranged from 25 to 49 cm. The gill lesions prevalences and gill histopathological assessment are presented in Table 6.1.

Table 6.1 - Lesions prevalences in gill and histopathological assessment in *Liza saliens* from Esmoriz-Paramos lagoon (N= 13).

<i>lesions</i>	<i>Prevalences (%)</i>	<i>Lesions assessment</i>
Aneurism	62	1.23 ± 1.73
Hyperplasia	92	3.17 ± 2.72
Lifting	69	2.37 ± 3.66
Vasodilation	92	1.76 ± 1.54

Gill epithelium of *L. saliens* showed several histopathological alterations; 77% of the fish presented 3 or 4 lesions. The main lesions observed were vasodilation and epithelial hyperplasia, occasionally resulting in lamellar fusion, both presented at 92% of fish. Epithelial lifting and lamellar capillary aneurisms were observed at 69% and 62% of fish, respectively. These histological alterations were observed at varying degrees of extension and severity. Hyperplasia and vasodilatation were rated with maximum severity (grade 3), whereas the maximum extension (grade 4) was observed for hyperplasia and lifting. The high mean assessment value of lesion was found for hyperplasia, followed by lifting, while the low was found for aneurisms and vasodilation.

Table 6.2 - Gill and liver copper and zinc contents in *Liza saliens* from Esmoriz-Paramos lagoon (N= 13).

Metal	(mg.Kg ⁻¹ d.w.)
Cu-liver	378.9 ± 122.5
Cu-gill	9.6 ± 1.6
Zn-liver	99.9 ± 30.9
Zn-gill	118.8 ± 26.5

Gill and liver metal contents in *L. saliens* from the lagoon are summarized in Table 6.2. The high Cu content was found in liver ($p < 0.01$), ranging from 125 to 547 mg.Kg⁻¹, and the high Zn content was observed in gill ($p < 0.05$), ranging from 88 to 134 mg.Kg⁻¹.

Gill and hepatic enzymes activities and lipid peroxidation in fish from the lagoon are presented in Table 6.3. This table also shows gill and hepatic CAT activities in fish from the sea.

Table 6.3 - Enzymatic activities, liver lipid peroxidation and liver protein levels from lagoon fish (N = 13). [CAT liver and gill activities from sea fish N=7].

Parameters		Liver	Gill
Enzyme activities	GST (nmol/min/mg prot).	85.8 ± 33.3	91.4 ± 33.0
	SOD (U/mg prot.)	3.8 ± 0.9	10.1 ± 4.3
	CAT (mmol/min/mg prot)	39.2 ± 16.6 [21.6 ± 5.3]	2.9 ± 2.6 [9.8 ± 4.5]
Lipid peroxidation (nmol MDA/g liver)		18.5 ± 7.5	
Protein (mg/g liver)		146.1 ± 49.7	

The GST activity levels in liver and gill from fish collected in the lagoon were similar. CAT activity was significantly higher in liver (13-69 mmol/min/mg prot) than in the gill ($p < 0.01$). SOD activity was higher in gill (6-23 U/mg prot) than in the liver ($p < 0.01$). Lipid peroxidation and total protein content in liver ranged from 9 to 38 nmol MDA/g and 90 to 266 mg/g, respectively.

Fish from the sea (22-35 cm of length) shows low CAT-liver activity when compared the values in fish from the lagoon ($p = 0.03$), ranged from 15.32 to 28.60 mmol/min/mg prot. In contrast, the CAT-gill activity was high in fish from the sea, ranging from 5.06 to 16.32 mmol/min/mg prot.

A negative correlation between gill CAT activity and both copper levels and lifting assessment value was found in fish from the lagoon (Fig. 6.1). Similar results were also found between gill CAT activity and lifting severity (Spearman's $r = -0.618$; $p = 0.032$). A positive correlation was found between fish age and SOD activity in gill, as well as between SOD and GST activities in gill (Fig. 6.1).

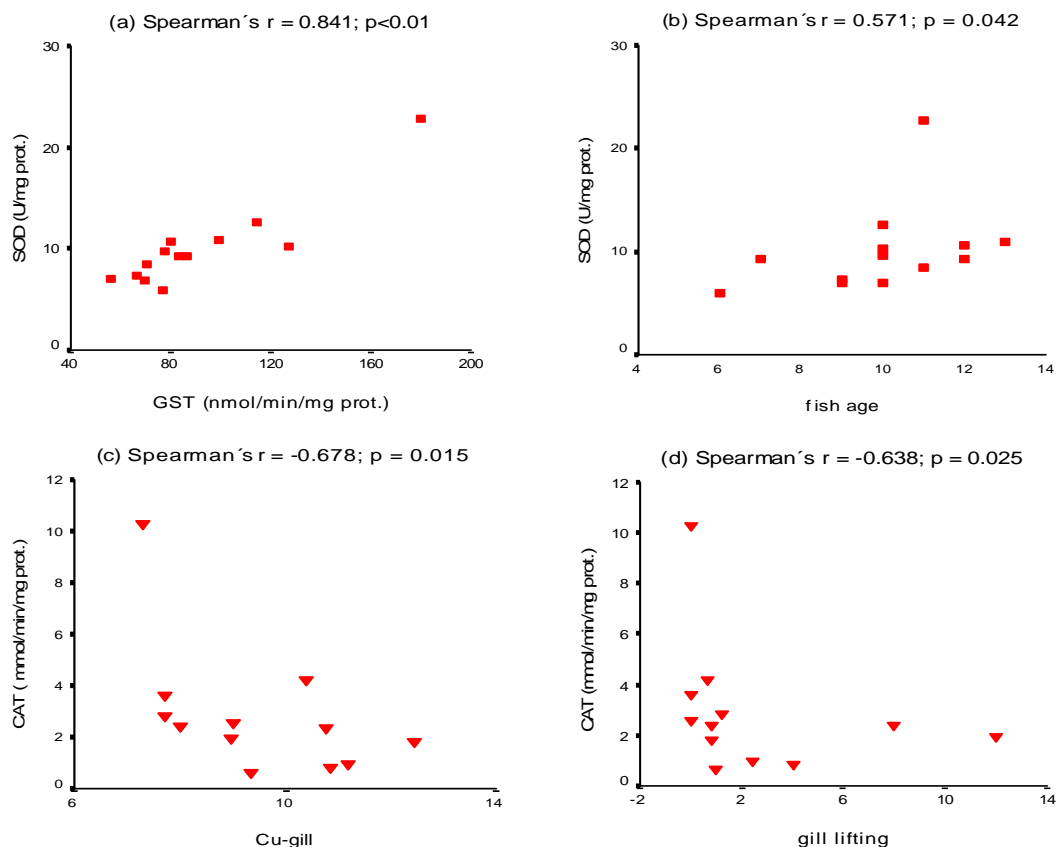


Fig. 6.1 - Relationships between SOD and GST activities (a), SOD and fish age (b), CAT and gill copper content (c), and CAT and gill alterations (d) in *Liza saliens* from Esmoriz-Paramos lagoon.

Fig. 6.2 shows a significant positive correlation between Cu-liver and lipid peroxidation. Similar results were found between Cu-liver and CAT activity. A negative correlation was found between GST activity and lipid peroxidation (Fig. 6.2). A negative relationship was also found between total liver protein content and fish length (Fig. 6.2) and fish age (Spearman's $r = -0.572$, $p = 0.04$). No significant relationship was observed with Zn.

