FUNDAÇÃO UNIVERSIDADE FEDERAL DO RIO GRANDE
PÓS-GRADUAÇÃO EM OCEANOGRAFIA BIOLÓGICA

A CORVINA *Micropogonias furnieri* (DESMAREST, 1823) COMO BIOMONITORA DA CONTAMINAÇÃO AQUÁTICA POR METAIS E DE EFEITOS BIOLÓGICOS DESTES POLUENTES NO ESTUÁRIO DA LAGOA DOS PATOS (RS)

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Apresentação

Esta tese foi apresentada na “Formatação Alternativa” conforme o Guia de Elaboração de Tese fornecido pelo Programa de Pós Graduação em Oceanografia Biológica. Neste formato, a tese pode ser apresentada de acordo com as publicações submetidas e/ou publicadas. Neste caso, o Corpo da Tese deve ser sempre redigido em português (resumo, palavras-chaves, introdução, material e métodos, conclusões, literatura citada) incluindo “abstract” e “keywords”. Os trabalhos em língua estrangeira deverão constar em anexo.

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RESUMO

Esta tese teve como objetivo avaliar a resposta da corvina *Micropogonias furnieri* coletada em diferentes áreas do estuário da Lagoa dos Patos (Rio Grande, RS) à exposição de metais através da medida da concentração tecidual (fígado e brânquias) de metais (Cu, Cd, Zn e Pb), bem como da análise de biomarcadores bioquímicos (proteínas semelhantes à metalotioneínas - PSMT, e peroxidação de lipídios - LPO no fígado e nas brânquias; genéticos (dano de DNA em hemáceas) e imunológicos (fagocitose e explosão respiratória em leucócitos). Para todos os parâmetros analisados, não foram observadas diferenças significativas entre os diferentes pontos de coleta e o sexo dos animais. Também não foram observadas correlações significativas entre os parâmetros analisados e a biometria (comprimento total) das corvinas. Porém, foram observadas diferenças sazonais significativas nas concentrações teciduais de metais e em todos os biomarcadores avaliados. A concentração dos metais foi geralmente maior no inverno e na primavera. A concentração de PSMT hepática foi maior nas estações mais frias, enquanto branquial foi maior nas estações mais quentes. No fígado, o conteúdo de LPO foi maior no inverno e na primavera, enquanto que nas brânquias o maior conteúdo de LPO ocorreu no outono e inverno. A freqüência de células micronucleadas foi maior na primavera. Tanto a atividade fagocitária quanto a explosão respiratória apresentaram seus valores mais baixos no outono e no inverno. Foram observadas correlações negativas significativas apenas entre a concentração de metais analisados nos tecidos e os biomarcadores imunológicos. As variações sazonais observadas nos outros biomarcadores não podem ser diretamente explicadas pela variação sazonal do acúmulo de metais nos tecidos, sugerindo que poluentes não analisados neste estudo podem estar envolvidos nas variações sazonais observadas nos

biomarcadores bioquímicos e genéticos. Finalmente, recomendamos o uso de juvenis de corvina *M. furnieri* como potencial bioindicador de poluição por metais traço para futuros programas de biomonitoramento no estuário da Lagoa dos Patos.

Palavras-chave: *Micropogonias furnieri*; estuário; Lagoa dos Patos; biomarcadores; bioindicador; metais.

ABSTRACT

This thesis aimed to evaluate the response of the white croaker *Micropogonias furnieri* collected at different sites of the Patos Lagoon estuary to metal exposure. Endpoints analyzed were tissue (liver and gills) metal burden (Cu, Cd, Zn and Pb) as well as biochemical (metallothionein-like proteins – MTLP and lipid peroxidation – LPO in liver and gills), genetic (DNA damage in erythrocytes) and immunological (phagocitosis and respiratory burst in leukocytes) biomarcarkers. For all endpoints analyzed, no significant differences were observed between sites of collection and fish sex. No significant correlations were observed between endpoints analyzed and the total length of croakers. However, significant seasonal variations were observed for metal tissue burden and all biomarkers analyzed. Tissue metal burdens were usually higher in croakers collected in winter and spring. Liver MTLP concentration was higher in cold seasons, while in gills the higher values were observed in hot seasons. In liver, LPO was higher in winter and spring, while in gills the higher values were observed in autumn and winter. The frequency of micronucleated erythrocytes was higher in spring. Phagocitic activity and oxidative burst in leukocytes were lower in croakers collected in autumn and winter. Significant and negative correlations were observed between metals analyzed and the immunological biomarkers. However, the seasonal variations observed in the other biomarkers analyzed seems not to be directly associated with the seasonal variations in the tissue burdens of metals analyzed. This finding suggests that other pollutants not evaluated in the present study could be involved in the seasonal responses showed by biochemical and genetic biomarkers. Finally, we recommend the use of the juvenile white croaker *M. furnieri* as a potential bioindicator of trace metal pollution for future biomonitoring programs in the Patos Lagoon estuary.

Key-words: *Micropogonias furnieri*; Patos Lagoon; estuary, biomarkers; bioindicator; metals.

1. INTRODUÇÃO

1.1. Contaminação aquática e monitoramento

Os contaminantes presentes em um ambiente podem ser de origem natural ou produzidas pelo homem. A grande maioria dos poluentes oriundos de atividades antropogênicas acaba sendo transportada direta ou indiretamente para zonas costeiras, o que leva à descarga no oceano de uma grande variedade de xenobióticos, substâncias estranhas aos organismos. Os xenobióticos podem ingressar no meio aquático através do despejo de efluentes industriais, esgoto doméstico, pluvial urbano e rural, deposição atmosférica, entre outros (Nipper, 2000). Desta forma, as regiões estuarinas e costeiras tornam-se zonas de contaminação ambiental críticas, pois recebem a descarga de uma grande variedade de xenobióticos oriundos de todos os rios que compõem sua bacia de drenagem (Corsi *et al*., 2003), além dos compostos tóxicos liberados durante operações portuárias (Stephensen *et al*., 2000). Estas diferentes fontes emissoras contribuem com diferentes tipos de poluentes, tanto orgânicos (hidrocarbonetos aromáticos policíclicos, pesticidas, bifenis policlorados, entre outros), quanto inorgânicos (metais como Hg, Cd, Pb, Zn e Cu), que atingem o ambiente estuarino sob a forma de misturas complexas capazes de provocar efeitos deletérios nos organismos que o habitam (Shailaja e D'Silva, 2003).

A poluição aquática traz sérias conseqüências tanto econômicas (redução da produção pesqueira) quanto ecológicas (diminuição da densidade e diversidade biológicas), pois os estuários são também importantes zonas de reprodução e crescimento para muitas espécies de peixes e invertebrados (Abreu e Castelo, 1998). Além disso, muitos poluentes são transferidos e acumulados ao longo das cadeias

alimentares, ameaçando também de forma indireta a saúde de seus consumidores, que podem ser tanto organismos aquáticos quanto seres humanos (Rodriguez-Ariza *et al*., 1999). Entretanto, as conseqüências em nível de ecossistema, normalmente só se fazem sentir em longo prazo. Assim, quando os efeitos se tornam visíveis, geralmente mais nenhum tipo de remediação é viável (Goksoyr, 1996). Por estas razões, tornou-se necessário o desenvolvimento de métodos de identificação, estimativa e manejo dos riscos impostos pela descarga indiscriminada de compostos químicos no ambiente aquático (Cajaraville *et al*., 2000).

Apesar dos estudos da avaliação e quantificação dos poluentes presentes no ambiente fornecerem informações importantes e fundamentais, sozinhos não permitem a avaliação do efeito tóxico provocado por estas substâncias aos organismos. Desta maneira, surgiu a necessidade de se detectar e avaliar o impacto destes poluentes nos organismos expostos. Esta necessidade originou o estudo e desenvolvimento de **biomarcadores** que detectem efeitos biológicos nos organismos.

Por definição, biomarcadores são alterações biológicas que expressam a exposição e os efeitos tóxicos dos s presentes no ambiente (Walker *et al*., 1996). Livingstone (1993) considera como biomarcadores os fluídos corpóreos, as células ou os tecidos que indicam, em termos bioquímicos ou celulares, a presença de contaminantes, bem como as respostas fisiológicas, comportamentais ou energéticas dos organismos expostos. Usualmente, as primeiras mudanças observadas devido à exposição a um xenobiótico em organismos aquáticos, são respostas bioquímicas conhecidas (Walker *et al*., 1996). Um biomarcador pode detectar a **exposição** de um organismo a um ou vários tipos de xenobióticos através de qualquer alteração biológica mensurável (biomarcadores de exposição) ou evidenciar **efeitos** tóxicos associados à exposição do organismo ao

xenobiótico (biomarcadores de efeito). Existem assim biomarcadores moleculares, celulares ou organísmicos, sendo alguns deles específicos para determinados contaminates**.** O estudo de biomarcadores tem sido recomendado pelo Conselho Internacional para a Exploração do Mar como uma metodologia complementar nos novos programas de monitoramento ambiental. Portanto, a utilização de biomarcadores em trabalhos como os de monitoramento ambiental é de suma importância, pois permite detectar precocemente a existência de contaminação por substâncias tóxicas biologicamente significativas, identificar espécies ou populações em risco de contaminação, avaliar a magnitude da contaminação e determinar o grau de severidade dos efeitos causados pelos contaminantes (Stegeman *et al*., 1992).

Em ecotoxicologia aquática, o uso de biomarcadores tem sido tradicionalmente aplicado em organismos biomonitores, os quais são selecionados a partir de uma comunidade residente em um ambiente poluído (Newman, 1998). O estudo de organismos como monitores de poluição apresenta várias vantagens quando comparadas a análises químicas de compartimentos abióticos. Organismos sempre estão presentes no ambiente e acumulam as formas biologicamente disponíveis do xenobiótico, permitindo desta forma o monitoramento contínuo de poluentes. Muitos dos artigos publicados avaliando organismos como bioindicadores de poluição têm sido concentrados em invertebrados, principalmente moluscos e crustáceos. Entretanto, o uso de peixes como indicadores para monitoramento de poluição marinha é amplamente reconhecido nos dias atuais (Bryan *et al*., 1985; Phillips e Segar, 1986, Reddy *et al*.2001, Amado *et al*.,2006a e b, Monserrat *et al*., 2007).

O uso de indicadores biológicos de poluição é eficiente quando informações básicas sobre aspectos biológicos e ecológicos dos ambientes avaliados estiverem

disponíveis. Algumas exigências são necessárias para a seleção de bioindicadores satisfatórios, entre elas estão a abundância e a facilidade de amostragem durante o ano todo, a natureza não-migratória, a identificação fácil dos organismos selecionados e a capacidade destes animais acumularem o poluente de interesse (Phillips e Segar, 1986, Markert, 2007).

Apesar de suas limitações como, por exemplo, uma mobilidade relativamente grande, muitas espécies de peixes tem atraído considerável interesse em estudos de avaliação de respostas biológicas a contaminantes ambientais. Isto se deve principalmente a características tais como a ubiqüidade no ambiente aquático e o importante papel ecológico dos peixes nas cadeias alimentares, já que estes funcionam como carreadores de energia dos menores para os maiores níveis tróficos. A compreensão dos efeitos dos contaminantes em peixes tem, portanto, um alto significado ecológico (Van der Oost *et al*., 2003).

1.2. Os metais e a biota

Entre os elementos químicos, os metais são os que constituem o maior grupo. O transporte desses elementos para o interior das células tem merecido bastante atenção a nível fisiológico, bioquímico e genético (Beveridge et al., 1997). Moléculas pequenas e sem carga provavelmente entram por difusão passiva de acordo com o gradiente de concentração. Já os íons carregados requerem energia para serem transportados. Esta pode ser fornecida na forma de ATP ou o íon pode ser co-transportado juntamente com outro íon, geralmente Na+ e H+, o qual é dirigido por um potencial trans-membrana (como as ATPases). Com relação aos requerimentos orgânicos, os metais podem ser classificados em essenciais e não essenciais. Os elementos essenciais aos seres vivos

são aqueles que têm importante papel no metabolismo dos organismos. Estes metais estão envolvidos em processos como catálise enzimática de hidrólise ou reações de oxidação e/ou redução (elementos de transição e o zinco). Estes elementos são igualmente importantes no transporte e armazenamento de moléculas menores, tais como oxigênio. Outras funções conhecidas são mecanismos de controle de reações, estabilização de estruturas e controle de pressão osmótica (cátions monovalentes). Já os metais não essenciais, não têm função biológica conhecida e são geralmente tóxicos a uma grande variedade de organismos (Roesijadi e Robinson, 1994). Deve-se, entretanto, lembrar que os metais essenciais também passam a ser tóxicos quando sua concentração está acima daquela requerida para o bom desempenho das atividades metabólicas. Assim, qualquer fator ambiental que reduza ou aumente a acumulação de um elemento essencial tem uma grande importância biológica (Di Giulio *et al*., 1995).

A obtenção, o acúmulo e a detoxificação de um metal por um organismo depende da espécie química deste elemento. Além de sua especiação, sua disponibilidade para os organismos pode ser influenciada pelas propriedades químicas e físicas da água e do sedimento, e também, pelas características fisiológicas e ecológicas dos animais, tais como a idade do indivíduo e a guilda alimentar da espécie (Di Giulio *et al*., 1995). Alguns fatores que influenciam na toxicidade dos metais em solução podem ser observados na Figura 1.