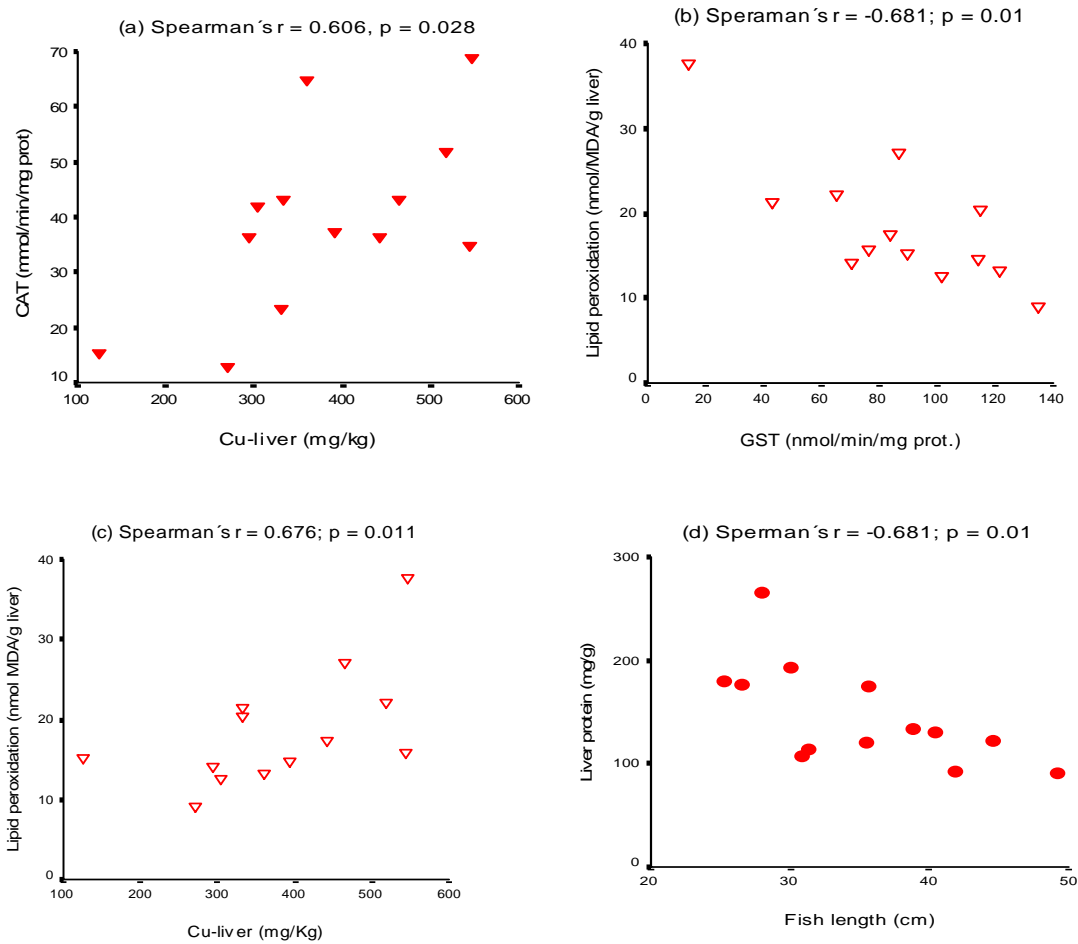


Fig. 6.2 - Relationships between hepatic CAT and Cu-liver (a), lipid peroxidation and hepatic GST activity (b) lipid peroxidation and Cu-liver (c) and liver protein and fish length (d), in *Liza saliens*, from Esmoriz-Paramos lagoon.

6.5. Discussion

Oxidative stress is defined as an adverse reaction resulting from the exposure of molecules, cells or tissues to excess levels of free radical oxidants, especially ROS (Li *et al.* 2005; Lesser 2006). ROS produced in biological systems are detoxified by antioxidant defences. One of the features of these antioxidant enzymes is their induction under conditions of oxidative stress, and such induction can be an important adaptation to pollutant-induced stress (Livingstone 2001). It is generally recognised that ROS production is associated with exposure to several metals (Ercal *et al.* 2001; Livingstone 2003), which can lead to induction of certain antioxidant enzymes (Livingstone 2001; Ozmen *et al.* 2004).

This study reveals tissue-specific changes in SOD and CAT activities. However, no significant differences were observed between gill and liver GST activities. Induction of antioxidant enzymes is a common mechanism of adaptive response in fish that vary among tissues (Oruc *et al.* 2004). CAT activity was high in liver, where the Cu content was high. Paris-Palacios *et al.* (2000) reported an increase of hepatic CAT activity in fish, *Brachydanio rerio* exposed to sublethal concentrations of Cu. The hepatic CAT activity in fish from the Esmoriz-Paramos lagoon was higher than in fish from the sea. These values were also high to that of *Mugil cephalus* caught in polluted environments (Orbea *et al.* 2002; Ferreira, *et al.* 2005). The high CAT activity may be a response to an increased H₂O₂ production (Ritola *et al.* 2002), to protect biological systems against ROS (Romeo *et al.* 2000). The high hepatic CAT activity found in this work suggests that a metabolic increase is achieved to cope with Cu oxidative stress. In contrast, the gill CAT activity is low, when compared with fish from the sea and shows a negative relationship with Cu and gill lifting. Previous studies revealed osmoregulatory disturbances in consequence of gill permeability and cell integrity change (Fernandes *et al.* accepted). Osmotic stress reduces rate of protein turnover and CAT, having an unusual rapid turnover, becomes affected, thus reducing its activity (Lesser 2006). According, the negative relationships found here suggest reduced gill-CAT activity due indirectly to gill epithelial lifting.

SODs are a group of metalloenzymes that plays a crucial antioxidant role and constitutes a defence system against the natural or chemically induced production of ROS (Roche and Bogé 1996; Livingstone 2001). Previous experimental studies in *Oreochromis niloticus* reported a decreased SOD activity in liver (Peixoto *et al.* 2006). However, other studies using different species exposed to several pollutants showed an increase of SOD activity (Palace *et al.* 1996; Figueiredo-Fernandes *et al.* 2006) and as well as when fish were exposed to Cu (Sanchez *et al.* 2005). The induction of hepatic SOD activity was also

described in several studies carried out in field polluted sites (Deviller *et al.* 2005; Ferreira *et al.* 2005).

A positive relationship between heavy metals (Cu and Zn) in gill and fish age in *L. saliens* caught in the Esmoriz-Paramos lagoon was demonstrated in a previous study (Fernandes *et al.* 2007). An increase of SOD gill activity with fish age was found in the present work and the higher SOD activity in gill than in the liver could be an indicator of compensatory tissue response. Oruc *et al.* (2004) also showed high SOD activity in gill when compared with other tissues as a result of pollutant exposure. SOD liver activity from our study was low when compared to that of *M. cephalus*, caught in polluted environments (Orbea *et al.* 2002; Ferreira *et al.* 2005).

The increase of hepatic CAT activity and the decrease of hepatic SOD activity agreed with the results observed in *Geophagus brasiliensis*, caught in a polluted area (Filho *et al.* 2001). Porte *et al.* (2002) also reported an increase in CAT activity and that there is no relationship between pollutants and hepatic SOD activity in wild mullet, *Mullus barbatus*.

The conjugation of phase I metabolites with GSH is catalyzed by the GSTs, one of the most widely studied conjugation enzymes in vertebrates. GST is involved in detoxification and excretion of foreign compounds and it may also show peroxydase activity (Paris-Palacios *et al.* 2000; Chung *et al.* 2004). This might explain the negative relationship between hepatic GST activity and lipid peroxidation found in the present study. The effects of pollutants on GST activity have been somewhat inconclusive, showing induction, no change, or inhibition of this enzyme (Stephensen *et al.* 2000). Some studies showed that exposure to pollutants can lead to an increase of hepatic GST activity (Sen and Kirikbakan, 2004; Camargo and Martinez, 2006), whereas others reported no variation (Porte *et al.* 2002) with respect to copper (Sanchez *et al.* 2005) and even decreased activity (Filho *et al.* 2001). Martinez-Lara *et al.* (1996) found a decrease of GST activity in gilthead seabream (*Sparus aurata*) exposed to pollutants and suggested that these results could be due to inactivation by ROS generated by pollutants. GST activity in liver of *L. saliens*, caught in a reference site is similar to those found in our work (Sen and Kirikbakan, 2004). In addition, hepatic GST activities in *M. cephalus* collected in a polluted site were high when compared with our data (Ferreira *et al.* 2006). This allowed us to think that the GST activity in liver obtained in this work did not vary with metal exposure. In turns, a positive relationship between gill SOD and GST activities was found that may reflect a reinforced response against oxidative stress in this organ.

No relationship between Zn content and oxidative stress enzymes activities was found in gill or liver of *L. saliens*. This might be related to the fact that Zn is apparently

regulated in *L. saliens* liver and its increase in gill over time is kept within a range (Fernandes *et al.* 2007).

MDA is a well-known oxidation product of polyunsaturated fatty acids, influencing cell membrane fluidity as well as the integrity of biomolecules (Ercal *et al.* 2001; Almroth *et al.* 2005) and is an important indicator of lipid peroxidation (Freeman and Crapo 1981). The present study reveals a positive relationship between lipid peroxidation measured as MDA, and Cu-liver content. The induction of hepatic lipid peroxidation caused by chronic dietary exposure to Cu was confirmed in grey mullet *Chelon labrosus* (Baker *et al.*). Our results suggest that antioxidant enzymes were not able to prevent the hepatic lipid peroxidation caused by chronic metal exposure.

The decrease of hepatic total protein was related with fish age and fish length, suggesting that is age-dependent. These data can be related to a metabolic activity reduction in older fish, as well as protein degradation due to ROS production.

In conclusion, the present study revealed that fish developed tissue-specific enzyme responses, adaptive by increasing CAT activity in liver and decreasing them in gill. CAT activity in liver can be an appropriate biomarker of oxidative stress, against copper exposure in Esmoriz/Paramos lagoon.

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CAPÍTULO 7

LIVER HISTOPATHOLOGICAL ALTERATIONS AND PLASMA TRANSAMINASE ACTIVITIES IN WILD MULLET *LIZA SALIENS* FROM ESMORIZ-PARAMOS LAGOON, PORTUGAL

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7. LIVER HISTOPATHOLOGICAL ALTERATIONS AND PLASMA TRANSAMINASE ACTIVITIES IN WILD MULLET *LIZA SALIENS* FROM ESMORIZ-PARAMOS LAGOON, PORTUGAL

7.1. Abstract

The Esmoriz-Paramos lagoon is an ecosystem located on the Northwest coast of Portugal, which quality has been degraded over the last years. Previous studies showed heavy metal concentrations in water and sediments, and evaluated their bioaccumulation in tissues of mullet (*Liza saliens*), living in the lagoon. In the present study, the occurrence of hepatic histological alterations and plasma blood biochemistry were determined in *L. saliens*. Blood parameters were compared between mullets collected from the lagoon and from the sea and included plasma enzyme activities (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP)), glucose (GLU) and total protein (TP).

A positive relationship between hepatocyte vacuolation index (HVI) and hepatosomatic index (HSI) was found, suggesting that lipid accumulation could have contributed to the increase liver weight. The hepatic alterations recorded included general diagnostic categories and additionally large areas of heterogenous parenchyma (HP), composed of hepatocytes with different spectrum of vacuolization. Liver histopathological evaluation revealed a sample prevalence of 34% of HP, 31% of foci of necrosis (FNs) and 9% of non-neoplastic lesions (NNL). No neoplastic lesions were found. Livers with HP showed higher Cu and Zn content than livers without this category of lesion, suggesting that metals accumulated could trigger this hepatic alteration.

AST was higher in mullets from the lagoon than in mullets from the sea, but no differences in ALP and ALT were found, suggesting an adaptive response, even though liver structural damage can not be neglected. The higher glycemia and proteinemia observed in mullets caught in the lagoon, is consistent with a stress response. Accordingly, the measurement of AST activity in blood could be a sensitive indicator of fish stress.

Keywords: hepatic lesions - plasma transaminase - heavy metals - chronic exposure - *Mugilidae* - coastal lagoon

7.2. Introduction

Fish have been widely used as models to evaluate the health of aquatic ecosystems and in toxicologic pathology (Law, 2003). Liver plays an important role in vital functions in basic metabolism and it is the major organ of accumulation, biotransformation, and excretion of contaminants in fish (Moon et al., 1985; Triebkorn et al., 1997). The study of liver histological alterations has been used for monitoring environmental exposure of fish to contaminants and is an indicator of environmental stress (Stentiford et al., 2003).

The impact of contaminants on aquatic ecosystems can also be assessed by measurement of biochemical parameters in fish that respond specifically to the degree and type of contamination (Petrivalsky et al., 1997). The measurement of fish cellular enzymes is an indicator of health condition and has been used as diagnostic tool in monitoring programs of aquatic pollution (Oluah, 1999; Bernet et al., 2001; Teles et al., 2003; Begun, 2004).

Toxicological studies of acute exposure have shown that the concentration of pollutants can change the enzyme activities and often directly induce cell damage in specific organs (Yang and Chen, 2003). Vertebrate transaminases are both mitochondrial and cytosolic enzymes involved in catabolism of amino acids, transferring amino groups to alfa-keto acids. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes used in the diagnosis of damage caused by pollutants in several fish tissues (De la Torre, et al., 2000). Based on the available literature data, it is evident that, despite different fish species respond differently to various environmental pollutants variations in serum transaminases could represent promising biomarkers (Stanic et al., 2006).

Esmoriz-Paramos is a lagoon located on the Northwest coast of Portugal that has ecological significance and is included in the National Ecological Reserve and classified as CORINE biotope. The quality of water and sediment within this ecosystem has been gradually degraded due to the discharges of mostly untreated industrial and domestic sewage. Contaminants include heavy metals that can be up-taken by fish from water, food, sediments, and suspended particulate material. However, despite these many inputs, the knowledge about the effects of pollutants in the watershed is scarce (Fernandes et al., 2007, accepted).

Over the past years fish population diversity in the lagoon has decreased. At present, leaping grey mullet (*Liza saliens*) is the dominant fish species living in this lagoon. It is a filter and detritus-mud feeder (Minos et al., 1995) that, through its feeding strategy, is in contact with pollutants in the water and sediments. Previous studies determined the heavy metal concentrations (copper and zinc) of water and sediments,

and evaluated their bioaccumulation in tissues of *L. saliens*. Results showed that time of exposure impaired the normal detoxification response for Cu, leading to liver bioaccumulation in *L. saliens* (Fernandes et al., 2007).

Since histopathology reveals the status of fish health, the aim of the present study was to examine the occurrence of hepatic alterations in wild leaping grey mullet (*Liza saliens*) living in the Esmoriz-Paramos lagoon and their relation with liver metal (Cu and Zn) concentrations. The histological observation was complemented with a selected blood biochemical profile, including glycemia, total protein and some plasma enzyme activities, which were compared with mullets collected from the sea.

7.3. Materials and methods

Fish collection and handling

Thirty-five mullets from lagoon and twenty-three mullets from sea, 14 Km northwards from the lagoon were captured by gill net in April 2004, at the post spawning period, immediately anaesthetized and their total weight and length recorded. Livers were removed and weighed to obtain hepato-somatic index (HSI = liver weight (g)/ body weight (g) x 100).

Liver samples from lagoon fish were randomly taken and immediately fixed for 24 h in 10% buffered formalin for histological observations. Blood, from a sub-sample of 10 lagoon fish and 23 sea fish, was drawn from the caudal vessels with heparinized syringes, for plasma enzyme activities, protein and glucose level determinations. Comparisons of blood biochemical parameters were made between 9 fish within the same size class.

Liver histology

Livers were routinely dehydrated after fixation in increasing concentrations of ethanol, and embedded in paraffin wax for light microscopy. Each tissue block was sliced in 3-5 µm-thick sections, and random sections were picked for analyses. Histological sections of liver tissue were stained with haematoxylin-eosin (H&E), mounted on glass slides, analyzed in a light microscope Olympus BX50, and photographed using an Olympus digital camera (Camedia 5050).

The hepatic alterations were classified according to Köhler et al. (2002) and included the following general diagnostic categories: non-neoplastic lesions (NNL) such as hyalinization, hepatocellular and nuclear polymorphism, multicellular hypertrophy and focal hepatocyte death; foci of necrosis (FN); granuloma and melanomacrophage centres

(MMC). Additionally an extensive area of heterogeneous parenchyma (HP), composed of hepatocytes with different spectrum of vacuolization was observed.

The hepatocyte vacuolation index (HVI) was calculated based on three levels of progressive increase vacuolization of liver cells (Stentiford et al., 2003).

Chemical and biochemical analyses

Copper and zinc concentrations in liver of lagoon fish were analysed elsewhere (Fernandes et al. 2007). Shortly, livers were freeze-dried and digested using nitric acid and hydrogen peroxide. Metal concentrations were measured using a Philips PU9100X flame atomic absorption spectrophotometer. Results were expressed in $\text{mg}\cdot\text{kg}^{-1}$ dry weight (d.w.).

Plasma was obtained by centrifugation (5 min, 10,000 g, 4°C) and analyses were conducted for enzyme activity (aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP)), total protein (TP) and glucose (GLU) content, using an automatic dry chemistry system analyzer (Clinical Diagnostics, VITROS 950). All the blood determinations were done according to the "National Committee for Clinical Laboratory Standards". Enzyme activities were colorimetric determined, AST activity was measured by dye formation via hydrogen peroxide (H_2O_2), ALT activity was measured by the NADH oxidation rate and ALP was measured by the *p*-nitrofenol formation. The results were expressed in U/L. The TP was determined by the Biuret reaction and the results were expressed in g/dL. The GLU concentration was colorimetric determined by H_2O_2 formation and the results were expressed in mg/dL.

Data analysis

The results were expressed as mean (standard deviation). Mann-Whitney U-Test was used to identify differences in liver metal levels and fish length between fish with and without a specific liver lesion, as well as to identify differences between plasma parameters from the two environments. Spearman's correlations were used to identify relationships between HVI and HSI. Calculations were performed with SPSS and a 5% significance level was employed throughout.

7.4. Results

The 35 mullets caught in the lagoon measured 23 to 49 cm, weighed 140 to 1060 g, presented an HSI between 1.80 and 4.38 % and had metal liver concentrations ranging from 51 to 547 $\text{mgCu}\cdot\text{kg}^{-1}$ d.w. and 26 to 190 $\text{mgZn}\cdot\text{kg}^{-1}$ d.w., as previously reported (Fernandes et al., 2007).

Table 7.1 summarizes the sample prevalences of the number and categories of hepatic histological alterations found in mullets caught at Esmoriz-Paramos lagoon. The sample prevalence regarding the diversity of hepatic alterations were as follows: 3% with four types of simultaneous alterations, 6% with three types of alterations, 14% with two types of alterations, 34% with one type of alteration, and 43% showed no alterations at all. Livers with no alterations were characterized mostly by a homogeneous parenchyma composed of polyhedric hepatocytes, with central nucleus, arranged in typical and complex tubular anastomosed architecture, in a quite similar structure to that described in control *Liza saliens* juveniles (D'Agati et al., 2006) and other control fish species (Paris-Palacios et al., 2000; Arellano et al., 1999; Visoottiviseth et al., 1999).

Table 7.1 – Sample prevalence of the number and categories of hepatic alterations in *Liza saliens* from Esmoriz-Paramos lagoon

<i>Parameters</i>		<i>Prevalence (%)</i>
Number of lesions	4	3
	3	6
	2	14
	1	34
	0	43
Categories	heterogenous parenchyma	34
	foci of necrosis	31
	granulomas	14
	non-neoplastic lesions	9
	melanomacrophagic centres	6

N=35; Number of lesions was estimated as the number of different liver lesions present simultaneously in each fish; Prevalence indicates the percentage of fish presenting a specific number of liver lesions, or a specific categories of lesion.

Heterogeneous parenchyma (HP), was the most prevalent hepatic alteration (34%), characterized by areas of macro and micro heterogeneous parenchyma vacuolization, consisting of hepatocytes either with a large spectrum of vacuolization contributing to parenchyma heterogeneity, or poorly vacuolated showing moderate to strong basophilia (Fig 7.1A and 7.1B). FNs were observed in 31% of fish, frequently associated with multifocal inflammatory processes, some of them spreading over large areas (Fig 7.1C), and with some FNs associated to parasitic lesions, whereas granulomas were observed in 14% of fish. NNLs were found in 9%, and included cell and nuclear polymorphism, focal hepato and nuclear hypertrophy (Fig 7.1D), hyalinization of cytoplasm, focal necrosis, frequent focal cell death (Fig 7.1E) and eventually inflammation, and lipidiosis. MMC was the histological alteration showing lowest prevalence (6%).