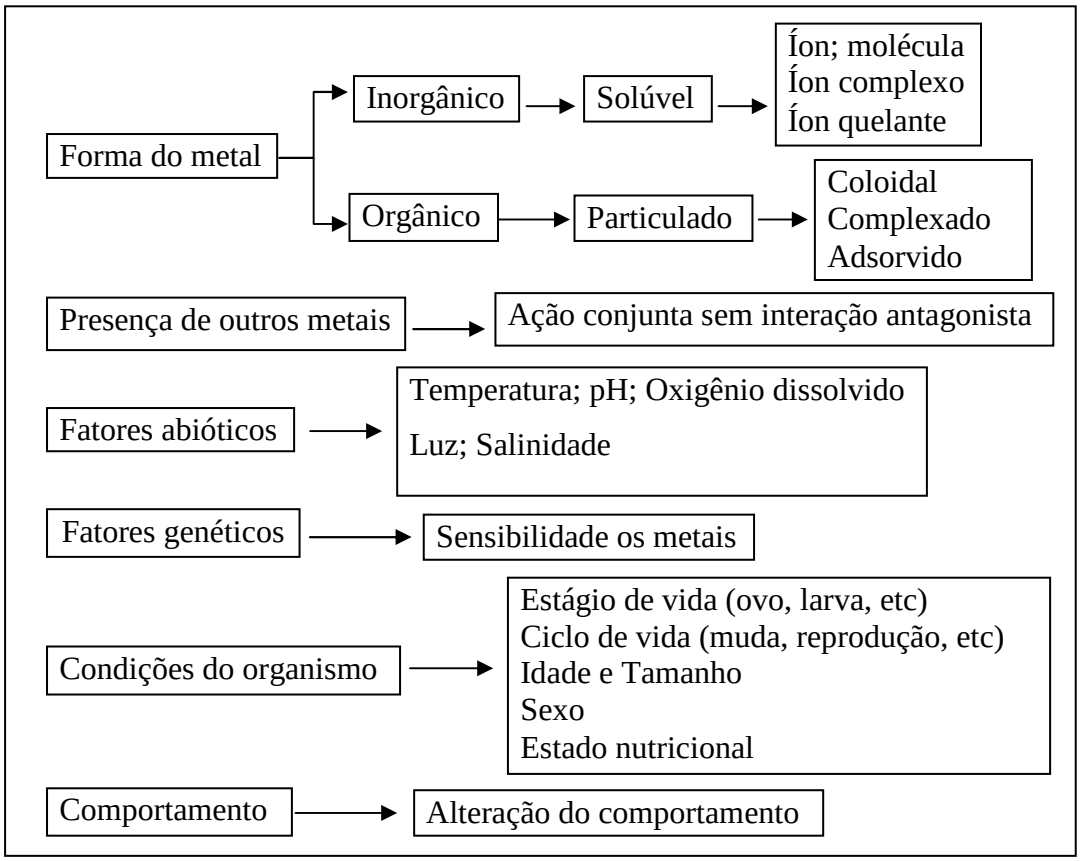

Figura 1. Fatores que influenciam a toxicidade dos metais (modificado de Förstner e Wittmann, 1983).

Em peixes, as principais vias de captação de metais são as brânquias e o intestino. Após serem absorvidos, os metais são transferidos das brânquias e intestinos ao sangue e distribuídos a outras partes do corpo. A distribuição corporal é inerente a cada metal, pois diferentes metais possuem diferentes padrões de distribuição. Alguns fatores intrínsecos dos peixes também determinam a distribuição de metais no organismo após serem assimilados, e a associação destes metais com diferentes ligantes celulares pode influenciar sua biodisponibilidade dentro da célula (Roesijadi e Robinson, 1994). Uma forma simplificada do caminho geral dos processos que resultam em acúmulo de metais nos tecidos dos organismos é apresentada na Figura 2.

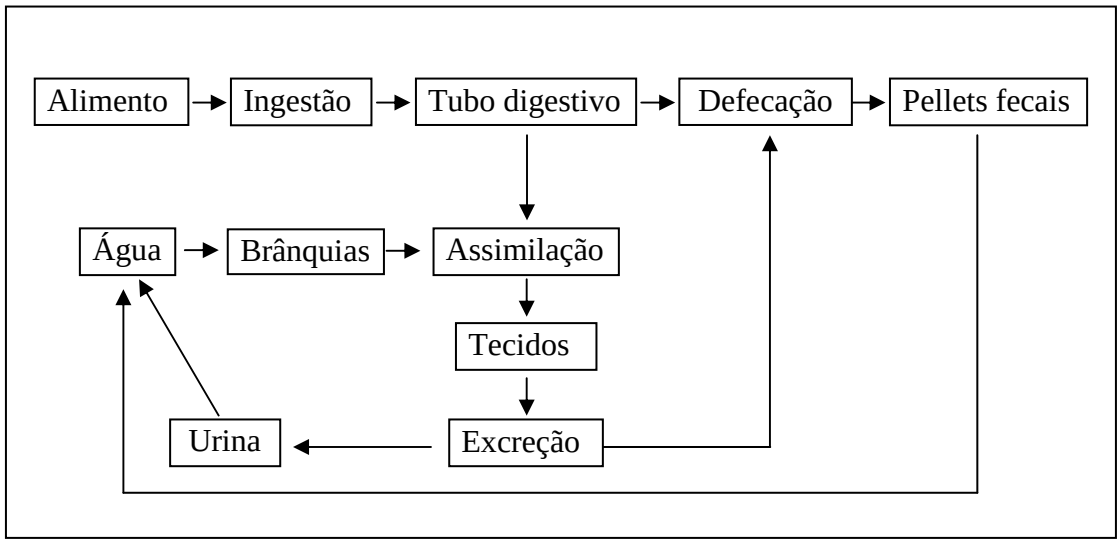

Figura 2. Fluxo geral de metais nos organismos.

A toxicidade de um xenobiótico pode ser afetada pelo metabolismo, o que, para o organismo, pode ser tanto benéfico (detoxificação) ou prejudicial (bioativação). No caso de contínua ou alta exposição, os metais são posteriormente transferidos do epitélio intestinal via sangue para outros tecidos, como fígado e rim, para armazenamento e detoxificação. Em peixes, o fígado é o principal órgão envolvido na metabolização de xenobióticos e, portanto, é o órgão alvo da grande maioria dos estudos (Viarengo *et al*., 2000). No fígado, durante a metabolização, os xenobióticos passam por duas fases de biotransformação: Fase I: alteração não sintética (oxidação, redução ou hidrólise) da molécula original que é conjugada na fase II e a seguir é eliminada. Portanto, o metabolismo é um importante determinante da atividade de um composto, da duração da atividade e da meia vida do composto no organismo. Após a assimilação, os metais de transição reagem com espécies reativas de oxigênio (EROs) formando complexos intermediários, que se decompõem formando radicais altamente reativos, que irão reagir

com moléculas orgânicas nos compartimentos celulares desencadeando danos severos ao organismo. As espécies reativas de oxigênio são átomos, íons ou moléculas que contêm oxigênio com um elétron não pareado em sua órbita externa. São caracterizadas por grande instabilidade e por isso elevada reatividade, e tendem a ligar o elétron não pareado com outros presentes em estruturas próximas de sua formação, comportando-se como receptores (oxidantes) ou como doadores (redutores) de elétrons. As espécies reativas de oxigênio são constantemente formadas no organismo humano por diversas atividades metabólicas, mas principalmente pela respiração celular.

1.3. Biomarcadores

Vários parâmetros estruturais e bioquímicos de diversas espécies de peixes têm sido testados para a avaliação da resposta a substâncias tóxicas e seu potencial uso como biomarcadores (Khangarot e Tripathi, 1991; Bols *et al*., 2001; Van der Oost *et al*., 2003).

A seguir, são listados os biomarcadores de exposição e efeito que foram utilizados na presente tese para a avaliação da resposta a contaminação por metais em corvinas *Micropogonias furnieri* coletadas no estuário da Lagoa dos Patos.

1.3.1. Biomarcadores de exposição:

a) Metalotioneínas (MTs)

As MTs constituem uma família de proteínas citosólicas de baixo peso molecular, ricas em cisteína (20-30%) e capazes de se ligar aos íons metálicos através de pontes metal-tiol. São amplamente distribuídas e já foram identificadas em todas as principais classes de vertebrados e invertebrados. As MTs atuam na regulação celular e metabólica

de metais essenciais, bem como na detoxificação destes e de outros metais não essenciais em ambientes contaminados (Klaassen *et al*., 1999). A relação positiva entre os níveis de metais no ambiente e a concentração de MTs nos tecidos dos animais, levou ao seu uso no monitoramento dos efeitos biológicos da exposição a metais, principalmente em ambientes estuarinos (Roesijadi, 1992). O mecanismo de detoxificação de metais pelas MTs ocorre via a ativação transcricional dos genes que codificam MTs.

O ataque dos radicais livres, principalmente às proteínas com grupamento sulfidril (−SH) e metionil, leva a oxidação e alteração conformacional protéica. As proteínas estruturais podem ser desnaturadas, sendo que as enzimas podem ter suas funções reduzidas ou aumentadas, acelerando, desta maneira, o dano tecidual.

1.3.2. Biomarcadores de efeito:

a) Peroxidação lipídica (LPO)

A LPO integra uma série de reações químicas envolvendo a deterioração oxidativa dos ácidos graxos poliinsaturados, que pode romper as estruturas celulares e destruí-las. A iniciação e a propagação da peroxidação lipídica são mediadas pelos radicais livres, moléculas muito reativas que têm um elétron não pareado. Esses radicais são formados, normalmente, durante o metabolismo e são neutralizados pelos antioxidantes (Oakes e Van der Kraak, 2003). A LPO ocorre na seguinte seqüência:

→O radical livre hidroxila capta um átomo de hidrogênio do ácido insaturado da membrana fosfolipídica e se transforma em água;

→O ácido graxo, atacado pelo radical livre, em presença de oxigênio transforma-se em um radical livre e dá início uma reação em cadeia que irá desestabilizar a membrana celular através da degradação dos seus constituintes;

→Um dos produtos da degradação, a lipofuscina, se acumula e torna as células lesadas. Desta maneira, a membrana perde sua flexibilidade e suas funções de barreira e informação, ocorrendo distúrbios na regulação iônica, alterações de permeabilidade e alterações entre receptor – ligante;

→O aumento da permeabilidade celular decorrente da lesão permite o influxo de cálcio na célula, ativando fosfolipases. Estas, por sua vez, continuam a lesar a célula, pois atacam a membrana lisossômica, permitindo a liberação das enzimas lisossômicas, acelerando a degradação.

Esta reação quando controlada (não excessiva) serve para renovar a membrana celular, sendo um passo essencial na biossíntese de mediadores químicos e desmonte de membranas intracelulares.

b) Micronúcleo (MN)

A formação de micronúcleos (Fig. 3) é outra conseqüência da reação de radicais livres no ambiente celular, onde ocorre a alteração dos ácidos nucléicos. Nas células, o radical peróxido reage com os íons ferro presentes nas moléculas de DNA, produzindo o radical hidroxila. Este ataca principalmente à pirimidina na ligação com a desoxirribose, rompendo a ligação açúcar-fosfato e liberando as bases livres dos nucleotídeos. Estas alterações produzem inativação ou disfunção genética, que causam alterações protéicas e até ativação de oncogenes com o desenvolvimento de neoplasias.

O teste de MN revela anomalias na formação do fuso acromático, quebras cromossômicas (clastogênese) e perda de cromossomos inteiros (aneugênese). Os MN são formados por fragmentos de cromossomos acêntricos ou por cromossomos inteiros que não foram incluídos no núcleo principal após a anáfase.

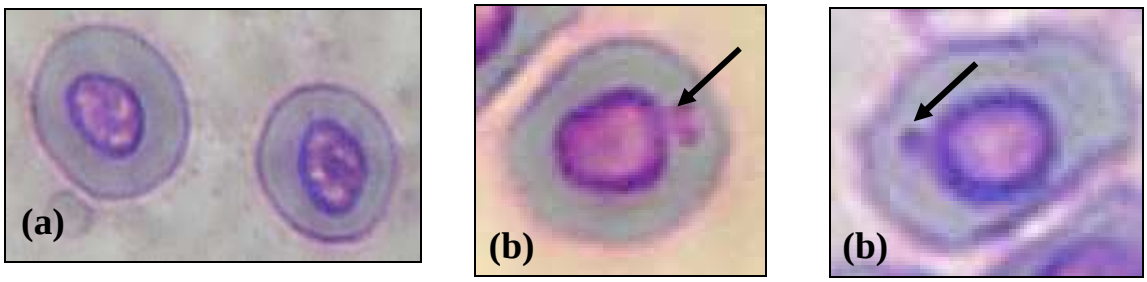

Figura 3. Fotomicrografia de hemácias normais (a); hemácia micronucleada (b), de corvinas *Micropogonias furnieri* coletadas no estuário da Lagoa dos Patos (Rio Grande, RS). Aumento 1000x. (Autor: Isabel Soares Chaves).

c) Fagocitose e Explosão respiratória

Além dos biomarcadores clássicos já descritos acima, nos últimos anos cresceu o interesse sobre o uso do sistema imune de peixes para detectar a toxicidade de poluentes ambientais (Khangarot e Tripathi, 1991; Bols *et al*., 2001). Dentre os sistemas bioquímico, celular e fisiológico de organismos monitores, o sistema imune tem algumas características atrativas únicas. Provavelmente, mais do que outros processos biológicos, o sistema imune está diretamente envolvido com a interação de uma espécie com outros organismos, o que é uma característica essencial da ecologia. Além disso, vários autores têm demonstrado efeitos imunossupressores em peixes provocados por uma grande maioria de xenobióticos (Aaltonen, 2000; Low, K. W. e Sin; Y. M., 1998; Bolls *et al*. 2001; Hoeger *et al*. 2004, Amado *et al.*, 2006).

Mudanças prejudiciais à imunidade têm o potencial para influenciar populações por afetar a susceptibilidade de indivíduos a doenças. Além disso, vários componentes do sistema imune são evolutivamente bem conservados (Ulevitch, 2000). Isto pode significar que a sensibilidade de um mecanismo imune a um determinado contaminante é similar entre diferentes espécies, possibilitando, assim, se predizer o impacto ambiental de um tóxico mais facilmente.

A imunidade não específica compreende a aço dos fagócitos. Embora estas células tenham vários papéis, a função mais freqüentemente examinada em ecotoxicologia é a **fagocitose**.

A **fagocitose** constitui um processo complexo que compreende as etapas de quimiotaxia (reconhecimento de um agente estranho ao organismo), engolfamento (início da internalização do um agente estranho ao organismo), ingestão (do um agente estranho ao organismo), desgranulação de lisossomos (com liberação de enzimas proteolíticas promovendo reações inflamatórias), morte e digestão intracelular (do agente estranho ao organismo). Entretanto, as duas etapas com maior enfoque em ecotoxicologia são a ingestão (do um agente estranho ao organismo), a qual é simplesmente chamada de fagocitose, e a digestão intracelular, denominada **explosão respiratória** ou explosão oxidativa, que compreende mecanismos microbicidas liberados no fagolisossomo com a finalidade de digerir o agente estranho ao organismo (Bols *et al*., 2001).

As células fagocitárias são geradoras de radicais livres durante os processos infecciosos e também em resposta ao estresse provocado pela ação dos contaminantes nos organismos. Os radicais livres atuam, portanto, como bactericidas e ajudam nas reações de produção de mediadores inflamatórios. A inflamação é um processo de

defesa do organismo contra agentes estranhos e/ ou patológicos (vírus, bactérias, partículas de pó) e resulta no recrutamento das células do sistema imune específico do corpo.

1.4. O estuário da Lagoa dos Patos e seus problemas ecotoxicológicos

A área estuarina da Lagoa dos Patos constitui-se no mais importante estuário do Rio Grande do Sul, e está limitada ao sul pelos molhes da barra do Rio Grande e ao norte por uma linha imaginária que une a Ilha da Feitoria à Ponta dos Lençóis (31º48'S, 52º05'W e 32º10'S, 52º15'W). A porção estuarina da Lagoa dos Patos é caracterizada por diferentes ambientes: zona de canal, zonas intermediárias, enseadas rasas protegidas, bancos, baixios, ilhas, marismas e praias arenosas (Calliari, 1998). Segundo Vieira *et al.*(1998), o gradiente de profundidade das zonas submersas propiciam vias de penetração e acesso ao ambiente estuarino, bem como áreas de proteção e alimentação abundante para o desenvolvimento e crescimento de alevinos, juvenis e sub-adultos de peixes. Em geral, após a época de reprodução, várias espécies utilizam o estuário da Lagoa dos Patos como uma área de crescimento e alimentação; e tem sido hipotetizado que a exploração de alimento abundante e o abrigo contra predadores, nas águas rasas e turvas deste estuário, são determinantes para a evolução das características migratórias apresentada por diversas espécies de peixes.