Among mullets from the lagoon, the livers showing HP had significantly higher metal contents accumulated (337 (128) mg·Cu and 107 (31) mg·Zn) when compared with fish without HP (223 (133) mg·Cu and 79 (29) mg·Zn).

The mean hepatocyte vacuolation index (HVI) was 1.09 (0.92) and a mild, but significant, correlation between HVI and HSI was found (Sperman's = 0.466, p= 0.006).

The comparison of biochemical blood parameters between mullets sampled at two different environments (lagoon and sea), within the same size class, is presented in Table 7.2. The AST levels were higher in fish from the lagoon than in mullet from the sea, whereas no significant differences were found for ALP and ALT. The TP and GLU concentrations were also higher in mullet from the Esmoriz-Paramos lagoon when compared with mullet caught in the sea.

Table 7.2 – Plasma parameters comparison between mullets sampled in lagoon and in sea, within the same size class (range 25-47 cm). Mean (sd)

<i>Variables</i>	<i>Lagoon</i>	<i>Sea</i>
	N=9	N=9
Length (cm)	36.95 (4.63)	32.43 (6.40)
ALP (U/L)	33.25 (17.50)	25.12 (18.38)
ALT (U/L)	4.28 (2.63)	12.62 (9.15)
AST (U/L)	263.22 (137.42) **	95.67 (44.97)
Glucose (mg/dL)	203.00 (64.21) *	97.00 (36.51)
Protein (g/dL)	6.73 (2.25) **	3.27 (1.57)

*<0.05; **<0.01

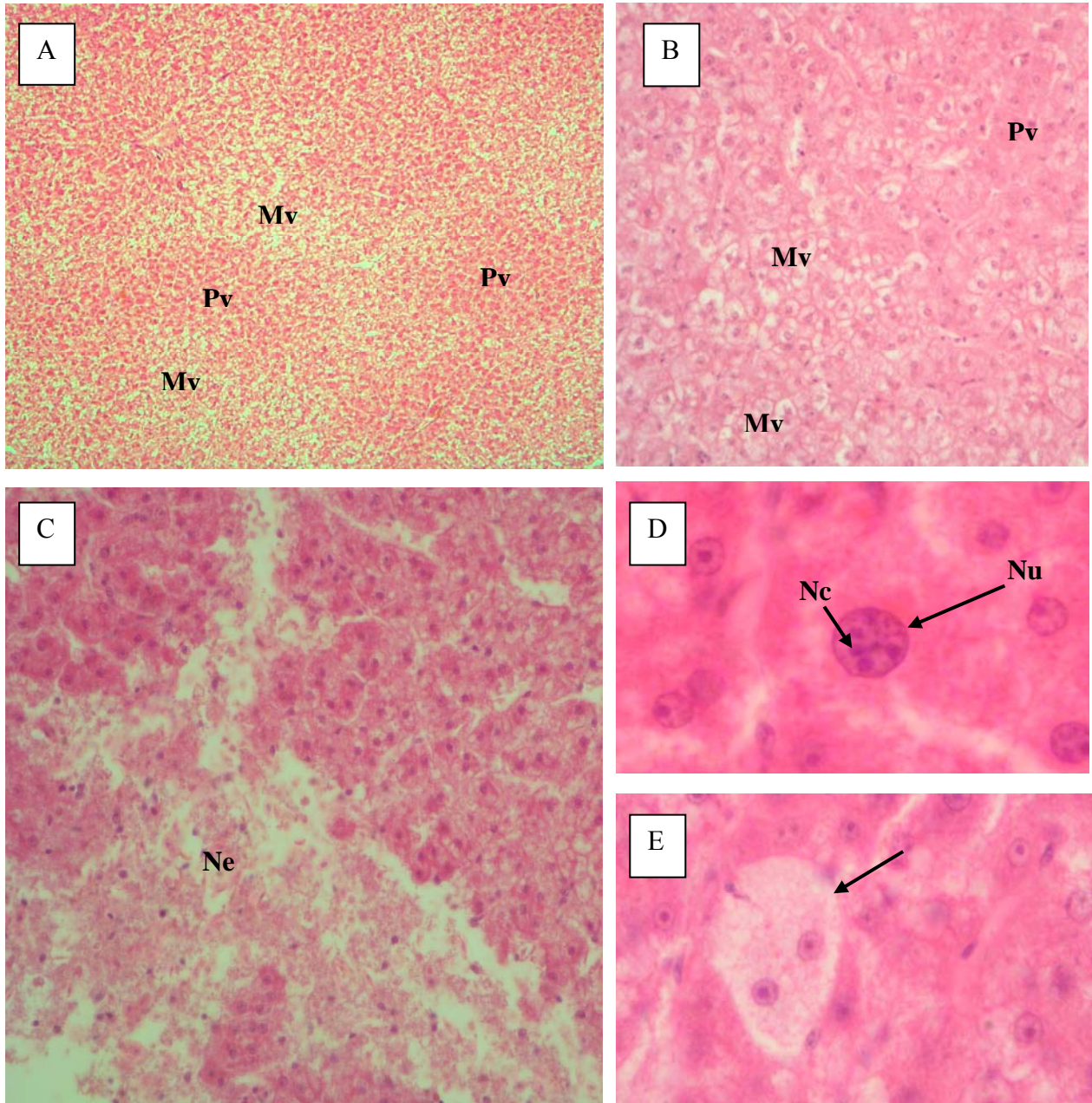


Fig. 7.1 - Histological sections of *L. saliens* liver showing: (A) extensive heterogeneous parenchyma with large spectrum of vacuolization (Mv) and poorly vacuolated (Pv) tissue 40x; higher magnification of heterogeneous parenchyma with hyaline tissue showing different spectrum of vacuolated hepatocytes (Mv) visible as white unstained areas and poorly vacuolated (Pv) visible as moderate to strong basophilia 100x; (C) large area of lytic necrosis (Ne) 100x; (D) nuclei hypertrofia (Nu) and simultaneous nucleolus (Nc) 100x; (E) focal hepatocyte death (arrow) 250x

7.5. Discussion

Liver histopathology has been used as an indicator of environmental stress since it provides a definite biological end-point of historical exposure (Stentiford et al., 2003), and the kind of injury is often dependent upon time of exposure to pollutants, such as metals (Yang and Chen, 2003; Au, 2004; Olojo et al., 2005). Although, liver histopathological alterations are not specific to pollutants, several studies have established a causal relationship between metals concentrations and fish liver lesions (Au, 2004). Liver lesions have been classified into several groups and ranked according to their relative importance as indicators of contaminant exposure. Non-neoplastic lesions, such as cellular and nuclear polymorphism have been considered as an initial toxicopathic lesion resulting from exposure to toxic agents (Stentiford et al., 2003). Hepatocellular necrosis has been observed as most pronounced in fish collected in contaminated ecosystems and was considered a response to metal exposure (Schwaiger et al., 1997; Olojo, et al., 2005). Therefore, NNs and FNs were recommended as hepatic histopathologic biomarkers for the assessment of fish chronic toxicity (Au, 2004).

The non-neoplastic lesions were found in a low number of mullets (9%). No differences in liver metals levels were found between fish showing hepatic FNs (31%) and fish without this kind of hepatic alteration (69%). However, the occurrence of HP in mullets caught at the Esmoriz-Paramos lagoon was associated with Cu and Zn. Previous studies revealed high Cu and Zn concentrations in liver from mullets caught in this lagoon (Fernandes et al., 2007). In fact in the present study, fish showing hepatic HP have higher metals content when compared with fish without this lesion, suggesting that the presence of this histopathological alteration could be a response to Cu and Zn accumulation in this organ.

The FNs and NNs have been described in literature however the HP found in this work, consisting of hepatocytes with a mixed vacuolization spectrum, or hepatocytes poorly vacuolated, was scarcely recorded. Paris-Palacios et al. (2000) found parenchyma heterogeneous, composed of two types of hepatocytes, a few large and clear hepatocytes, and very numerous small basophilic hepatocytes, related with Cu accumulation in zebrafish raised in laboratory conditions.

Several studies demonstrated that fish livers from both contaminated and reference sites show occasional MMC and granulomas (Teh et al., 1997; Stentiford et al., 2003). Although high prevalence of hepatic MMCs may occur in fish collected in contaminated sites due to the storage of foreign material (Marshall et al., 1999), they can not be linked to direct contaminant exposure without being integrated with other data, since they are also involved in other metabolic functions (Stentiford et al., 2003). In addition,

granulomas are likely to be associated to parasitic infections. Thus, granulomas and MMCs observed in this study were not considered alterations with toxicopathic aetiology.

Pre-neoplastic lesions, such as hepatic foci of cellular alterations, characterized by basophilic, eosinophilic, vacuolated and clear cells, are an early stage in the formation of hepatic neoplasia and provide a histopathological biomarker of exposure (Köhler et al., 2002; Stentiford et al., 2003; Au, 2004). Neoplastic changes, such as hepatocellular adenoma, are considered important in the development of carcinogenesis, since they represent transitional lesions to malignant lesions (Stentiford et al., 2003). However, pre-neoplastic or neoplastic lesions were not found in the present study, and therefore mullets from the lagoon may have liver carcinogenesis under development.