A região estuarina da Lagoa dos Patos destaca-se não só por sua importância ecológica, sendo zona de produção biológica e de biodiversidade, mas também por sua relevância sócio-econômica, já que reúne atividades portuárias, industriais, agrícolas, pesqueiras e turísticas (Asmus e Tagliani, 1998). Entretanto, o equilíbrio ecológico desta região vem sendo ameaçado pelo aumento da poluição orgânica e inorgânica

causada pelo crescimento populacional e pela expansão do pólo industrial próximo a cidade do Rio Grande (Santos *et al,*. 1997; Baumgarten *et al.*, 1998; Baumgarten e Niencheski, 2000; Niencheski *et al.* 2006). Embora existam regiões consideradas não contaminadas (Baumgarten e Niencheski, 1990, 1998, Niencheski *et al.* 2006), existem algumas regiões apresentando problemas ecotoxicológicos (Baumgarten *et al.*, 1998; Amado, 2006a e b).

Os efluentes domésticos e de indústrias de pescado contribuem com a introdução de carga orgânica e bacteriana de origem fecal, e com o aumento de outros compostos, tais como fosfatos e amônio (Almeida *et al*., 1993, Baumgarten *et al*., 1998), o que pode trazer prejuízos para a fauna aquática. As indústrias de fertilizantes também contribuem para o acréscimo de metais no ambiente estuarino através dos resíduos minerais. Segundo Baisch *et al*. (1988), os sedimentos de fundo da região sul do estuário revelaram maior contaminação por metais, sendo observados valores elevados de cádmio ao longo das áreas industriais e portuárias, e de zinco, cobre e chumbo, associados às fontes urbanas e industriais. Por sua vez, em estudo feito no litoral sul do estado do Rio de Janeiro, Souza (1986) mostrou que em áreas eutrofizadas ou contaminadas por resíduos urbanos, ocorreu um aumento da fração de metal potencialmente disponível para a bioacumulação. Baumgarten e Niencheski (1990) determinaram que as concentrações dos metais associados ao material em suspensão no estuário da Lagoa dos Patos não obedecem a um padrão de sazonalidade, mas se relacionam ao pH e a salinidade, bem como as características, concentrações e constituições do material em suspensão de cada local.

Apesar da relativa abundância de dados referentes a aspectos químicos da água e sedimento (Niencheski e Baumgarten, 2000; Barbosa, 2007; Niencheski *et al.* 2006) e

da existência de estudos enfocando a estruturação de comunidades bentônicas em ambientes impactados (Geracitano *et al.* 2004 a, b), ainda são poucos os trabalhos voltados para a avaliação dos efeitos da exposição de peixes aos diversos poluentes em menores níveis de organização biológica.

Segundo Amado *et al.* (2006a), linguados (*Paralichthys orbignyanus*) coletados em um ambiente da Lagoa dos Patos considerado poluído (Coroa do Boi) apresentaram aumento nos níveis de pró-oxidantes do tecido hepático, o que provocou danos em lipídios de membranas e no DNA. Em estudos preliminares para o Projeto RECOS: Uso e Apropriação de Recursos Costeiro (Instituto do Milênio) foi verificado que espécimes de *M. furnieri* coletados no Estuário da Lagoa dos Patos, oriundos de local apontado como contaminado (Saco da Mangueira), apresentaram depressão do sistema imunológico e dano de DNA, entre outros efeitos adversos (Amado *et al.*, 2006b). Estes resultados sugerem a utilização desta espécie como biomonitora em estudos desta natureza. Segundo estes autores, os efeitos registrados podem ser provocados pela exposição aos poluentes historicamente encontrados nos locais de coleta (Baumgarten e Niencheski, 1998, Barbosa, 2007). Estes resultados reforçam a necessidade de se conhecer os efeitos biológicos dos poluentes presentes no estuário da Lagoa dos Patos, em diferentes compartimentos deste ecossistema.

Espécie Biomonitora

A corvina *Micropogonias furnieri* (Desmarest, 1823) (Fig. 4) é uma espécie costeira de ampla distribuição e grande importância econômica nas pescarias costeiras do Brasil, Uruguai e Argentina. Esta espécie forma aglomerados distribuídos na plataforma do Rio Grande do Sul entre as isóbatas de 30-50 m de profundidade (no

inverno, ocasionalmente, próximos à isóbata de 100 m), associados a substratos arenosos e lodosos, em hábito demersal, não apresentando deslocamentos verticais (Vazzoler, 1975).

Figura 4. A corvina *Micropogonias furnieri* do estuário da Lagoa dos Patos. Autor: Isabel Soares Chaves.

As condições produtivas ideais para a corvina na plataforma sul do Brasil parecem estar associadas, assim como para outros peixes demersais presentes na região, aos deslocamentos latitudinais das águas da Convergência Subtropical, que é formada pela Corrente do Brasil que flui em direção ao sul, transportando Água Tropical (T>20 ºC, S>36), e pelo ramo costeiro da Corrente das Malvinas, rumo ao norte, que transporta Água Subantártica (T 4-15 ºC, S 33,70 – 34,15) (Garcia 1998). As outras massas que colaboram para as ricas condições do hábitat da espécie na região são os deságües de água doce da Lagoa dos Patos (média anual de 700-3000 m^3 s^{-1}, máximo no inverno/primavera) e da bacia de drenagem do Rio da Prata (média 20000–25000 m^3 s^{-1}) que atuam na formação da água costeira (Castelo *et al.* 1998; Odebrecht e Castelo, 2001).

A corvina adulta apresenta padrão migratório ao longo da costa sul do Brasil, concentrando-se no extremo sul (33º S) no verão e no extremo norte (28º S) no inverno

(Vazzoler e Santos, 1965), nitidamente acompanhando os deslocamentos da Convergência Subtropical.

A desova de *M. furnieri*, do tipo parcelada (Vazzoler, 1970) acontece na região da barra do Rio Grande durante a primavera-verão (Vazzoler, 1971). A maior concentração de ovos e larvas de cianídeos foi encontrada bem próximo à abertura da Lagoa dos Patos (Ibagy e Sinque, 1995). Weiss (1981) deduz, pela grande densidade de ovos de corvina no estuário da Lagoa dos Patos, que os adultos desovantes se encontram em águas muito próximas ou até mesmo no canal de acesso.

A corvina que desova na costa do Rio Grande do Sul pode pertencer a uma população limitada entre 29º S e 33 º S separada por critérios de abundância relativa genéticos (Vazzoler, 1971; Isaac, 1988; Vazzoler, 1991), ou outra de maior distribuição entre 23º S e 33 º S, com alta diversidade genética, molecular e heterogeneidade haplóica (Puchnick, 2001). Levy *et al.* (1998) não rejeitam a hipótese de única população panmítica, sugerindo que a política de manejo da corvina a defina assim, mas que considere os diferentes parâmetros da dinâmica populacional.

Haimovici e Umpierre (1996) identificam que a corvina capturada na costa do Rio Grande do Sul pertence a dois grupos populacionais, um que desova em frente à Lagoa dos Patos e outro na proximidade do Rio da Prata no Uruguai.

No Estuário da Lagoa dos Patos, *M. furnieri* é a espécie de peixe mais abundante na zona estuarina (Chao *et al.*, 1985), a mais abundante na pesca artesanal (Reis e Pawson, 1999) e apresenta uma alta comercialização. Com base em seu ciclo de vida e quanto ao uso do estuário, *M. furnieri* foi classificada como estuarino dependente no Estuário da Lagoa dos Patos (Vieira *et al.*, 1998). Esta espécie utiliza, ao longo do ano, a totalidade do ambiente estuarino, mostrando sua ampla adaptabilidade às diferenças de

salinidade que ocorrem no ambiente. Além disso, apresenta diferenças na preferência por determinados ambientes (zonas rasas/águas profundas) segundo o hábito trófico dos indivíduos, o qual está relacionado com a ontogenia da espécie e o tamanho dos indivíduos em suas classes de tamanho (Castelo, 1986). Os juvenis do ano vivem nas águas rasas do estuário, mas após alcançar tamanhos maiores (>15 cm), estes indivíduos podem mover-se para águas mais profundas do canal da lagoa, de onde podem seguir para cima em direção a água doce da lagoa (Vieira e Castello, 1996). Tem sido hipotetizado que parte desta população permanece nos lugares de água doce até os adultos maduros começarem a migrar para fora da lagoa para se reproduzir em águas marinhas. Os dados de Garcia *et al.* (2007), analisando isótopos estáveis de C e N, em exemplares coletados na zona estuarina e em regiões de água doce da Lagoa dos Patos, fornecem evidência indiretas de que indivíduos menores (<15 cm) permanecem no estuário da Lagoa dos Patos sem movimentarem-se para regiões profundas ou de água doce, e que indivíduos maiores (>15 cm), após moverem-se para as regiões de água doce, permanecem nestes locais por períodos prolongados.

No Brasil, a desova de *M. furnieri* ocorre na primavera e no verão em águas costeiras sob o aporte de água doce da Lagoa dos Patos (Vazzoler, 1991), e seus ovos são distribuídos em todo o estuário da Lagoa dos Patos, que serve como área de alimentação e crescimento. As larvas são planctófagas, e os juvenis e os adultos são consumidores bentônicos, em fundo de lama e areia, com preferências por pequenos invertebrados, principalmente poliquetas e crustáceos, e em menor proporção, por moluscos e pequenos peixes, conferindo a esta espécie grande importância na cadeia trófica do estuário da Lagoa dos Patos (Vieira *et al.*, 1998). Segundo Figueiredo e Vieira (2005), a corvina parece ter seu padrão de alimentação regulado pelo

fotoperíodo, alimentando-se mais intensivamente durante o dia. Entretanto, no estuário da Lagoa dos Patos a alta turbidez da água parece prevenir os peixes de responder aoo fotoperíodo. Portanto, durante os períodos em que a transparência da água é alta, as corvinas podem se alimentar mais intensivamente e ter alta taxa de consumo alimentar.

A corvina *M. furnieri* foi utilizada anteriormente como espécie sentinela em trabalho de monitoramento ambiental realizado pelo RECOS: Uso e Apropriação de Recursos Costeiro (Instituto do Milênio), e foi também recomendada como indicador biológico em programas de monitoramento no estuário do Rio da Prata, Argentina (Marcovecchio, 2004).

1.6. OBJETIVOS

Objetivo geral:

O objetivo geral deste trabalho foi avaliar a resposta da corvina *Micropogonias furnieri* do estuário da Lagoa dos Patos (Rio Grande, RS) à exposição a metais, através da análise de biomarcadores de efeito e de exposição.

Objetivos específicos:

1. Avaliar a concentração de Cu, Cd, Zn e Pb no fígado e brânquias de *M. furnieri* coletados sazonalmente em cinco diferentes pontos do estuário da Lagoa dos Patos;

2. Avaliar a resposta sazonal de biomarcadores bioquímicos de exposição relacionados com o processo de detoxificação de metais, através da quantificação de proteínas semelhantes à metalotioneínas (PSMT) no fígado e brânquias de *M. furnieri* provenientes de cinco diferentes pontos do estuário da Lagoa dos Patos;

3. Avaliar a resposta sazonal de biomarcadores bioquímicos de efeito, através da análise da peroxidação lipídica no fígado e brânquias de *M. furnieri* provenientes de cinco diferentes pontos do estuário da Lagoa dos Patos;

4. Avaliar a resposta sazonal de biomarcadores genéticos de efeito, através do teste de micronúcleo (dano de DNA) em hemáceas de *M. furnieri* provenientes de cinco diferentes pontos do estuário da Lagoa dos Patos;

5. Avaliar a resposta sazonal de biomarcadores imunológicos de efeito, através da atividade fagocitária e de explosão respiratória em leucócitos de *M. furnieri* provenientes de cinco diferentes pontos do estuário da Lagoa dos Patos;

6. Determinar as possíveis correlações entre as concentrações branquiais e hepáticas dos metais (Cu, Cd, Zn e Pb) e os biomarcadores analisados.

Paralelamente ao desenvolvimento desta tese, tendo em vista a relevância do assunto abordado, foi desenvolvido um estudo apresentado em forma de revisão científica enfocando o uso de biomarcadores de poluição em animais estuarinos. Como resultado, foi gerado o artigo de revisão intitulado “Pollution biomarkers in estuarine animals: Critical review and new perspectives”, sendo que a autora desta tese foi responsável por parte da redação do item 5. “The employment of new biomarkers: genotoxic and immune responses” (Anexo I).

2. MATERIAL E MÉTODOS: aspectos gerais

2.1. Coleta e amostragem:

Exemplares da corvina *Micropogonias furnieri* foram capturados em 4 diferentes pontos do estuário da Lagoa dos Patos (Fig. 5) historicamente caracterizados por

diferentes padrões de contaminação (Baumgarten e Niencheski, 1990, 1998) e um ponto considerado como não contaminado em estudos anteriores (Amado *et al.* 2006).

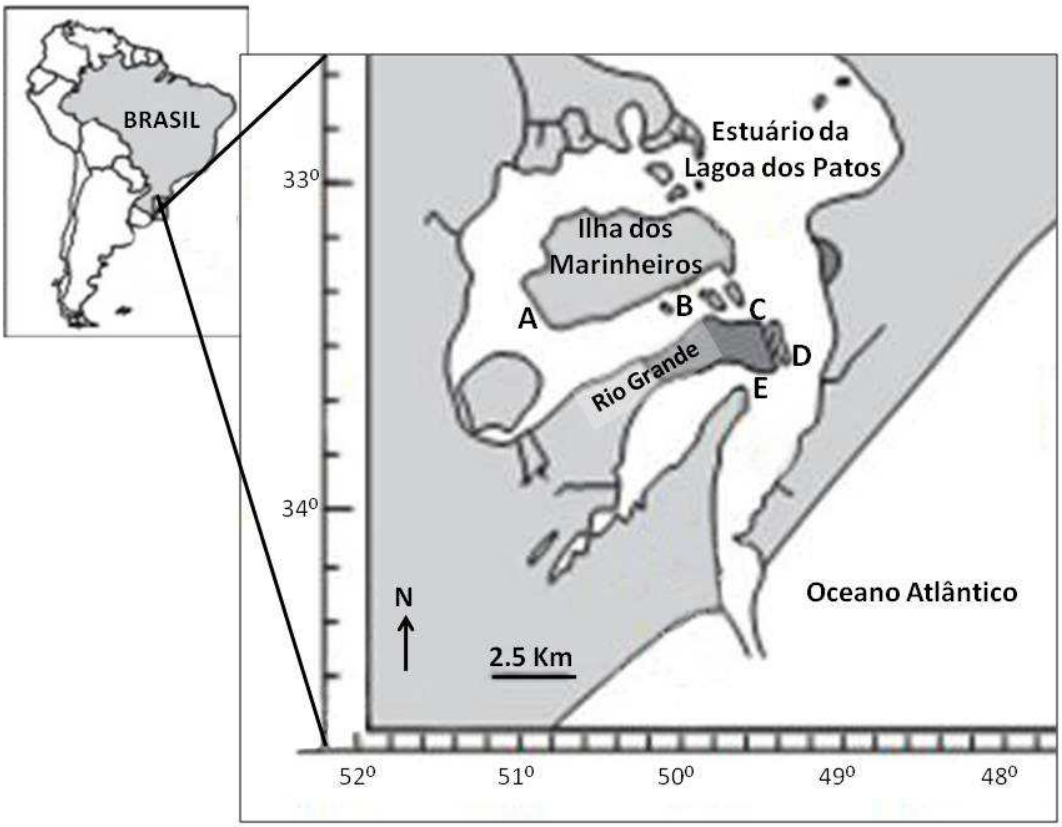

Figura 5. Locais de coleta de corvinas *Micropogonias furnieri* (Teleostei:Scianidae) no estuário da Lagoa dos Patos (Sul do Brasil). (A) Ilha dos Marinheiros; (B) Saco do Rio Grande; (C) Porto Velho; (D) Porto Novo; (E) Coroa do Boi.

Um mínimo de 5 corvinas (8-15 cm) foi obtido em cada ponto de coleta e estação do ano. O comprimento das corvinas coletadas nas diferentes estações do ano foi fixado em no máximo 15 cm, para não haver interferência de peixes que por ventura já tenham migrado para a costa e voltado ao estuário, ou migrado para zonas profundas e áreas de água doce do estuário (Grarcia *et al.* 2007). A coleta dos peixes foi realizada

sazonalmente, através de arrastos de fundo com duração de 5 minutos em águas rasas (~2m). Apesar da relativa proximidade entre os locais de coleta, nós assumimos que nenhuma migração de corvinas aconteceu, e que nossas amostras são representativas de *M. furnieri* em áreas rasas do estuário, e trabalhos anteriores suportam esta posição (Figueiredo e Vieira, 2005; Grarcia *et al.* 2007).

Após a captura, as corvinas foram rapidamente anestesiadas com benzocaína (200 ppm), e coletado o maior volume possível de sangue por punção dos vasos caudais. O sangue foi extraído usando-se seringas heparinizadas para as análises de dano de DNA (teste de micronúcleo em hemáceas) e imunológicas (ensaios de fagocitose e explosão respiratória em leucócitos). Em cada local de coleta foram registradas temperatura e salinidade da água, no momento da coleta. Após a coleta de sangue, os animais foram mantidos sob gelo e encaminhados para o Laboratório de Zoofisiologia do Departamento de Ciências Fisiológicas (DCF) da Universidade Federal do Rio Grande (FURG), para avaliação biométrica (peso, comprimento padrão e comprimento total), identificação do sexo (quando possível) e coleta dos órgãos (brânquias e fígado) para as demais análises de biomarcadores. Cada amostra de tecido foi colocada em um tubo plástico do tipo Eppendorf, devidamente identificado. As amostras de fígado e brânquias foram divididas em 3 sub-amostras para análise de LPO, PSMT, e concentração de metais. Para as amostras destinadas às análises de metais, os tubos foram previamente lavados em HNO_3 (1%). As amostras sanguíneas utilizadas para os ensaios de fagocitose, explosão respiratória e teste de micronúcleo foram processadas no prazo máximo de 15 h após a coleta.

2.2. Determinação da concentração de metais

Foi utilizado um mínimo de 5 peixes por local de coleta para a análise de metais nos tecidos. Alíquotas de fígado e brânquias, de cada peixe, foram pesadas em tubos plásticostipo eppendorf previamente lavados com HNO_3 1% (72 horas), e o peso úmido foi registrado. Após as amostras serem secas por 48 h (80 ºC em estufa), o peso seco foi calculado. Posteriormente ao resfriamento dos tubos, os tecidos foram digeridos em HNO_3 (Suprapur) por 48 h e então diluídas em água MilliQ. Foram determinadas as concentrações de cobre, cádmio, zinco e chumbo em espectrofotometria de absorção atômica (GBC - AAS 932 Plus). Para cada metal analisado, foram utilizadas as soluções padrões em diferentes concentrações, preparadas a partir de uma solução estoque de referência (CELM; São Paulo, Brasil) calibradas a partir de padrão internacional (NIST, EUA). Os dados de metais foram expressos em μg/g de peso seco do tecido

2.3. Análises dos Biomarcadores:

2.3.1. Peroxidação de Lipídeos (LPO)

A LPO foi determinada empregando-se o método de TBARS descrito por Oakes and Van der Kraak (2003). Alíquotas do tecido branquial ou hepático foram homogeneizadas em solução tampão contendo 1,15% KCl e 35 mM de hidroxitolueno butilado (BHT). Uma alíquota do homogeneizado foi adicionada à mistura de reação contendo 12,4 mM de dodecil sulfato de sódio (SDS), 0,8% de ácido tiobarbitúrico (TBA), 20% de ácido acético ajustado a pH 3,5 com NaOH, e água bidestilada. Uma alíquota adicional de 67 mM de BHT (em etanol) foi adicionada antes de aquecer a mistura em banho-maria à 95ºC por 60 min. Após as amostras resfriarem, foi adicionada água deionizada e n-butanol. Após centrifugação, a camada orgânica não miscível foi removida e a fluorescência medida por espectrofotometria (Perkin-Elmer; excitação =

515 nm; emissão = 553 nm). Como padrão externo foi utilizada uma solução 1,1,3,3-tetramethoxypropane (TMP). A LPO foi então expressa como nmol TMP/mg de peso úmido do tecido.

2.3.2. Proteínas Semelhantes à Metalotioneínas (PSMT)

A concentração de PSMT foi determinada empregando-se o método descrito por Viarengo *et al.*, (1997). Alíquotas do tecido branquial ou hepático foram homogeneizadas em solução tampão resfriada (pH ajustado a 8,60), preparado com sacarose (500 mM), tris-HCL (20mM), PMSF (100 mM) e β-mercaptoetanol (0,01%), na proporção 1:5 (peso:volume). O homogeneizado foi centrifugado a 30.000 *x* g durante 45 min. Foram transferidos 250 μL do sobrenadante para outro tubo e adicionados 265 μL de etanol absoluto (resfriado a –20ºC) e 20 μL de clorofórmio. A suspensão resultante foi homogeneizada e centrifugada a 6.000 *x* g durante 10 min a 4ºC. Uma alíquota de 300 μL do sobrenadante foi transferida para outro tubo e foram adicionados 20 μL de HCl puro (37%) e 900 μL de etanol puro resfriado. A solução resultante foi mais uma vez homogeneizada e as amostras mantidas a –20ºC por 1 h. Após mais uma centrifugação a 6.000 *x* g durante 10 min a 4ºC, o sobrenadante foi descartado e o precipitado obtido homogeneizado com 1 mL de etanol 87% e clorofórmio 1% diluídos em tampão Tris-HCl (20 mM). A seguir, a solução resultante foi centrifugada a 6.000 *x* g durante 10 min a 4ºC. O sobrenadante foi novamente descartado, o novo precipitado foi homogeneizado em 150 μL de NaCl (250 mM) e adicionados 150 μL de solução EDTA (4 mM)-HCl (1N). A seguir, 100 μL de cada amostra (em duplicata) foram adicionados a 1,4 mL de solução de 5,5-ditio-bis (2-ácido nitrobenzóico) (DTNB) preparada em tampão (pH 8,0) contendo $Na_2HPO_4.7H_2O$ (200

mM); NaCl (2 M) e DTNB (0,43 mM). Após homogeinização, 350 μL desta solução foram transferidos, em duplicata, para os poços de uma microplaca. O conteúdo de PSMT foi estimado espectrofotometricamente por absorbância (412 nm) usando-se DTNB (0,43 mM). Diferentes concentrações de glutationa (GSH) (0 a 500 μM) foram empregadas como padrões. A concentração de PSMT foi expressa em termos de equivalentes de GSH (μmol GSH/g de tecido úmido).

2.3.3. Teste de Micronúcleo (MN)

A análise de micronúcleo foi realizada empregando-se o método descrito por Hooftman e de Raat (1982). Com o sangue heparinizado coletado, foram feitos 2 esfregaços em lâminas de vidro por peixe coletado. Após secar a temperatura ambiente, os esfregaços foram fixados em metanol por 10 minutos e deixados secar em temperatura ambiente por 24 h. As lâminas foram coradas por 20 min com Giemsa 5% diluída em tampão fosfato (KH_2PO_4 0,06 M e Na_2HPO 0,06 M; pH 6,8) e lavadas com água destilada. A análise foi feita em microscópio óptico sob aumento de 1000x (lente de imersão). Em cada lâmina foram analisadas 1000 células, registrando-se o número de células com MN e células normais. Os resultados foram expressos em freqüência relativa de células micronucleadas.

2.3.4. Ensaios imunológicos:

Os ensaios da atividade fagocitária e explosão respiratória foram realizados com leucócitos extraídos do sangue periférico de *M. furnieri.* Para a obtenção dos leucócitos, as hemácias foram lisadas com solução de NH_4Cl. Após a lise das hemácias, os leucócitos foram suspendidos em HBSS suplementado com RPMI 1640, heparina

sódica e soro fetal bovino inativado, e então contados em câmara de Neubauer. A viabilidade celular foi determinada pelo teste de exclusão de Azul de Trypan, e foi sempre maior que 90%.

O ensaio da atividade fagocitária foi adaptado de Chilmonczyk e Monge (1999). Resumidamente, uma alíquota de 10^7 células foi incubada por 1 hora em uma microplaca (placa branca de 96 poços de fundo chato) para permitir a adesão dos leucócitos. Após o período de aderência, o sobrenadante foi removido e solução HBSS adicionada. As células aderidas foram então incubadas com bactérias opsonizadas *Escherichia coli* marcadas com fluoresceína por 1 h a 22º C, para proceder à fagocitose. Após o período de fagocitose, as poças foram imediatamente lavadas com solução Azul de Trypan para apagar a fluorescência das bactérias que não foram fagocitadas, e imediatamente após, as células foram lavadas com solução HBSS. A fluorescência foi lida espectrofluorometricamente (excitação = 485 nm; emissão =530 nm). Os resultados foram expressos em unidades arbitrárias de fluorescência.

O ensaio de explosão respiratória foi adaptado de Brubacher e Bols (2001). Uma alíquota de 10^7 células foi incubada por 1 h em uma microplaca (placa branca de 96 poços de fundo chato) para permitir a adesão dos leucócitos. Após o período de aderência, o sobrenadante foi removido e solução HBSS adicionada. As células foram então incubadas com 10 μM de diacetato de diclorodihidrofluoresceína (H_2DCFDA) e 30 nM forbol 12-miristato-13-acetato (PMA) como agente estimulador da explosão respiratória. A fluorescência foi lida espectrofluorometricamente (excitação = 485nm; emissão = 530nm). Os resultados foram expressos em unidades arbitrárias de fluorescência.

2.4. Análise estatística dos dados:

Os dados foram expressos como média ± erro padrão. Diferenças sazonais e entre os pontos de coleta foram avaliadas por análise de variância (ANOVA), seguida pelo teste de Tukey (α=0,05). Os pressupostos da ANOVA (homogeneidade de variância e normalidade) foram previamente testados (Zar, 1984). Tendo em vista que tanto para machos quanto para fêmeas não foram observadas variações significativas nas concentrações de metais entre as corvinas coletadas nos diferentes pontos da Lagoa dos Patos, todos os dados foram agrupados por estação de coleta para cada sexo. As possíveis relações entre as concentrações dos metais nos tecidos e os biomarcadores analisados foram avaliadas pelo índice de correlação de Pearson (α = 0,05).

3. SÍNTESE DOS RESULTADOS:

3.1. Características ambientais e parâmetros morfométricos.

Os períodos em que foram realizadas as coletas, bem como as características químicas da água (temperatura e salinidade) medidas nos locais de coleta são apresentados na Tabela 1. Os mais altos valores de salinidade e temperatura forma registrados no verão, e os mais baixos no inverno.

Tabela 1. Parâmetros registrados durante o período amostral (2006-2007) nos locais de coleta no estuário da Lagoa dos Patos (Rio Grande, RS). Os dados são expressos como média ± erro padrão (n: número de medidas).

Estação	Temperatura (ºC)	Salinidade	*n*
Verão (02 e 12/2007)	24,25 ± 0,37	15,75 ± 3,27	8
Outono (03-05/2007)	20,00 ± 1,03	6,00 ± 1,34	5
Inverno (06-08/2007)	16,33 ± 1,48	1,92 ± 0,27	6
Primavera (09-11/2007)	23,80 ± 0,58	7,00 ± 3,30	5

Os parâmetros morfométricos (tamanho e peso) dos peixes amostrados são listados em função do sexo na Tabela 2. Os machos coletados no verão apresentaram os maiores pesos corpóreos, enquanto que na primavera, as fêmeas apresentaram-se com maior peso. Como o tamanho dos peixes foi padronizado, acredita-se que as diferenças nos valores de pesos médios entre os sexos possam estar relacionadas aos fatores de turbidez da água, como os citados por Figueiredo e Vieira (2005), ou com o aumento do metabolismo dos peixes nestas estações, o que levaria a um aumento na dieta. Levando-se em consideração que os espécimes amostrados apresentavam-se em fase juvenil, os dados coletados foram analisados agrupando-se os dados de machos e fêmeas. Os dados biométricos totais dos peixes amostrados são apresentados na Tabela 3.

Tabela 2. Dados morfométricos de corvinas (*Micropogonias furnieri*) em machos e fêmeas coletados no estuário da Lagoa dos Patos (Rio Grande, RS). Os dados são expressos como media ± erro padrão. Letras diferentes indicam diferenças significativas ($P < 0,05$) entre os valores médios em peixes do mesmo sexo, nas diferentes estações do ano (n: número de animais coletados).