The high HSI found in mullets (Fernandes et al., 2007 accepted), and its positive relationship with hepatocyte vacuolation index (HVI) means that lipid accumulation could contribute to increase liver weight. Other studies revealed lipid accumulation in hepatocytes from fish exposed to toxic compounds, including Cu, Cd and Zn (Arellano et al., 1999; Van Dyk et al., 2007) and are considered as general indicator of liver toxic injury (Visoottiviset et al., 1999; Köhler et al., 2002; Van Dyk et al., 2007). Gül, et al. (2004) have related the higher HSI found in fish living in a polluted area to liver damage that ranges from increased liver weight and fat content to cell necrosis.

It is generally accepted that changes in enzyme activity in the extracellular fluid or plasma is a sensitive indicator of even minor cellular damage, since the level of these enzymes will be higher than normal (Van der Oost et al., 2003). Thus, the measurement of phosphatase and transaminase activities in the circulating fluid is frequently used as a diagnostic tool in water pollution studies (Palanivelu et al., 2005).

The mechanism responsible for the maintenance of free amino acid pool, during the process of protein synthesis, is transamination, which involves two types of transaminases, i.e, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Transamination is a key reaction affecting various metabolic processes such as the formation of nonessential amino acids and waste products and gluconeogenesis. The blood parameters comparisons were made between mullets of similar size caught in lagoon (N=9, length range 27-42 cm) and mullets caught in sea (N=9, length range 25-47 cm), to avoid age differences, since age could influence metabolic rate. Nevertheless plasma AST activity was higher in fish from lagoon than that of fish from sea. This increase in AST activity may suggest a change in protein metabolism, in consequence of metals exposure. Some studies revealed that metal content (Zn and Cu) increase plasma AST activity (Folmar et al., 1992). An increase in plasma AST and ALT activities due to metals (Zn, Cu and Cd) were also found in experimental conditions (Oluah, 1999;

Varanka et al., 2001; Zikic et al., 2001), as well as in fish chronically exposed to metals (Levesque et al., 2002).

The higher concentrations of protein and glucose found in lagoon mullets can be due to contaminants. Blood glucose is a sensitive indicator of fish stress and some studies concluded that metals can induce hyperglycemia in different fish species (Varanka et al., 2001; Zikic et al., 2001). Plasma total protein (TP) was used as a general index of fish health. Although TP may be influenced by the diet, high values may reflect haemoconcentration, impaired water balance (Zsigmond et al., 2002) or structural liver alterations (Bernet et al., 2001). Our data is compatible with a toxicant stress induced higher glycemia and proteinemia.

In conclusion, besides the heterogeneous parenchyma observed, the other abnormal conditions in liver of mullets (*Liza saliens*) from Esmoriz-Paramos lagoon resembled other pathological responses described in fish from contaminated sites. The significant higher Zn- and Cu-liver content associated with the occurrence of heterogeneous parenchyma suggest that metal chronic exposure in the lagoon may affect the liver, causing some degree of structural damage. Also, the observed increase in AST activity could be a manifestation of the general adaptive response, although generation by liver structural damage can not be neglected. The results of this study suggest that plasma AST activity could be used as biomarker of metal contamination.

Finally, the present study provided information about the nature of adverse effects on fish and the ecological impact that the pollutants are having in Esmoriz-Paramos lagoon.

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CAPÍTULO 8

DISCUSSÃO GERAL

8. DISCUSSÃO GERAL

A Barrinha de Esmoriz/Lagoa de Paramos é uma laguna costeira classificada como prioritária na directiva habitat e actualmente integrada na Reserva Ecológica Nacional, tendo sido classificada como biótopo CORINE no quadro da Comissão da União Europeia e integrada na rede Natura 2000 (SIMRIA, 2002).

Na área da sua Bacia Hidrográfica existem diversas unidades industriais, designadamente dos sectores da cortiça, curtumes, calçado, madeira, papel, têxtil, metalurgia e metalomecânica, reparação automóvel e também unidades agropecuárias, bem como da indústria de produtos químicos que utilizam produtos abrasivos, detergentes, tintas e vernizes (Almeida, 1998; SIMRIA, 2002).

A acumulação de água nesta laguna resulta essencialmente dos volumes drenados da sua bacia hidrográfica por duas linhas de água principais. A ligação da laguna com o mar era efectuada ocasionalmente por um canal com tendência a assorear devido à marcada deposição de areias. Nos últimos anos, a Barrinha de Esmoriz/Lagoa de Paramos tem sido o destino final de diversas descargas não controladas de águas residuais domésticas e industriais, bem como de situações difusas, referentes a actividades agrícola e agropecuária, levando a que a carga poluente tenha sido muito diversa, sendo frequente a presença de metais pesados (SIMRIA, 2002).

Pelos motivos expostos, considerou-se prioritário monitorizar o estado de contaminação da laguna resultante da actividade humana. A entrada das diferentes classes de poluentes pode ocorrer nos ecossistemas aquáticos por diversas vias, designadamente através da água, do alimento, dos sedimentos e dos materiais em suspensão e, em última análise, pode exercer efeitos nos peixes e na saúde humana.

Os metais pesados são uma das classes de poluentes preocupantes para esta laguna, porque apresentam ao mesmo tempo persistência, capacidade de bioacumulação e toxicidade. A entrada de metais neste ecossistema pode implicar a sua acumulação na água, sólidos em suspensão, sedimentos e biota. Os sedimentos funcionam como repositórios estratificados deste tipo de poluentes a partir da coluna de água, através da adsorção às partículas, precipitação e/ou co-precipitação (Chapman et al., 2003). Deste modo, os sedimentos constituem um bom indicador de poluição ambiental, tanto actual, como remota. Além disso, diversos processos bióticos e abióticos podem levar à remobilização dos metais, comprometendo os sedimentos como fonte de poluição secundária.

Neste contexto, a presente dissertação abordou em primeiro lugar a contaminação do meio abiótico, analisando as concentrações totais dos metais Cr, Cu, Pb e Zn, na água e nos sedimentos da Barrinha de Esmoriz/Lagoa de Paramos.

As amostras da água, num total de 12, provenientes de 3 locais e analisadas ao longo dum ano, revelaram que o Cr não foi detectado e que os restantes metais foram quantificados predominantemente na fracção particulada, embora o Zn tenha ocorrido ocasionalmente em ambas as fracções e em concentrações significativamente maiores do que o Cu (Tabela 2.2). Por outro lado, não se registaram diferenças na concentração total dos metais na água recolhida nas 3 estações, revelando igual contributo de contaminação dos afluentes da Barrinha. As concentrações médias de metais na água excederam ocasionalmente os valores de referência sugeridos pela USEPA (1999), em situações de exposição crónica ($0.11 \text{ mg Zn}\cdot\text{L}^{-1}$, $0.012 \text{ mg Cu}\cdot\text{L}^{-1}$ e $0.0032 \text{ mg Pb}\cdot\text{L}^{-1}$).

A análise dos metais nos sedimentos mostrou que a maior contaminação ocorreu nas estações E8 e E9, e em Pb na E6, que correspondem aos locais mais próximos de afluentes ocasionais, resultantes da ocupação limítrofe da Barrinha. Apesar disso para cada metal, a comparação da média das estações ao longo da profundidade não mostra diferenças (Tabelas 2.3 e 3.2), sugerindo um padrão de deposição semelhante ao longo do tempo. Assim, a média das concentrações de metais nos sedimentos da laguna, até aos 30 cm de profundidade, foi: $241 \text{ mg Zn}\cdot\text{Kg}^{-1}$, $83 \text{ mg Cu}\cdot\text{Kg}^{-1}$, $87 \text{ mg Pb}\cdot\text{Kg}^{-1}$ e $47 \text{ mg Cr}\cdot\text{Kg}^{-1}$. Dos resultados, conclui-se que a ordem relativa de contaminação nos sedimentos e na água foi "Zn > Cu ~ Pb > Cr".

A análise granulométrica revelou sedimentos maioritariamente compostos por areia grossa (2-0.25 mm; 63%), seguida de areia fina (0.25-0.063 mm; 19%) e partículas finas que também incluem silte e argila (<0.063 mm; 18%). Não se registaram correlações positivas entre o tamanho das partículas dos sedimentos e o teor em metais, embora se tenham observado variações na percentagem de distribuição granulométrica entre as estações de amostragem. A matéria orgânica nos sedimentos foi positivamente correlacionada com o Cu, Pb e Zn, entre os 0 a 20 cm de profundidade e a maior percentagem foi encontrada na estação mais contaminada, E9, com 19%.

A segunda vertente de estudo envolveu a avaliação do impacto da contaminação por metais no biota. Actualmente, a utilização de algumas espécies de peixes como organismos bioindicadores na monitorização do impacto da poluição aquática é generalizada (Chen e Chen, 1999; Marcovecchio, 2004). Os mugilídeos têm sido usados como organismos sentinela em vários estudos de carácter ambiental porque apresentam várias características importantes num bioindicador estuarino (Ferreira et al., 2005; Ferreira et al., 2006). Com efeito, são peixes versáteis do ponto de vista alimentar, tolerantes a uma grande variedade de condições ambientais e resistentes à presença de substâncias tóxicas, naturais ou de origem antropogénica, adaptando-se a condições aquáticas muito adversas.

Actualmente, a tainha (*Liza saliens*) é a espécie dominante na laguna e a interrupção do canal de comunicação desta com o mar dificultou a migração destes peixes. A presença de um determinado metal pesado em concentrações elevadas no meio não envolve necessariamente um risco toxicológico para os peixes, em particular, na ausência de bioacumulação. Neste contexto, considerou-se relevante monitorizar a bioacumulação dos metais nas tainhas da laguna, dado que é considerado um indicador temporal e específico de contaminação e que, adicionalmente, sugere o impacto potencial na saúde do organismo (Kotze et al., 1999).