Estação	Sexo	Comprimento (cm)	Peso (g)	*n*
Verão	Macho	11,73 ± 0,43 a	38,64 ± 3,91 b	31
	Fêmea	12,31 ± 0,32 a	34,67 ± 2,16 ab	43
Outono	Macho	11,10 ± 0,36 a	26,92 ± 2,40 ab	39
	Fêmea	11,63 ± 0,44 a	30,03 ± 3,91 ab	21
Inverno	Macho	11,01 ± 0,27 a	24,87 ± 1,67 ab	31
	Fêmea	10,68 ± 0,30 a	23,38 ± 1,50 a	37
Primavera	Macho	12,06 ± 0,37 a	24,00 ± 0,74 a	31
	Fêmea	12,43 ± 0,31 a	34,67 ± 2,16 b	44

Tabela 3. Dados morfométricos de juvenis de corvinas (*Micropogonias furnieri*) coletadas em diferentes estações do ano no estuário da Lagoa dos Patos (Rio Grande, RS). Os dados são expressos como media ± erro padrão. Letras diferentes indicam diferenças significativas (*P < 0,05*) entre os valores médios nas diferentes estações do ano (n: número de animais coletados).

Estação	Comprimento (cm)	Peso (g)	*n*
Verão	12,07 ± 0,26	36,33 ± 2,06 b	74
Outono	11,11 ± 0,28	27,04 ± 2,13 ab	60
Inverno	10,83 ± 0,20	24,06 ± 1,11 a	68
Primavera	12,28 ± 0,44	30,08 ± 1,58 b	75

3.2. Acumulação de metais

As concentrações hepáticas e branquiais de Cu, Cd, Zn e Pb não apresentaram diferenças significativas (*P<0,05*) entre machos e fêmeas em cada estação do ano (Tabela 4), mas os dados agrupados mostraram uma importante e significativa variação sazonal (Tabela 5).

Tabela 4. Concentração de metais traço em fígado e brânquias de corvinas *Micropogonias furnieri* do estuário da Lagoa dos Patos (Rio Grande, RS). A concentração de metais está expressa em μg/g de peso seco do tecido. Os dados são expressos como média ± erro padrão (n: número de animais amostrados). Não foram observadas diferenças significativas entre machos e fêmeas em cada estação do ano.

Fígado

Estação	Sexo	Cu	Cd	Zn	Pb	*n*
Verão	Macho	28.52 ± 4.51	0.69 ± 0.29	76.51 ± 5.13	16.60 ± 3.49	12
	Fêmea	31.17 ± 6.07	0.44 ± 0.15	83.64 ± 9.54	19.61 ± 3.65	17
Outono	Macho	50.58 ± 8.53	0.53 ± 0.11	114.29 ± 9.19	18.51 ± 2.35	17
	Fêmea	40.27 ± 8.96	0.28 ± 0.05	102.91 ± 10.77	21.15 ± 3.39	16
Inverno	Macho	107.41 ± 34.01	0.39 ± 0.14	86.48 ± 15.11	17.30 ± 3.99	09
	Fêmea	121.37 ± 20.64	0.60 ± 0.21	101.32 ± 10.71	21.57 ± 3.26	16
Primavera	Macho	82.90 ± 15.25	1.99 ± 0.33	134.82 ± 12.16	23.19 ± 4.14	13
	Fêmea	83.22 ± 13.13	3.07 ± 0.56	131.15 ± 11.55	19.31 ± 3.83	18

Brânquias

Estação	Sexo	Cu	Cd	Zn	Pb	*n*
Verão	Macho	3.16 ± 0.41	0.41 ± 0.04	57.75 ± 5.96	20.06 ± 2.22	12
	Fêmea	3.89 ± 0.38	0.45 ± 0.03	57.24 ± 3.44	21.39 ± 1.78	17
Outono	Macho	4.35 ± 0.27	0.57 ± 0.04	71.35 ± 6.93	20.04 ± 1.81	17
	Fêmea	4.12 ± 0.28	0.62 ± 0.07	69.49± 3.65	19.28 ± 2.98	16
Inverno	Macho	4.92 ± 0.34	0.34 ± 0.05	58.76 ± 3.89	23.90 ± 2.17	09
	Fêmea	5.39 ± 0.52	0.47 ± 0.04	63.70 ± 6.59	27.44 ± 2.58	16
Primavera	Macho	3.73 ± 0.30	0.94 ± 0.08	73.90 ± 4.40	24.58 ± 0.92	13
	Fêmea	2.82 ± 0.28	0.82 ± 0.04	57.58 ± 3.10	21.80 ± 3.51	18

3.3. Biomarcadores

Assim como concentração dos metais nos tecidos, os biomarcadores analisados nas corvinas mostraram importante variação sazonal, como é mostrado a seguir:

3.3.1. Concentração de Proteínas Semelhantes à Metalotioneínas (PSMT)

A concentração de PSMT no fígado e brânquias de corvinas apresentou variações sazonais significativas (*P<0,05*). No fígado, a menor média (± erro padrão) foi de 1,46 ± 0,07 (primavera) e a maior de 3,16 ± 0,17 (outono) μmol GSH/g de peso úmido (Fig. 6A). Nas brânquias, a menor média foi de 0,21 ± 0,11 (inverno) e a maior de 0,80 ± 0,13 (verão) μmol GSH/g de peso úmido (Fig. 6B). Quando o conjunto total dos dados foi analisado, não foram observadas correlações significativas entre as concentrações dos metais e a concentração de PSMT, tanto no fígado quanto nas brânquias.

Tabela 5. Variação sazonal da concentração de metais traço em fígado e brânquias de corvinas *Micropogonias furnieri* do estuário da Lagoa dos Patos (Rio Grande, RS). As concentrações de metais são expressas em μg/g de peso seco do tecido. Os dados são expressos como média ± erro padrão. Letras diferentes indicam diferenças significativas (*P < 0,05*) entre os valores médios nas diferentes estações do ano. n: número de animais amostrados.

	Fígado				
Estação	Cu	Cd	Zn	Pb	*n*
Verão	29,85 ± 5,29 a	0,57 ± 0,22 a	80,08 ±9,56 a	18,10 ± 3,56	29
Outono	45,42 ± 8,74 b	0,41 ± 0,10 a	108,60 ± 9,97 bc	19,83 ± 2,87	33
Inverno	114,39 ± 27,32 c	0,49 ± 0,17 a	93,90 ± 12,91 ab	19,44 ±3,62	25
Primavera	83,06 ± 14,19 c	2,53 ± 0,22 b	132,98 ± 11,85 c	21,25 ± 3,98	31
	Brânquias				
Estação	Cu	Cd	Zn	Pb	*n*
Verão	3,52 ± 0,39 a	0,43 ± 0,03 a	57,49 ± 4,70 a	20,72 ± 2,00 a	29
Outono	4,23 ± 0,27 b	0,59 ± 0,05 b	70,42 ± 2,28 b	19,66 ± 2,39 a	33
Inverno	5,16 ± 0,43 c	0,41 ± 0,04 a	61,23 ± 5,24 ab	25,67 ± 2,37 b	25
Primavera	3,27 ± 0,29 a	0,88 ± 0,06 c	65,74 ± 3,75 b	23,19 ± 2,21 ab	31

3.3.2. Conteúdo de lipídeos peroxidados (LPO)

Variações sazonais significativas na LPO foram observadas (*P<0,05*) em fígado e brânquias das corvinas amostradas. No fígado, a menor média foi de 0,52 ± 0,06 (verão) e a maior de 1,29 ± 0,12 (primavera) nmol TMP/mg de peso úmido (Fig. 7A). Nas brânquias, a menor média foi de 0,19 ± 0,01 (primavera) e a maior de 0,33 ± 0,04 (outono) nmol TMP/mg de peso úmido (Fig. 7B). Quando o conjunto total dos dados foi analisado, não foram observadas correlações significativas entre as concentrações dos metais e os valores de LPO, tanto no fígado quanto nas brânquias.

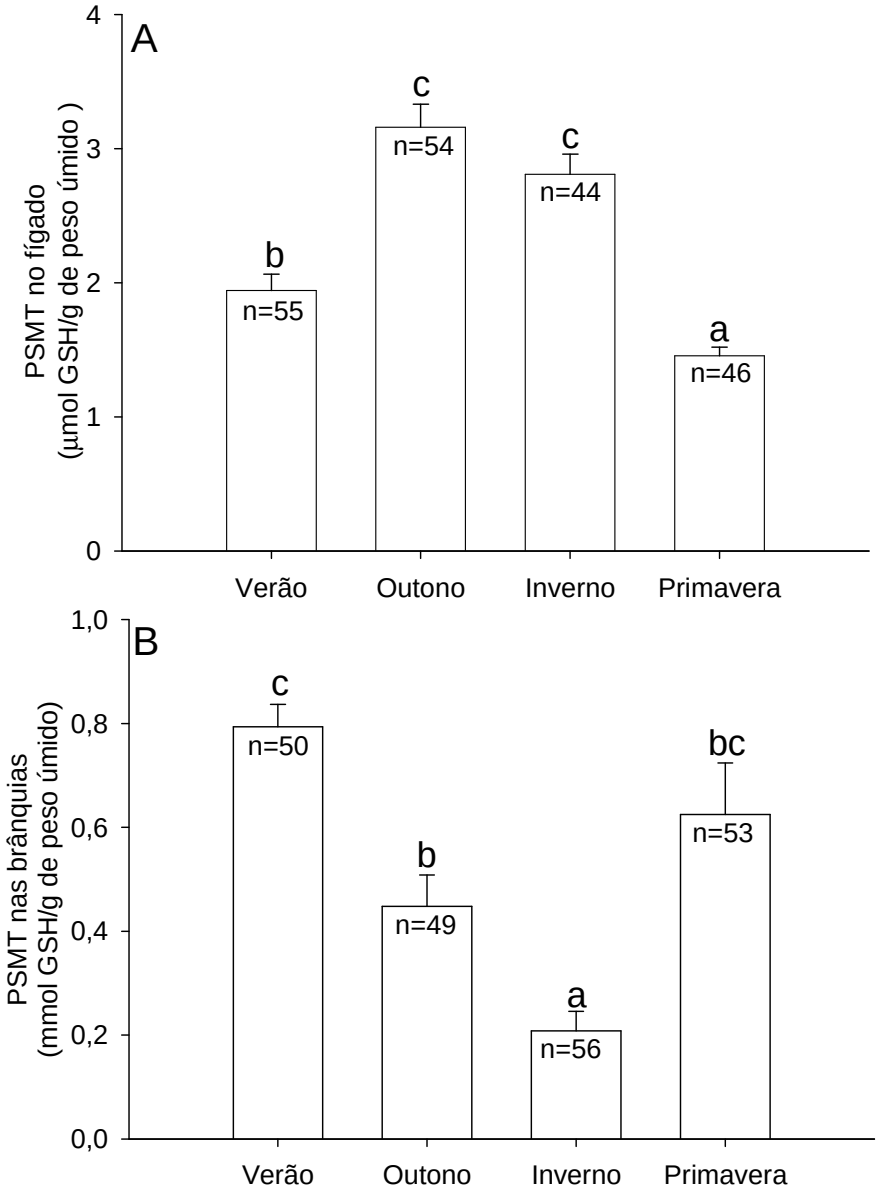

Figura 6. Proteínas semelhantes à metalotioneínas (PSMT) em fígado (A) e em brânquias (B) de corvinas (*Micropogonias furnieri*) coletadas no estuário da Lagoa dos Patos (Rio Grande, RS). Letras diferentes indicam diferenças sazonais significativas (*P <0,05*). Os dados são expressos como média ± erro padrão. n= número de animais amostrados.

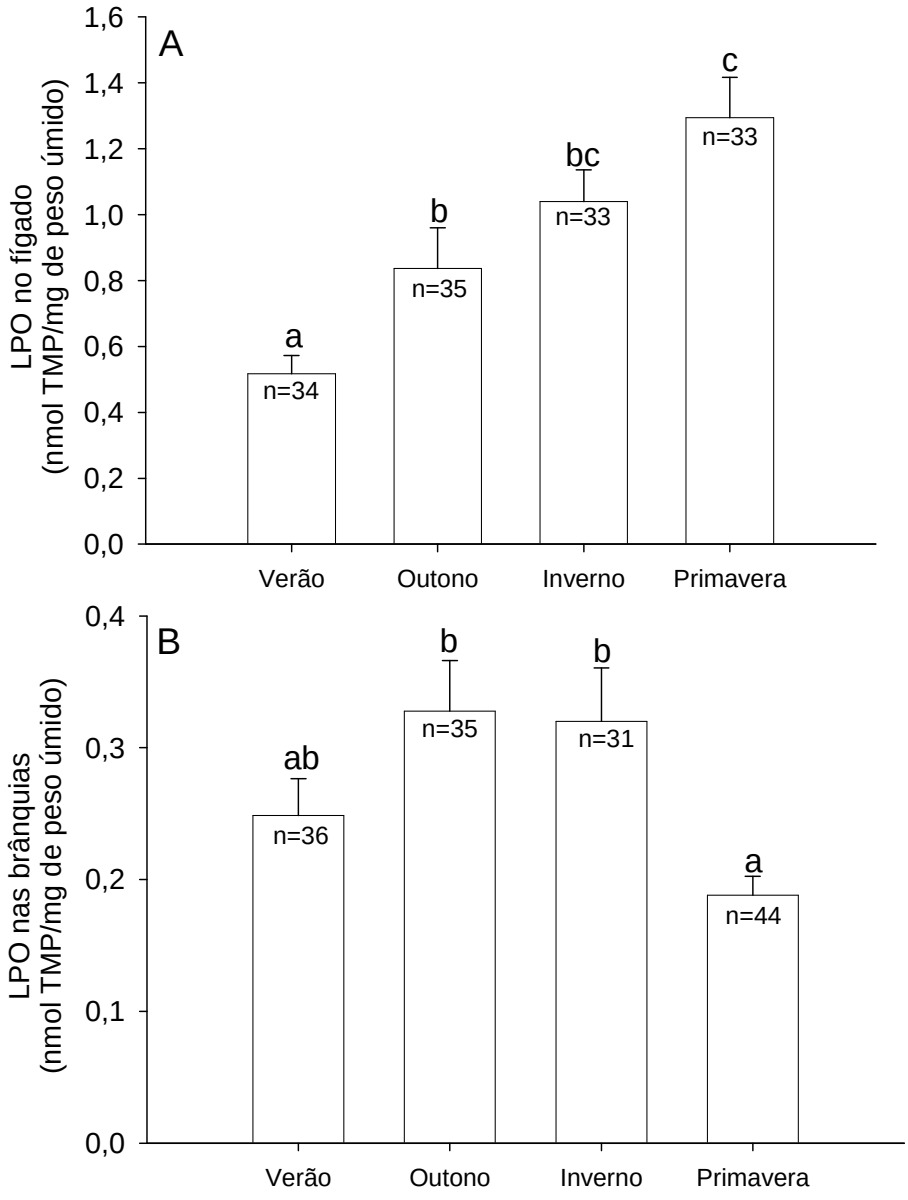

Figura 7. Peroxidação de lipídios (LPO) em fígado (A) e brânquias (B) de corvinas (*Micropogonias furnieri*) coletadas no estuário da Lagoa dos Patos (Rio Grande, RS). Letras diferentes indicam diferenças sazonais significativas ($P < 0,05$). Os dados são expressos como média ± erro padrão. n= número de animais amostrados.

3.3.3. Teste de Micronúcleo

O teste de micronúcleo mostrou maiores níveis de dano de DNA (*P<0,05*) em corvinas coletadas na primavera. A menor média foi de 1,28 ± 0,14 (outono) e a maior de 1,95 ± 0,17 (primavera) MN/1000 céls. (Fig. 8). Quando o conjunto total dos dados foi analisado, não foram observadas correlações significativas entre as concentrações dos metais e os valores de freqüência de células micronucleadas nas hemácias.

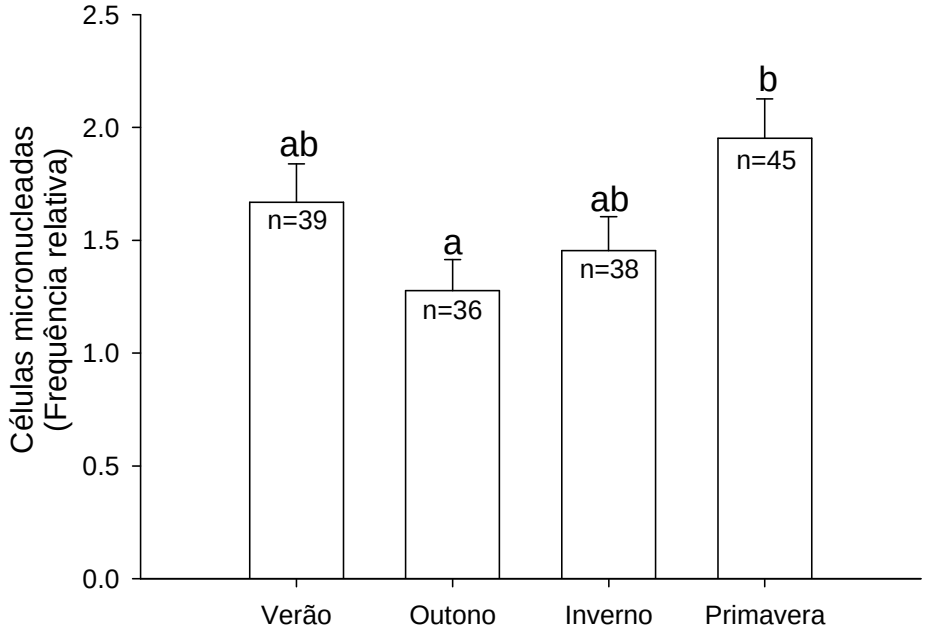

Figura 8. Variação sazonal da freqüência relativa de células micronucleadas em corvinas (*Micropogonias furnieri*) coletadas no estuário da Lagoa dos Patos (Rio Grande, RS). Letras diferentes indicam diferenças sazonais significativas (*P < 0,05*). Os valores são expressos como média ± erro padrão. n= número de animais amostrados.

3.3.4. Ensaios imunológicos

A fagocitose (FG) (Fig. 9A) e a explosão respiratória (ER) (Fig. 9B) apresentaram variações sazonais e significativas (*P<0,05*). Ambos biomarcadores apresentaram os valores mais baixos no inverno (FG=6676,86 ± 602,96; ER=31,00x10^4 ± 3,33x10^4), enquanto no outono foram observados os valores mais altos (FG=3105,4 ± 2856,27; ER=66,37x10^4 ± 5,73x10^4).

Quando o conjunto total dos dados foi analisado, observou-se que os valores de fagocitose mostraram uma forte correlação significativa e negativa com as concentrações de Zn (*r*=-0,4798, P=0,024) e Pb (*r*=-0,5938, *P*=0,004) no fígado, enquanto que a explosão respiratória correlacionou-se negativamente com as concentrações de Cu (*r*=-0,5439, *P*=0,005) e Zn (*r*=-0,4643, *P*=0,019). As concentrações de Cu, Zn e Pb nas brânquias mostraram altos índices de correlação negativa com os valores fagocitose (*r*=-0,6356, *P=0,001*; *r*=-0,5456, *P=0,013* e *r*=-0,7011, *P=0,000*, respectivamente). Em relação à explosão respiratória, somente foi observada uma correlação significativa e também negativa com o Cu (*r*=-0,3913, *P=0,048*).

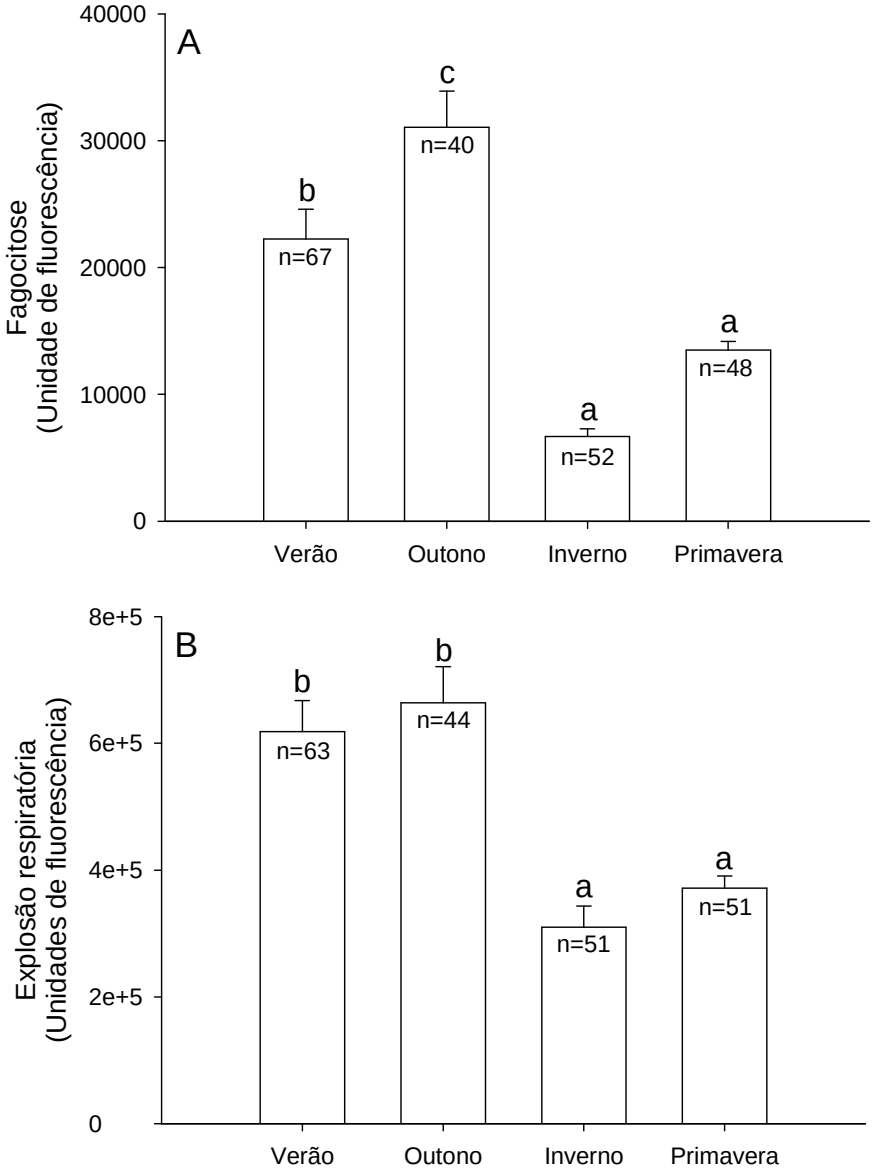

Figura 9. Atividade fagocitária (A) e explosão respiratória (B) em leucócitos sanguíneos de corvinas (*Micropogonias furnieri*) coletadas no estuário da Lagoa dos Patos (Rio Grande, RS). Letras diferentes indicam diferenças sazonais significativas ($P < 0,05$). Os dados são expressos como média ± erro padrão. n= número de animais amostrados.

4. CONCLUSÕES E CONSIDERAÇÕES GERAIS

1. Os metais mostraram uma tendência de maior acumulação em fígado e brânquias de corvinas coletadas no inverno e primavera, sugerindo uma maior biodisponibilidade de metais em condições de mais baixas salinidades nestas estações do ano.

2. No fígado, a maior acumulação de Cu no inverno estimulou a síntese de PSMT. Por sua vez, as maiores concentrações de Zn e Cd na primavera, associada a uma redução na concentração de PSMT pode ser responsável, pelo menos em parte, pela maior resposta dos biomarcadores de efeito observadas nestas estações do ano.

3. Os maiores valores de LPO no fígado durante o inverno e primavera mostram que as defesas antioxidantes não foram suficientemente eficientes para proteger o dano em lipídios de membranas nas corvinas coletadas nestas estações do ano.

4. A concentração de PSMT nas brânquias parece ter sido mais influenciada pelos fatores ambientais, como o aumento de temperatura no verão, do que com a concentração de metais acumulados no tecido, mostrando uma baixa especificidade deste tecido para a análise deste biomarcador de exposição.

5. Nas estações mais frias (outono e inverno), as brânquias de corvinas apresentaram-se mais susceptíveis ao dano de lipídios, enquanto na primavera apresentaram uma maior freqüência de dano de DNA.

6. As corvinas apresentaram imunossupressão tanto de sua capacidade fagocitária quanto de sua resposta oxidativa, mostrando uma clara resposta ao estresse da exposição aos metais analisados no presente estudo.

7. O uso de juvenis de corvina *M. furnieri* (<15 cm) coletados no estuário da Lagoa dos Patos é fortemente recomendado como potencial bioindicador de poluição

por metais traço para futuros programas de biomonitoramento no estuário da Lagoa dos Patos.

8. Como nesta tese tratam-se de corvinas juvenis, as conclusões sobre estes juvenis não devem ser extrapoladas para os indivíduos adultos, pescadas na costa do Brasil, Uruguai e Argentina.

5. PERSPECTIVAS

1. Identificar as isoformas de PSMT em fígado e brânquias de corvinas coletadas nas diferentes estações do ano no estuário da Lagoa dos Patos;

2. Determinar as concentrações de contaminantes orgânicos (pesticidas e hidrocarbonetos) em amostras biológicas de corvinas coletadas no estuário da Lagoa dos Patos (RS);

3. Identificar e desenvolver marcadores fisiológicos e bioquímicos potenciais para o uso na avaliação de impacto e monitoramento de contaminantes orgânicos (pesticidas e hidrocarbonetos) em corvinas presentes no estuário da Lagoa dos Patos (RS).

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ANEXOS

Anexo I

7.1. Pollution biomarkers in estuarine animals: Critical review and new perspectives.

Monserrat, J. M., Martínez, P.E., Geracitano, L. A. Amado, L. L., Martins, C. M. G., Pinho, G. L. L., Chaves, I. S., Ferreira-Cravo, M. Ventura-Lima, J. Bianchini, A.

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Anexo II

7.2. The croaker *Micropogonias furnieri* as bioindicator of trace metal pollution in the Patos Lagoon estuary (Southern Brazil). Part I: Tissue metal accumulation

Isabel Soares Chaves & Adalto Bianchini

Artigo submetido à Marine Pollution Bulletim

Anexo III

7.3. The croaker *Micropogonias furnieri* as bioindicator of trace metal pollution in the Patos Lagoon estuary (Southern Brazil). Part II: Biomarker responses

Isabel Soares Chaves; Pablo Elias Martínez and Adalto Bianchini

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Review

Pollution biomarkers in estuarine animals: Critical review and new perspectives ☆

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Abstract

In this review, recent developments in monitoring toxicological responses in estuarine animals are analyzed, considering the biomarker responses to different classes of pollutants. The estuarine environment imposes stressful conditions to the organisms that inhabit it, and this situation can alter their sensitivity to many pollutants. The specificity of some biomarkers like metallothionein tissue concentration is discussed in virtue of its dependence on salinity, which is highly variable in estuaries. Examples of cholinesterase activity measurements are also provided and criteria to select sensitive enzymes to detect pesticides and toxins are discussed. Regarding non-specific biomarkers, toxic responses in terms of antioxidant defenses and/or oxidative damage are also considered in this review, focusing on invertebrate species. In addition, the presence of an antioxidant gradient along the body of the estuarine polychaete *Laeonereis acuta* (Nereididae) and its relationship to different strategies, which deal with the generation of oxidative stress, is reviewed. Also, unusual antioxidant defenses against environmental pro-oxidants are discussed, including the mucus secreted by *L. acuta*. Disruption of osmoregulation by pollutants is of paramount importance in several estuarine species. In some cases such as in the estuarine crab *Chasmagnathus granulatus*, there is a trade off between bioavailability of toxicants (e.g. metals) and their interaction with key enzymes such as Na^+–K^+-ATPase and carbonic anhydrase. Thus, the metal effect on osmoregulation is also discussed in the present review. Finally, field case studies with fish species like the croaker *Micropogonias furnieri* (Scianidae) are used to illustrate the application of DNA damage and immunosuppressive responses as potential biomarkers of complex mixture of pollutants.

Keywords: Estuarine organisms; Biomarkers; Metallothionein; Cholinesterase activity; Antioxidant defenses; Osmoregulation; DNA damage; Immune responses

Contents

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1. General introduction

Pollution results from the direct or indirect introduction by man of molecules or energy that induce deleterious effects for living resources and even human health (Livingstone, 1993, 1998). In recent years, some authors have recognized the paradoxical role of water as simultaneously being a vital resource and a vehicle for pollutant elimination (Schnurstein and Braunbeck, 2001). This paradox can be considered as a part of the present environmental crisis that arises from the conflict between nature and technology. According to Krüger (2001), the paradigm for the establishment of a homeostatic condition for natural systems should include a monitoring process in order to take corrective actions. This paradigm is accomplished in some situations by the employment of biomarkers, previously defined as the measurements of body fluids, cells, or tissues that indicate in biochemical or cellular terms the presence of contaminants (Livingstone, 1993). The definition has been broadened in order to include behavioural parameters (Depledge et al., 1995; López-Barea and Pueyo, 1998), now recognized to be extremely important in establishing ecological inferences with observed biochemical and/or physiological responses. For example, Scott and Sloman (2004) reported that neurological dysfunctions induced by toxicants can trigger behavioural changes, some of them ecologically meaningful, such as changes in reproductive, feeding and social behaviours. Measurements at the biochemical or physiological level detect more quickly and specifically the presence of several toxic compounds, allowing earlier identification of change, before deleterious effects reach higher organization levels (Monserrat et al., 2003a).