Os metais (Cr, Cu, Pb e Zn) foram pesquisados no fígado e na brânquia, como órgãos com maior potencial para a acumulação de metais, e no músculo, como exemplo de um tecido não implicado activamente na acumulação de metais.

Dos metais analisados, o Pb não foi detectado nem no músculo, nem no fígado e na brânquia o máximo quantificado foi $1.14 \text{ mg}\cdot\text{Kg}^{-1}$; o Cr não foi detectado em nenhum dos tecidos de *Liza saliens*. Na brânquia, a ordem relativa de contaminação foi semelhante à do meio, sendo de $114 \text{ mg Zn}\cdot\text{Kg}^{-1}$ ($69\text{-}191 \text{ mg}\cdot\text{Kg}^{-1}$) e $8.55 \text{ mg Cu}\cdot\text{Kg}^{-1}$ (3.21 a $15.83 \text{ mg}\cdot\text{Kg}^{-1}$). No fígado, a ordem relativa de contaminação apresentou um perfil oposto ao da brânquia, sendo de $262 \text{ mg Cu}\cdot\text{Kg}^{-1}$ (51 a $547 \text{ mg}\cdot\text{Kg}^{-1}$) e $89 \text{ mg Zn}\cdot\text{Kg}^{-1}$ (26 a $190 \text{ mg}\cdot\text{Kg}^{-1}$). Por sua vez, no músculo o teor em Cu foi inferior a $3 \text{ mg}\cdot\text{Kg}^{-1}$ e o teor em Zn foi de $26 \text{ mg}\cdot\text{Kg}^{-1}$ (Tabela 2.4 e Figura 3.2).

Os resultados mostram diferenças na bioacumulação de cada metal, nos diferentes tecidos. É conhecido que a bioacumulação é mediada por factores bióticos e abióticos, os quais podem influenciar a entrada de metais em larga extensão (Rajotte et al., 2003). Com efeito, a concentração e tempo de exposição, a actividade metabólica e também outros factores como especiação e biodisponibilidade, entre outros, afectam a entrada do metal no organismo. A bioacumulação dos metais não essenciais, como são os casos do Pb e Cr, reflecte a sua biodisponibilidade, dado que não são activamente regulados (Chattopadhyay et al., 2002). Apesar disso, nos tecidos pesquisados, os teores de Cd e Pb foram muito baixos (Pb na brânquia) ou não foram detectados.

Por outro lado, a bioacumulação dos metais nos órgãos do peixe é variável com as suas funções metabólicas, com o comportamento da espécie, com os hábitos alimentares e com a capacidade reguladora, entre outros (Chattopadhyay et al., 2002; Clearwater, 2002). Os metais essenciais são pouco acumulados, comparando com os não essenciais, pois são passíveis de regulação. No entanto, a acumulação dos metais essenciais, como Cu e Zn, foi maior do que Cr e Pb e nos tecidos a maior acumulação ocorreu na brânquia e no fígado. Estes órgãos são activos no metabolismo dos metais e por isso podem acumular níveis mais elevados do que o músculo, como o que se verificou neste estudo. No entanto, a elevada concentração de Cu observada no fígado, comparando com a

brânquia, sugere que pode haver transferência do metal da brânquia e doutros tecidos, para desintoxicação neste órgão, visto que é principalmente o fígado o responsável pela acumulação, biotransformação e excreção de poluentes nos peixes.

Estes resultados sugerem também que vários factores bióticos e abióticos medeiam a bioacumulação dos metais e que os mais preocupantes neste ecossistema são o Cu e o Zn, pelas elevadas concentrações encontradas na brânquia e no fígado.

Sendo *L. saliens* detritívora os sedimentos com elevadas concentrações de metais devem ser uma importante fonte de contaminação. Considerando a área da laguna (56 ha), a taxa de transporte sólido anual ($14000\text{m}^3/\text{ano}$) e assumindo que a deposição de sedimentos é uniforme em toda a área, conclui-se que uma deposição de 30 cm de profundidade corresponde, no mínimo, ao registo de 12 anos. Como não foram observadas diferenças na média de metais das estações ao longo da profundidade, os valores de $241\text{ mg Zn. Kg}^{-1}$ e 83 mg Cu.Kg^{-1} podem ser estimados como a concentração média nos sedimentos, a que as tainhas amostradas (6 a 13 anos) estiveram expostas.

A análise dos factores de bioacumulação (BFAs), em função da contaminação dos sedimentos, revelou que os valores extremos para o Cu foram registados no fígado, 305%, e no músculo, <3%. O Zn apresentou o valor mais elevado de BFA na brânquia, 47%, enquanto o mais baixo se registou no músculo, 11%. Os BFAs mostraram ser diferentes quando são comparados entre tecidos e entre metais. Em síntese, os valores de BFAs foram registados de acordo com a ordem seguinte: "Cu no fígado" > "Cu na brânquia" > "Cu no músculo" e "Zn na brânquia" > "Zn no fígado" > "Zn no músculo" (Tabela 3.3).

Apesar da concentração do Cu e do Zn poder ser regulada pelos peixes, em especial durante a exposição crónica, os resultados sugerem que a exposição prolongada das tainhas, nas condições da Barrinha, resulta na alteração da capacidade homeostática de regulação do Cu. O aumento dos BFAs do Cu na brânquia e no fígado em função da idade do peixe revelam que o tempo de exposição afecta a regulação deste metal em *Liza saliens* levando à bioacumulação do Cu (Figura 3.3). O fígado e a brânquia nos peixes são órgãos alvos para o Cu (Arellano et al., 1999) e a acumulação dos metais nestes tecidos varia com a taxa de entrada, acumulação e eliminação. No entanto, o facto do BFA do Cu ser mais elevado no fígado do que na brânquia, revela que é este o principal órgão envolvido no metabolismo do cobre. Além disso, a correlação positiva dos BFAs do Cu na brânquia e no fígado ($r=0.364$; $p=0.0034$ para $N=34$), apoia a hipótese de que as tainhas da laguna têm a capacidade de transferir Cu da brânquia para o fígado, onde este se vai acumulando com o tempo de exposição. Este metabolismo do Cu, envolvendo a bioacumulação do metal maioritariamente no fígado com o tempo de exposição, permite prevenir a acumulação do metal no músculo. Assim, os níveis de Cu no fígado

poderão ser um bom indicador de contaminação por este metal. Como consequência, as concentrações de Cu encontradas no fígado, ultrapassando os níveis considerados normais em peixes (Paris-Palacios et al. 2000), sugerem potenciais efeitos tóxicos neste órgão.

No que diz respeito ao Zn, o fígado do peixe parece regular a sua concentração ao longo do tempo. Na brânquia, o elevado BFA e o seu aumento com a idade indicam alteração da capacidade de regulação do Zn (Figura 3.3). No entanto, verificou-se um aumento do BFA no músculo com o aumento do BFA na brânquia, dependendo da idade do peixe ($r=0.365$; $p=0.036$ para $N=31$). Esta relação sugere que o metabolismo do Zn pode estar relacionado também com o tecido muscular, sendo o aumento do Zn na brânquia distribuído para o tecido muscular. Segundo Marcovechio e Moreno (1993) e McGeer et al. (2003), a relação entre a exposição crónica e a acumulação de metais nas espécies aquáticas mostra que usualmente o teor em Zn está bem regulado.

Marcovechio (2004) e Yilmaz (2003) registaram valores médios de Zn no músculo de mugilídeos de locais contaminados, respectivamente, de $49 \text{ mg}\cdot\text{Kg}^{-1}$ e de $51 \text{ mg}\cdot\text{Kg}^{-1}$ de peso húmido. No presente estudo, a concentração do Zn no músculo foi de $99 \text{ mg}\cdot\text{Kg}^{-1}$ de peso húmido, aproximadamente o dobro, o que indica a possibilidade de ocorrerem efeitos tóxicos.

Dado que a variação dos parâmetros de condição pode ser um indicador de efeitos tóxicos de contaminação (Mayer, et al., 1992), os valores do índice hepatossomático (IHS) e do factor de condição de Fulton (K) de tainhas da Barrinha foram comparados com tainhas recolhidas no mar, a cerca de 14 Km ao largo do local de estudo, numa zona potencialmente não poluída (Tabela 2.5). Os valores de K observados nas tainhas da Barrinha foram mais elevados, quando comparados com os obtidos nas tainhas capturadas no mar. Em geral, a presença crónica de metais conduz a uma diminuição dos valores de K (Laflamme et al., 2000; Eastwood e Couture, 2002; Rajotte et al., 2003). Factores nutricionais, como abundância de alimento na laguna, e a dificuldade de migração dos peixes que implica economia energética, podem no entanto, ter contribuído para estas diferenças. Apesar disso, um aumento do K pode ser indicativo de efeitos tóxicos (Figueiredo-Fernandes et al., 2006).

Os resultados mostraram também que existe um aumento dos valores de IHS nas tainhas da Barrinha, comparando com as de mar. Este aumento do peso relativo do fígado pode estar relacionado quer com questões nutricionais, quer com modificações metabólicas causadas pela exposição crónica aos metais. Com efeito, Stephensen et al. (2000) consideram que valores mais elevados de IHS registados em *Myoxocephalus scorpius* recolhidos em locais poluídos podem ser um indicador de um aumento da actividade das enzimas de biotransformação. Estes resultados também estão de acordo

com os obtidos em estudos efectuados em condições laboratoriais (Arnold et al., 1995; Gadagbui e Goksoyr, 1996).