Usually, biomarkers are classified as *specific* or *non-specific* ones. The use of toxicant-specific biomarkers such as metallothionein has been widely employed to indicate the presence of heavy metals (Giguère et al., 2003), although as will be shown in Section 2, new evidence points to the confounding effects that some abiotic factors like salinity can exert on this parameter. Also, the measurement of cholinesterase activity is considered a specific biomarker of organophosphorus and carbamate pesticides and neurotoxins such as anatoxin-a(s) (Monserrat et al., 2001, 2003a; Hyne and Maher, 2003). On the other hand, since several pollutants can modify directly or indirectly the balance between the concentration of pro-oxidants and antioxidants, the determination of oxidative stress (DNA damage, protein oxidation, lipid peroxidation) and/or antioxidant responses in aquatic species is commonly employed as a non-specific biomarker (Bainy et al., 1996; Geracitano et al., 2004a). The determination of these biomarkers has been successfully employed in field studies aimed to characterize impacted areas, where complex mixtures of pollutants are usually present (Bainy et al., 1996; Geracitano et al., 2004a,b; Amado et al., 2006a,b).

Pollution in estuarine environments is considered a critical environmental issue because of the high variation in several abiotic factors that impose severe restrictions to organisms living in these areas (Matthiessen and Law, 2002; Amado et al., 2006a). In the classical paper of Magnum and Towle (1977), the term "enantiostasis" was introduced for the first time being defined as a type of regulation occurring when the effect of a change in one chemical and/or physical property experienced by the animal is counteracted by an opposite change in another variable(s), preserving the stability of a particular physiological system. Among these responses, the homeoviscous adaptation is well known, representing changes in membrane lipid composition to maintain a relatively constant physical state during thermal acclimation (Hochachka and Somero, 2002). A result of this strategy is a higher proportion of unsaturated fatty acids (UFA) in biological membranes to maintain their fluidity during cold periods. However, the homeoviscous adaptation could synergize the effects of pollutants that directly or indirectly generate oxidative stress, since lipid peroxidation (LPO) occurs mainly on UFA. This effect has been observed even in invertebrates (mussels) collected in non-polluted areas during winter (Viarengo et al., 1991). In addition, estuarine environments are characterized by intense variation of water physico-chemical parameters, such as salinity, pH and temperature, that can alter the bioavailability and, by consequence, the toxicity of pollutants (Witters, 1998).

Taking into account the importance and potential severity of aquatic pollution in estuaries, the present review will focus mainly on toxicological responses at several biochemical and physiological levels in organisms living in these environments. The main objectives were to identify and discuss, whenever possible, the specificity of biomarker responses to several toxic molecules taking into account the influence of abiotic factors on these responses since, as previously mentioned, their variability is one the main characteristic of estuarine environments, and that any biomonitoring program to be conducted in these environments should consider this fact. Some key physiological responses, such as osmoregulation were also analyzed due to the importance of salinity as a changing variable in estuarine environments. Several examples of studies in invertebrates are discussed in virtue of their abundance and, in the case of benthic organisms, because they are in close contact with the sediment,

where usually most of pollutants are trapped and concentrated. The fact that these animals have little mobility is also useful in biomonitoring programs since they can reflect local pollution (Monserrat et al., 2003b).

2. Specific biomarkers

2.1. Metallothionein proteins: how specific are they?

Metallothioneins (MT) constitute a family of low molecular weight, cysteine-rich proteins, which are capable of binding metals. First reported in mammals in the late 1950s, MT have been studied in many aquatic invertebrates, especially molluscs (Langston et al., 1998; Soazing and Marc, 2003) and crustaceans (Schlenk and Brouwer, 1993; Syring et al., 2000; Brouwer et al., 2002).

The behavior of MT is dominated by the chemistry of the thiol (–SH) group. The metal–thiolate clusters within the MT molecules allow rapid exchanges of metallic ions between clusters and with other MT molecules. These characteristics of binding and transference of metals appear to be unique to MT and fundamental to their biological role (Viarengo et al., 2000; Amiard et al., 2006). Biological functions of MT include homeostasis of physiological important metals (Cu, Zn), detoxification of both essential metals and non-essential metals and antioxidant defense (Roesijadi, 1996; Viarengo et al., 2000; Amiard et al., 2006; and references therein). The possibility that MT can act as oxyradical scavenger can be predicted by the high sulfhydryl content present in this protein. It should be noted that MT could protect the cells from oxidative stress not only acting as oxyradical scavenger, but through metal binding/release dynamics (Viarengo et al., 2000). Other functions for MT can be predicted, since MT expression is rapidly induced by a variety of substances including metals, hormones, cytokines, oxidants, stress and irradiation (Andrews, 2000; Haq et al., 2003).

Binding of MT during an excess of harmful metals protects the organism against toxicity by limiting availability of these cations at undesirable sites (Langston et al., 1998). Generally, MT expression increases with the elevation of tissue concentrations of MT-inducing metals, reflecting metal bioavailability in the environment (Leung et al., 2002; Ross et al., 2002). For example, Pedersen et al. (1997) reported a clear induction of MT in gills of the crab *Carcinus maenas* related to the presence of copper in the field; whereas Schlenk and Brouwer (1991) demonstrated that copper induced MT synthesis in hepatopancreas of the blue crab *Callinectes sapidus* both in the field and in the laboratory. In fact, it has been established that increases in MT concentrations are associated with decreases in the sensitivity of an organism to metals (Pavicic et al., 1994).

The induction of MT synthesis in many marine species by metal contaminants (Ag, Cd, Cu, Hg) has led to the proposed use of these proteins as potential specific biomarkers for metal exposure and toxicity in aquatic biomonitoring (Langston et al., 1998; Cajaraville et al., 2000; Soazing and Marc, 2003; Amiard et al., 2006), provided that natural and physiological factors have been take into account (Rainbow, 1998; Legras et al., 2000). Recently, evidence of a metal-specific induction of different MT isoforms has been described, possibly enhancing the specificity of MT as biomarkers of metal exposure (Syring et al., 2000; Brouwer et al., 2002; Soazing and Marc, 2003) and clarifying any double role of MT in homeostasis or detoxification by detecting specific MT gene expression devoted to either role. Schlenk and Brouwer (1993) found several MT isoforms playing different physiological roles in the blue crab *C. sapidus*. These authors described two isoforms for Cd associated with Cd detoxification, the CdMT-I showing three-fold higher binding to Cd than the CdMT-II. They also reported three isoforms for Cu and suggested that CuMT-I and CuMT-II are associated with Cu metabolism while CuMT-III is related to Cu detoxification.

MT expression can also be influenced by natural factors that may affect accumulation of metal, such as salinity, and this constitutes an important factor to be considered when MT is measured and employed as a specific biomarker of metal pollution (Fig. 1). Salinity affects the speciation and bioavailability of trace metals, influencing their uptake by aquatic organisms (Bianchini and Gilles, 2000; Bianchini et al., 2002). Thus, it directly controls the amount of incoming metal that potentially needs to be bound by MT (Fig. 1). Thus, there should be a clear link between variations in environmental salinity and MT concentration in the organism (Legras et al., 2000; Mouneyrac et al., 2001; Leung et al., 2002). Usually, decreases in salinity are associated with increased uptake rates of many trace metals by marine organisms. This increased uptake rate can result from increases in the free metal ion concentration, a consequence of decreased metal complexation by chlorides in lower salinities (Bianchini and Gilles, 2000; Paquin et al., 2002; Bianchini et al., 2002; Janssen et al., 2003). Leung et al. (2002) have shown that the induction of MT was higher in the dog whelk *Nucella lapillus* exposed to cadmium (500 μg/l) in low salinity (22 psu) respect the control salinity (33 psu). Monitoring programs for trace metals often use organisms from saltwater environments, such as estuaries and intertidal zones, where salinity can fluctuate on hourly, daily, weekly and seasonal time scales (Leung et al., 2002; Amado et al., 2006a). Therefore, if MT measurements are to be incorporated into biomonitoring programs, salinity influence on MT concentration should be considered (Leung et al., 2002). In fact, changes in salinity, typically cause metabolic changes in crustaceans. Responses of euryhaline crab species, including the blue crab *C. sapidus*, to low salinities are characterized by an increased gill oxygen consumption rate (Péquex, 1995) as a consequence of the higher energy expenditure related to ionic and osmotic regulation. In turn, increases in the oxygen consumption rate and high mitochondria density in tissues are closely related to the generation of reactive oxygen species (see Section 3 and Fig. 1). In this way, the induction of MT in this scenario should be adaptive in virtue of its antioxidant properties (Viarengo et al., 2000).

2.2. Cholinesterase activity: a specific biomarker with an old history and new uses

Some pesticides, including organophosphorus and carbamates, are known to selectively inhibit cholinesterase (ChE)

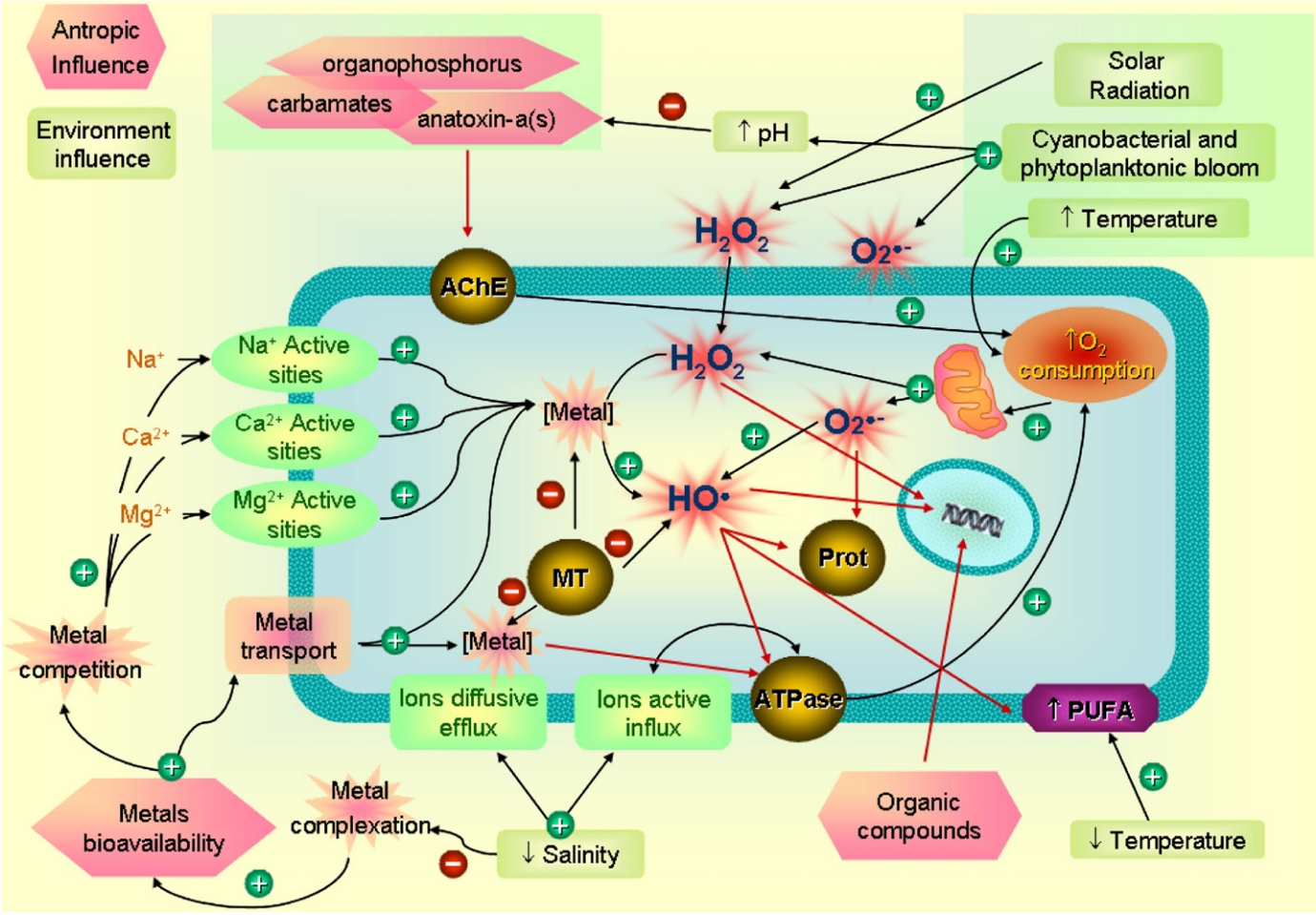

Fig. 1. Examples of how several biochemical and physiological responses of estuarine organisms are influenced by inter-relationship between abiotic parameters and anthropogenic pollution. The symbols (+) and (−) shown in black arrows indicate the influence of biochemical, physiological or environmental factors over biotic and abiotic variables. For example, higher temperature augments (+) oxygen consumption, whereas metallothioneins (MT) diminish (−) free metals concentration. AChE: acetylcholinesterase. Prot: proteins. Reactive oxygen species: hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$). PUFA: polyunsaturated fatty acids. Red arrows indicate deleterious effects of pollutants or other chemical species on target molecules.

activity (Valbonesi et al., 2003). When directly released into the environment, these chemicals can reach rivers and sometimes the sea, leading to the contamination of various aquatic ecosystems (Mora et al., 1999). The relationship between the presence of these compounds in aquatic environments and tissue ChE activity has been widely studied and employed as a biomarker in aquatic invertebrate and vertebrate species (Rodriguez-Fuentes and Gold-Bouchot, 2000; De La Torre et al., 2002; Tortelli et al., 2006). Since organophosphorus and carbamates have a relatively short half-life, the assessment of cholinesterase (ChE) inhibition is a useful tool to evaluate their impact on aquatic biota (Fig. 1), even when the chemical themselves are not longer detectable in the environment (Valbonesi et al., 2003). Authors like Cunha Bastos et al. (1991) employed rat brain homogenates in order to detect *in vitro* anticholinesterase compounds in environmental samples. The conversion of thionophosphate insecticides to their oxo-form is performed through the liver microsomal P450 mixed oxidase system, an enzymatic system also present in other mammal tissues such as the lung and brain. In this way, the use of rat brain homogenates should provide simultaneously the enzymatic oxidative system to activate a thionophosphate pesticide and the enzyme (ChE) that can be inhibited by the oxo-form of the pesticide.

The *in vitro* approach has been used extensively because dissimilar P450 levels in aquatic species can result in marked differences in resistance to pollutants that need to be metabolically activated (Livingstone, 1998). This methodology is not restricted to the organophosphorus and carbamate pesticides since acetylcholinesterase inhibitors produced by soil microorganisms (*Streptomyces antibioticus*) and anticholinesterase activity of marine zoanthids pigments has been reported (Neumann and Peter, 1987; Sepcic et al., 1998). Also, cyanobacterium anatoxin-a (s) was considered as an organophosphorus pesticide analogue (Fig. 1). Its chemical structure was determined by Matsunaga et al. (1989) and, as expected, its physicochemical characteristics are similar to organophosphorus pesticides, that is, low stability at high pH and temperature (Barros et al., 2004). This toxin resembles an activated oxo-form of a thio-organophosphorus molecule, allowing detection by *in vitro* enzymatic systems without P450 mixed oxidase systems, since anatoxin-a(s) is a 'ready to act' molecule, and can inhibit ChE activity without

further metabolization. In this context, neurotoxins like anatoxin-a(s) would not depend on the oxidative metabolism capabilities of the target organism to exert a noxious effect. Thus, differential affinities of neurotoxins to ChEs should be the key factor determining their toxicity. This feature has been considered for the *in vitro* detection of anatoxin-a(s), since authors like Devic et al. (2002) obtained mutant acetylcholinesterases that presented high sensitivity against anatoxin-a(s) and resistance against several organophosphorus and carbamate pesticides, lowering the probability of obtaining false positives.