A análise histopatológica de tecidos de *Liza saliens* permitiu avaliar a extensão do impacto da contaminação da laguna, ao nível celular. A brânquia dos peixes constitui o primeiro órgão de contacto com os poluentes e onde são exercidos os primeiros efeitos tóxicos (Playe et al., 1992; Perry e Laurent, 1993), pelo que as alterações da brânquia são consideradas respostas a agentes de *stress* físico e químico (Mallat, 1985; Au, 2004).

A análise microscópica, qualitativa e quantitativa, de cortes seriados da brânquia mostraram elevada incidência de lesões, como aneurismas dos capilares das lamelas, hiperplasia do epitélio filamentar, muitas vezes com fusão das lamelas, destacamento do epitélio e vasodilatação. Verificou-se que todos os peixes analisados mostraram alterações histopatológicas da brânquia. Além disso, a prevalência de três ou quatro alterações em simultâneo na amostra foi mais elevada (76%) do que a prevalência de uma ou duas alterações (24%). A prevalência para as diferentes lesões foi também elevada, sendo a vasodilatação a alteração que foi registada em maior número de peixes amostrados (85%) e os aneurismas em menor número (65%). Quando comparadas com as tainhas recolhidas no mar, as tainhas amostradas na laguna mostraram uma prevalência mais elevada quer em relação ao número de lesões, quer em relação às diferentes lesões (Figura 4.1 e Tabela 4.1).

As lesões foram caracterizadas por grau de extensão e grau de severidade, resultando para cada uma um valor de classificação global (grau de extensão x grau de severidade) que variou entre 1 e 12. Foi ainda atribuído um factor de impacto para cada lesão, quando o valor da classificação global foi ≥ 6 . À excepção do destacamento do epitélio branquial, registou-se um predomínio de lesões com baixo a moderado grau de severidade e de extensão. As lesões com maior factor de impacto e por isso consideradas mais graves, foram o destacamento, seguido da hiperplasia, devido essencialmente ao elevado grau de extensão (Tabela 4.2).

O destacamento, pela contribuição de elevados graus de extensão e severidade, aparece como a lesão que eventualmente mais concorre para alterações irreversíveis da capacidade respiratória e osmoreguladora normais da brânquia. A relação negativa desta lesão com a idade do peixe parece indicar que os peixes mais velhos não conseguem sobreviver com esta lesão, devido à falência das funções fisiológicas normais da brânquia.

As alterações histopatológicas observadas na brânquia de *L. saliens* são semelhantes às observadas noutros peixes expostos ao Cu ou Zn (Skidmore e Tovell, 1972; Arellano et al., 1999; Chen e Lin, 2001; De Boeck et al. 2001; Mazon et al., 2002). Apesar disso, estas alterações não são especificamente induzidas pela presença destes metais e a

ausência de relações positivas entre as lesões e os metais na brânquia de *L. saliens* sugerem que outros factores podem estar implicados e que a realização de mais estudos poderá ser necessária.

Os resultados do estudo das alterações da função de regulação osmótica e iónica da brânquia mostraram que as concentrações plasmáticas do potássio e do fósforo estão elevadas em *L. saliens*, quando comparadas com os níveis de tainhas mar (Tabela 5.1). Apesar disso, não foram encontradas relações directas destes electrólitos com o teor em metais neste órgão.

A exposição crónica aos metais pode ter desencadeado o aumento dos teores em potássio, dada a correlação positiva encontrada com os teores de Cu no fígado. Considerando que a concentração de Cu no fígado revelou ser um bom indicador de exposição a este metal, a relação encontrada pode sugerir que a acumulação do Cu afecta a concentração plasmática de potássio. Outros investigadores também encontraram um aumento dos níveis plasmáticos de K⁺ na exposição aguda ao Cu (Mazon et al., 2002).

De acordo com Mercaldo-Allen et al. (2004), o aumento do K⁺ plasmático pode resultar de um ajuste osmótico, como forma de compensação do declínio de outros componentes plasmáticos, ou como perda da capacidade reguladora. O aumento do K⁺ no presente estudo pode também ser devido à alteração da integridade da membrana, provocada pelas lesões registadas, tais como o destacamento epitelial, já que se registou uma correlação positiva entre o destacamento (em severidade e extensão) e os teores de potássio.

Neste estudo não se registaram alterações dos teores de sódio, ao contrário do observado por alguns autores (Grosell et al. 2003; Taylor et al. 2004). Também não se verificaram alterações assinaláveis dos teores plasmáticos de cálcio e cloro.

Quanto ao teor em fósforo, o seu aumento no plasma pode ser um mecanismo compensatório no equilíbrio osmótico (Mercaldo-Allen et al., 2004) e/ou no equilíbrio ácido-base. No entanto, a perda da integridade celular pode também ser um contributo importante, visto o aumento plasmático do fósforo estar associado à severidade da hiperplasia.

Em síntese, a exposição crónica aos metais mostra efeitos indirectos nos níveis plasmáticos dos electrólitos, revelando aumentos que traduzem uma resposta de *stress*.

Os metais podem exercer efeitos tóxicos relacionados com o *stress* oxidativo, no entanto os peixes possuem diversos mecanismos que permitem a sua desintoxicação e excreção. Nesses mecanismos incluem-se a acção de enzimas antioxidantes, como a superóxido dismutase (SOD) e a catalase, (CAT) e reacções de conjugação com

determinadas substâncias, como a glutathiona que promovem uma rápida excreção. As enzimas envolvidas na biotransformação de poluentes são encontradas na brânquia e particularmente no fígado.

Neste contexto, foram comparadas as actividades das enzimas envolvidas no *stress* oxidativo, nos dois órgãos alvo, brânquia e fígado. Este estudo mostrou um aumento da actividade da SOD branquial (10.1 U/mg proteína) e da CAT hepática (39.2 mmol/min/mg proteína). Todavia, não se registaram diferenças de actividade da glutathiona-S-transferase (GST) nos tecidos analisados (Tabela 6.3).

A indução da actividade de enzimas antioxidantes é um mecanismo comum de resposta adaptativa nos peixes, sendo variável com o tipo de tecido (Oruc et al., 2004). Dos resultados observados, pode-se concluir que a actividade da CAT foi maior no fígado, onde os teores de Cu também eram os mais elevados. Importa ainda registar que a actividade hepática da CAT das tainhas da Barrinha foi mais elevada do que a das tainhas capturadas no mar. A correlação positiva da actividade da CAT com o Cu hepático sugere que este aumento da actividade metabólica pode estar relacionado com o *stress* oxidativo devido à presença do metal. Este aumento de actividade pode ser uma resposta a um aumento da produção de H₂O₂ (Ritola et al. 2002), para proteger os sistemas biológicos contra as espécies reactivas de oxigénio (Romeo et al. 2000). Paris-Palacios et al. (2000) também referem um aumento da CAT em peixes zebra expostos a concentrações sub-letais de Cu.

Em contraste, a actividade da CAT na brânquia é mais baixa do que no fígado e do que nas brânquias das tainhas recolhidas no mar e além disso, mostra uma correlação negativa com o destacamento epitelial e com o Cu acumulado. O *stress* osmótico reduz a taxa de renovação das proteínas, podendo afectar particularmente a CAT e por consequência a sua actividade, visto que esta enzima apresenta uma taxa de renovação muito rápida (Lesser 2006). Assim, a diminuição da actividade da CAT branquial resulta provavelmente das alterações observadas na regulação osmótica que ocorrem com o destacamento do epitélio e não como uma inibição devido à presença do Cu.

Quanto à actividade da SOD, registaram-se valores mais elevados na brânquia, comparando com o fígado, e um aumento da sua actividade neste órgão com a idade do peixe. De facto, é também na brânquia que a acumulação de Cu e Zn aumenta com a idade. Assim, o aumento da actividade da SOD na brânquia pode ser considerado um indicador de uma resposta compensatória deste tecido, face à acumulação dos metais com o tempo de exposição. Oruc et al. (2004) também observaram um aumento da actividade da SOD na brânquia como resultado da exposição a poluentes. No fígado e apesar de não existir nenhuma correlação da actividade da SOD com os metais, os elevados teores de Cu poderão ser os responsáveis pelo decréscimo da actividade da

SOD. Estudos anteriores revelaram também um decréscimo da actividade hepática da SOD em *Oreochromis niloticus* exposta a poluentes (Peixoto et al. 2006). O aumento da actividade da CAT e o decréscimo da SOD no fígado, observado no presente estudo, está de acordo com os resultados verificados em trabalhos realizados em áreas poluídas, nomeadamente em *Geophagus brasiliensis* (Filho et al., 2001).

A conjugação dos metabolitos da fase I com GSH é catalisada pela GST, uma das enzimas mais estudadas nos vertebrados. Trata-se de uma enzima que está envolvida no processo de desintoxicação de moléculas estranhas e que também pode mostrar actividade peroxidase (Paris-Palacios et al. 2000; Chung et al. 2004). Este facto pode explicar a correlação negativa entre a actividade hepática da GST e a peroxidação lipídica observada no presente estudo. De um modo geral, os estudos realizados até ao presente sobre o efeito dos poluentes na actividade da GST hepática não podem ser considerados conclusivos, visto que os resultados mostraram poder existir uma indução, inibição ou ausência de efeito (Stephensen et al., 2000; Filho et al. 2001; Porte et al., 2002; Sen e Kirikbakan, 2004; Camargo e Martinez, 2006). Apesar disso na Barrinha, a actividade hepática da GST em *Liza saliens* parece semelhante à actividade hepática encontrada por Sen e Kirikbakan (2004) na mesma espécie, capturada em local de referência. Este facto leva-nos a concluir que a actividade da GST hepática nas tainhas da laguna não variou com a exposição aos metais. Sanchez et al. (2005) também não encontraram variação da actividade da GST hepática em peixes (*Gasterosteus aculeatus*) com a exposição ao Cu.

A peroxidação dos lípidos que decorre da formação de radicais, tendo como resultado malonaldeído (MDA), é considerada uma das principais manifestações de *stress* oxidativo que pode ser induzida por diversos poluentes, designadamente pelos metais (Livingstone, 2003; Lesser, 2006). Este parâmetro tem vindo a ser utilizado, com grande incidência, como biomarcador (Sayeed et al. 2003; Ozmen et al. 2004; Almroth et al. 2005; Durmaz et al. 2006).

Os resultados deste estudo revelam uma correlação positiva entre a peroxidação lipídica, medida como MDA, e o teor em Cu no fígado. A indução da peroxidação lipídica no fígado, provocada pelo cobre, foi observada anteriormente em *Chelon labrosus* por Baker et al. (1998). Estes resultados em *L. saliens* sugerem que a actividade das enzimas antioxidantes, provavelmente, não consegue prevenir a peroxidação lipídica causada pela exposição crónica aos metais.

Não foi encontrada qualquer correlação do Zn com os parâmetros do *stress* oxidativo, reflectindo talvez a regulação deste metal através de um mecanismo de adaptação que envolve os 3 tecidos, fígado, brânquia e músculo. Apesar disso, não se exclui a influência do Zn na variação da actividade das enzimas estudadas.

Desta investigação conclui-se que o peixe consegue desenvolver respostas adaptativas em tecidos específicos, aumentando as actividades quer da CAT hepática, quer da SOD branquial, como resposta ao *stress* oxidativo devido à acumulação de metais.

Na sequência destes resultados, tornou-se necessária uma análise histopatológica do fígado para averiguar a extensão da contaminação ao nível celular. A histopatologia do fígado tem sido muito utilizada como indicador de *stress* ambiental (Stentiford et al., 2003), dependendo o tipo de alteração do tempo de exposição aos poluentes (Yang e Chen, 2003; Au, 2004; Olojo et al., 2005).

A análise qualitativa e semi-quantitativa de cortes seriados do fígado, revelou lesões geralmente descritas na bibliografia centros melanomacrofágicos (MMC), como lesões não-neoplásicas (NNL), granulomas e focos de necrose (FN), cujas prevalências foram baixas a moderadas (Tabela 7.1). Adicionalmente foram observadas extensas zonas de parênquima heterogeneo (HP) compostas por focos de hepatócitos apresentando largo especto de vacuolização ou hepatócitos fracamente vacuolizados, com basofilia moderada a fortemente acentuada (Figura 7.1A e 7.1B). De um modo geral a prevalência do número de lesões foi baixa.

Dos fígados observados, 43 % não apresentaram qualquer tipo de alterações, mostrando um parênquima homogéneo, composto por hepatócitos com estrutura semelhante à descrita por outros autores em *L. saliens* controlo (D'Agati et al., 2006).

A presença dos granulomas e dos MMC, nos fígados observados no presente estudo, não foi considerada de natureza toxicopática. Com efeito, diversos estudos revelam que o fígado de peixes provenientes de locais contaminados e mesmo de locais de referência mostram, ocasionalmente, este tipo de lesões (Teh et al., 1997; Stentiford et al., 2003). Além disso, visto que os MMC se encontram envolvidos em várias funções metabólicas, para além do armazenamento de compostos estranhos, nem sempre a maior prevalência deste tipo de lesões, em peixes de locais poluídos, se pode relacionar com a contaminação (Marshall et al., 1999; Stentiford et al., 2003).

O HP foi a lesão hepática que apresentou uma maior prevalência (34%) e verificou-se que os fígados evidenciando esta lesão apresentavam maior concentração de Cu e Zn quando comparados com os fígados sem esta lesão, sugerindo que a presença deste tipo de alteração pode ter sido desencadeada pela acumulação destes metais no tecido. Este tipo de lesão, ao contrário das restantes observadas, aparece pouco descrita na bibliografia. Apesar disso, Paris-Palacios et al. (2000) encontraram focos de parênquima heterogéneo, com características similares ao deste estudo, em peixe zebra exposto ao Cu em condições controladas.

Na avaliação qualitativa da histopatologia do fígado não foram encontrados adenomas ou carcinomas hepatocelulares, permitindo concluir que o fígado de *L. saliens* não está invadido por processos malignos. Por outro lado, a moderada prevalência do número de lesões e das diferentes lesões, sugere que as funções metabólicas do fígado, como as associadas aos parâmetros do *stress* oxidativo, não se encontram comprometidas.

Comparando com a brânquia, o fígado encontra-se estruturalmente menos afectado, sendo menor a prevalência do número de lesões e das diferentes lesões. Estes resultados levam à conclusão de que as elevadas concentrações de Cu no fígado devem estar em parte sequestradas por metaloproteínas.

A relação do índice de vacuolização dos hepatócitos (IVH) com o peso relativo do fígado (IHS) mostra uma correlação positiva, sugerindo que a acumulação de gordura pode ser um contributo para o aumento do peso relativo do fígado, observado em *L. saliens*. Esta acumulação de gordura nos hepatócitos também pode ser considerada como um indicador geral de *stress* no fígado. Vários estudos mostraram que a acumulação lipídica no fígado ocorre em peixes expostos a tóxicos, incluindo Cu, Cd e Zn (Arellano et al., 1999; Van Dyk et al., 2007) e é considerado um indicador geral de toxicidade neste órgão (Visoottiviseth et al., 1999; Köhler et al., 2002; Van Dyk et al., 2007). O aumento do peso relativo do fígado pode também estar relacionado com as lesões no fígado e Gül, et al. (2004) relacionaram o elevado IHS, encontrado em peixes de zonas poluídas, com as lesões no fígado que variaram desde a infiltração lipídica à necrose celular. Foram descritas diferenças no IHS entre machos e fêmeas (Noris et al., 2000), no entanto neste trabalho não foi possível avaliar esse contributo porque das tainhas capturadas na laguna só foi viável retirar gónadas a sete peixes que se encontravam em estado atrésico.

Os parâmetros hematológicos, nomeadamente a actividade das enzimas aspartato aminotransferase (AST), alanina aminotransferase (ALT) e fosfatase alcalina (ALP) são usados no diagnóstico de danos celulares no fígado e outros órgãos, causados por diferentes poluentes em peixes (Oluah, 1999; De la Torre, et al., 2000; Bernet et al., 2001; Varanka et al., 2001; Zikic et al., 2001; Yang and Chen, 2003; Van der Oost et al., 2003), tendo mesmo sido considerados biomarcadores de poluição promissores (Palanivelu et al., 2005; Stanic et al., 2006). Nos vertebrados a AST e ALT encontram-se envolvidas na transaminação que afecta também vários processos metabólicos como a formação de aminoácidos não essenciais, produtos de excreção e gluconeogénese. Neste estudo, os níveis plasmáticos da actividade da AST foram mais elevados nas tainhas amostradas na laguna, em comparação com as de mar, não tendo sido encontradas diferenças nas actividades da ALP e ALT (Tabela 7.2). Estes resultados sugerem uma alteração no metabolismo proteico.

Alguns investigadores referem um aumento da actividade plasmática da AST em peixes recolhidos de locais poluídos (Beyer, 1996; Van der Oost et al., 1998). Igualmente, outros autores sugerem que os metais (Zn e Cu) podem estar associados a um aumento da actividade plasmática da AST (Folmar et al., 1992) e aumentos da actividade desta enzima, bem como da ALT plasmática foram observados em peixes expostos a metais, designadamente Zn, Cu e Cd, quer em condições laboratoriais (Oluah, 1999; Varanka et al., 2001; Zikic et al., 2001), quer em exposição crónica (Levesque et al., 2002). Os resultados obtidos nesta investigação apontam para a utilização da actividade da AST no plasma como um biomarcador de contaminação por metais.

Os níveis plasmáticos da glucose e da proteína total foram também mais elevados nas tainhas amostradas na laguna, comparando com as de mar, apontando para um aumento da glicemia e da proteinemia, situações que podem ser induzidas por *stress*.

Em suma, os resultados de natureza química, bioquímica hematológica e histológica, obtidos no presente estudo, são indicadores das frágeis condições ambientais da área de estudo, Barrinha de Esmoriz/Lagoa de Paramos, e indicadores de danos na população piscícola. Apesar disso, os peixes residentes, em paralelo com uma selecção natural que quase é certo tenha ocorrido, mostraram capacidade de adaptação desenvolvendo algumas estratégias que lhes permitiram sobreviver.

Por último, considera-se que o resultado dos esforços desenvolvidos para a revitalização da Barrinha de Esmoriz/Lagoa de Paramos que consistiram na entrada em funcionamento do Sistema Multimunicipal de tratamento de águas residuais e na conclusão da obra de gestão controlada da abertura da laguna ao mar, resultaram na visível melhoria da qualidade da água dos afluentes da laguna. Assim, prevê-se uma diminuição da entrada de poluentes, como os metais, para esta laguna que deve no entanto ser monitorizada, bem como a recuperação da população piscícola.

8.1. Referências

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