Previous *in vitro* assays showed that the sensitivity of silverside fish *Odontesthes argentinensis* ChE was higher than that showed by the crab *C. sapidus* (Monserrat et al., 2001) in terms of the concentration of the carbamate eserine needed to inhibit 50% of ChE activity (IC_{50}). In fact existing conspicuous differences in sensitivity against anticholinesterase compounds have been reported between several fish and invertebrate species (Table 1). Differences in sensitivity between species can arise due to several factors. One of them is the ability of the active site of the enzyme to fit the alkyl chain of substrates such as acetylthiocholine iodide (AcSCh) and butyrylthiocholine iodide (BSCh). For example, Monserrat and Bianchini (2000) found that in the crab (*C. sapidus*) cholinesterase showed a lower sensitivity to the inhibitory effects of eserine than in fish (*O. argentinensis*) ChE (Table 1). Crab ChE showed a higher affinity to AcSCh than to BSCh when compared to that recorded for fish ChE, suggesting that the active site of fish enzyme allow an easier access to bigger substrate molecules such as BSCh (Monserrat and Bianchini, 2000). Note that, however, some authors have reported low *in vitro* ChE sensitivity to carbamates and organophosphorus pesticides in fish. Silva Filho et al. (2004) found different levels of ChE inhibition in neotropical fishes, using methyl paraoxon. These authors reported IC_{50} values ranging from 3.34 μM in *Paralonchurus brasiliensis* to 0.123 μM in *Prochilodus lineatus*.

The enzyme (E) inhibition by an inhibitor (I), like carbamate or organophosphorus molecules can be summarized as follows:

$$E + I \underset{k-1}{\overset{k1}{\rightleftharpoons}} (EI)^{R} \xrightarrow{kc} (EI)^{I} \quad (1)$$

where $(EI)^R$ represents a reversible enzyme–inhibitor complex and $(EI)^I$ an irreversible one. The affinity equilibrium constant (k_a) is defined as $k_a = k\text{–sub } {}_1/k_1$ and k_c represents the carbamylation, if we considered inhibition by a carbamate. The bimolecular inhibition constant (k_i) is defined as $k_i = k_c/k_a$. Comparison of the inhibition kinetic parameters is useful for visualizing sensitivity of different ChEs, as depicted in Table 1. Note that some fish species like *Oreochromis niloticus* and *Odontesthes bonariensis* are particularly sensitive to the carbamate eserine, whereas other aquatic species including the mollusc *Perna perna* show high resistance, as observed by its IC_{50} value (Table 1). In terms of inhibition constant, this is also observed. For instance, the fish species *Cyprinus carpio* possesses a much higher k_i than the oyster *Crassostrea rhizophorae* (Table 1). Thus a trend seems to exist towards a higher sensitivity of ChE to anticholinesterase compounds in fish species compared to invertebrate species, with the only and striking exception of the estuarine croaker *Micropogonias furnieri* (see Table 1).

3. Non-specific biomarkers: antioxidant and oxidative damage in invertebrate species

The superoxide anion ($O_2^{\cdot -}$), the hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$) are the dark side of the "oxygen

Table 1
Concentration of eserine that inhibits 50% of cholinesterase activity (IC_{50}, in μM) in different fish (F), molluscs (M) and crustacean (C) species

Species	k_a	k_c	k_i	IC_{50}	Reference
Oreochromis niloticus (F)	–	–	–	9.76×10^{-4}	Rodríguez-Fuentes and Gold-Bouchot (2004)
Odontesthes bonariensis (F)	–	–	–	1×10^{-3}	Monserrat et al. (2002)
Cnesterodon decemmaculatus (F)	3.4×10^{-3}	0.39	137.5	1.43×10^{-3}	De la Torre et al. (2002)
Cyprinus carpio (F)	2.1×10^{-3}	1.18	244.2	5×10^{-3}	De la Torre et al. (2002)
Odontesthes argentinensis (F)	–	–	–	4.6×10^{-2}	Monserrat and Bianchini (2000)
Callinectes sapidus (C)	–	–	–	0.16	Monserrat and Bianchini (2000)
Crassostrea rhizophorae (M)	1.6×10^{-2}	0.83	51.0	0.91	Monserrat et al. (2002)
Perna perna (M)	–	–	–	4.58	Monserrat et al. (2002)
Micropogonias furnieri (F)	–	–	–	4472.00	Tortelli et al. (2006)

Inhibition kinetic parameters are also shown. k_a: affinity equilibrium constant (mM). k_c: carbamylation constant (in min^{-1}). k_i: bimolecular inhibition constant (mM^{-1} min^{-1}).

paradox", where opposite to the energetic benefits of the aerobic metabolism appeared the curse of reactive oxygen species (ROS) and also the reactive nitrogen species (RNS) that can induce several deleterious effects at cellular level (Hermes-Lima, 2004). Among the RNS, nitric oxide, nitrogen dioxide and peroxynitrite radical are the most known. Recently, it has been estimated that about 0.1% to 0.4% of all oxygen consumed by vertebrates (and possibly by higher invertebrates) produces superoxide anion, being the mitochondria the main site of its production by means of the "leaky" electron transport system (Fridovich, 2004). However, in the intracellular environment, the presence of many enzymatic and non-enzymatic antioxidant defenses keeps ROS and RNS at a low concentration (Hermes-Lima, 2004). Oxidative stress can be defined as the disturbance between ROS concentration and the antioxidant defenses concentration, favoring the first. This situation can lead an organism to suffer oxidative damage in terms of lipid, proteins or DNA oxidation (Fig. 1), and often causes a general disturbance of the cellular redox balance, *i.e.* the ratio of reduced to oxidized glutathione (GSH/GSSG) and the NADH/NAD^+ ratio (Abele and Puntarulo, 2004).

As mentioned in the General introduction, several molecules can alter the pro-oxidant/antioxidant balance, leading to organisms becoming susceptible to oxidative stress. In recent years, the measurement of antioxidant responses has been conducted in terms of the overall tissue capacity to scavenge different forms of ROS, according to the TOSC (total oxyradical scavenging capacity) method (Regoli and Winston, 1999; Regoli et al., 2002). Interestingly, this method gives a general picture of the oxidative status of a particular tissue, a difficult goal to reach when either an individual or small group of antioxidant defenses are measured. As stated by Regoli et al. (2002), the advantage of TOSC (or an equivalent methodology) is the capacity to establish an integrated antioxidant response of an organism or tissue against a particular type of ROS, like peroxyl, hydroxyl and peroxynitrite radicals. Its use has been successfully employed to detect the influence of pollution in depleting the antioxidant defenses in mussels (*Mytilus galloprovincialis*) transplanted to polluted areas, this effect being correlated with oxidative DNA damage (Regoli et al., 2004). Furthermore, gills of the estuarine crab *Chasmagnathus granulatus* showed an augmented antioxidant competence towards peroxyl radicals after exposure to the cyanotoxin microcystin, a response that Vinagre et al. (2003) considered adaptive, since no oxidative damage was observed, at least in terms of lipid peroxidation (LPO).

The nereidid polychaete *Laeonereis acuta* has been well studied in terms of its antioxidant responses and oxidative damage induced by pollutants, both under experimental and field conditions. The Nereididae family is described as anoxic and hypoxia tolerant (Abele-Oeschger et al., 1994). Studies of Geracitano et al. (2002, 2004a,b) demonstrated the effectiveness of the use of the antioxidant responses and oxidative damage as biomarkers in *L. acuta* exposed naturally and experimentally to pollutants occurring in Patos Lagoon estuary (Southern Brazil). Geracitano et al. (2004a) found different responses after acute and chronic exposure of worms sampled at a reference and a polluted site, indicating a higher capacity of worms from the unpolluted site to face oxidative stress, since no oxidative damage (measured through LPO) was observed after experimental exposure to copper. Other environmental stressful conditions have been shown to affect the pro-oxidant/antioxidant balance in the polychaete *L. acuta*, including cyanobacteria blooms. The study of Rosa et al. (2005a) revealed that the occurrence of a non-toxic bloom induced oxidative damage in *L. acuta*, a result that the authors related to hyperoxia/anoxia cycles induced by the cyanobacteria through photosynthetic and respiratory processes (Fig. 1). These cycles should resemble the ischemic/reperfusion process triggering an increase in the production of reactive oxygen species, leading to oxidation of cellular components after the turn back of oxygen in the reperfusion (Lushchak et al., 2001).

Morphological and physiological alterations have been reported along body regions of annelids exposed naturally or experimentally to different pollutants that can induce potential differences in the antioxidant and oxidative stress responses. The earthworms *Dendrodrilus rubidus* and *Lumbricus rubellus* sampled in soil contaminated with cadmium showed major accumulation of this metal within the posterior alimentary canal (Morgan et al., 1989). In the posterior region of the freshwater oligochaete *Tubifex tubifex*, higher concentrations of copper and lead were observed compared to the metals accumulated in the anterior region (Lucan-Bouché et al., 1999), a situation that should favor the generation of hydroxyl radicals through Fenton reaction if metals are free. In the polychaete *Heteromastus filiformis*, Abele et al. (1998) observed that superoxide dismutase (SOD: catalyzes the reaction $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2$) activity and haemoglobin quantity were higher in the tail compared with the head of the worm, a fact that the authors correlated with an external gradient of PO_2 and pH. In the polychaete *L. acuta*, a gradient of antioxidant enzyme activity along the anterior, middle, and posterior regions of the worm (Rosa et al., 2005b) was seen, exhibiting higher catalase (CAT: catalyzes the reaction $H_2O \rightarrow H_2O + 1/2O_2$) and SOD activities in the posterior region, ensuring the degradation of inductors of lipid peroxidation products such as hydrogen peroxide and superoxide anion (Table 2). Posterior studies have also verified a higher antioxidant competence in the posterior region against hydroxyl and peroxyl radicals (Ferreira-Cravo et al., in press; Table 2). At least in part, some of the differences in antioxidant defenses along the body of *L. acuta* can be related to cuticle width, since in the posterior region it is thinnest (Table 2), which should facilitate oxygen and/or environmental ROS (like H_2O_2) entry, imposing higher antioxidant defenses to cope with this more pro-oxidant condition.

Several complex biological interactions can alter the pro-oxidant/antioxidant balance, including mutualistic-like relationships, as reported for the mucus secreted by the polychaete *L. acuta* (Moraes et al., 2006). This secretion shows higher activity of antioxidant enzymes (with the only exception of SOD) and higher antioxidant competence against peroxyl and hydroxyl radicals than the anterior and posterior regions of *L. acuta* (Table 2). Thus, it can be considered that the mucus secretion contributes substantially to the antioxidant defense system of

Table 2
Antioxidant enzyme activity, total antioxidant scavenging capacity (TOSC) against hydroxyl and peroxyl radicals and cuticle width determined in different body regions of the estuarine worm *Laeonereis acuta* (Polychaeta, Nereididae) and in its mucus secretion

	Anterior region	Middle region	Posterior region	Mucus secretion
Catalase [1,2]	1.88 ± 0.26^{a}	3.54 ± 0.31^{b}	4.99 ± 0.58^{c}	12.71 ± 3.28^{d}
Glutathione peroxidase [1,2]	$7.71 \times 10^{-3} \pm 1.30 \times 10^{-3\ a}$	$8.94 \times 10^{-3} \pm 1.81 \times 10^{-3\ a}$	$7.11 \times 10^{-3} \pm 0.86 \times 10^{-3\ a}$	$5.20 \times 10^{-2} \pm 1.50 \times 10^{-2\ b}$
Superoxide dismutase [1,2]	12.65 ± 0.65^{a}	19.96 ± 1.93^{b}	36.50 ± 0.73^{c}	$15.96 \pm 3.74^{a,b}$
Glutathione-*S*-transferase [1,2]	$8.79 \times 10^{-2} \pm 0.43 \times 10^{-2\ a}$	$3.21 \times 10^{-2} \pm 0.71 \times 10^{-2\ b}$	$3.00 \times 10^{-2} \pm 0.72 \times 10^{-2\ b}$	Not observed
TOSC (hydroxyl radicals) [2,3]	128.47 ± 21.74^{a}	248.44 ± 11.33^{b}	486.81 ± 97.73^{c}	1699.97 ± 455.57^{d}
TOSC (peroxyl radicals) [2,3]	453.22 ± 58.74^{a}	633.21 ± 45.65^{a}	1477.50 ± 405.64^{b}	1781.35 ± 550.32^{b}
Cuticle width [1]	5.03 ± 0.14^{a}	–	2.46 ± 0.06^{b}	–

Enzyme activities are expressed in catalytic units, whereas TOSC values are expressed in units/mg of total protein. Cuticle width is expressed in μm.
In all cases, data are shown as mean ± 1 S.E.M. Similar letters indicate absence of statistical differences.
[1] Data from Rosa et al. (2005b).
[2] Data from Moraes et al. (2006).
[3] Data from Ferreira-Cravo et al. (in press).

the worm against environmental ROS and represents one of the several traits that organisms inhabiting stressful environments such as estuaries have acquired.

4. Key physiological responses in estuarine organisms: osmoregulation

When different sources and kinds of pollutants are considered, metals are arguably the most studied (Péquex et al., 1996). In general, studies involve metal accumulation and effects on survival, growth, feeding, and reproduction (Forget et al., 1998; Santos et al., 2000; Hook and Fisher, 2001; Bianchini et al., 2005a). While it has been reported that estuarine species are sensitive to metals, several studies have also emphasized the influence of the salinity stress on metal toxicity. In euryhaline animals, it has been demonstrated that changes in salinity affect metal bioavailability, and consequently its uptake from solution (Wright, 1995). Thus, attempts to understand factors affecting metal toxicity in aquatic animals have evolved the development of many models. For instance, the "Biotic Ligand Model" (BLM) has been proposed to value quantitatively how the water chemistry, including salinity, affects metal speciation and bioavailability in aquatic systems, and therefore to predict metal toxicity to aquatic organisms (Paquin et al., 2002).

Osmoregulation is the ability to actively maintain osmotic concentrations in extracellular fluids, in spite of the osmolarity (salinity) of the surrounding environment. It is a fundamental physiological adaptation of animals living in estuarine environments. However, organisms living in brackish-water ecosystems are influenced not only by spatio-temporal variations in hydrochemical parameters and tidal dynamics, but also by the input of several types of toxicants. Therefore, they may be exposed to both salinity and pollution stress during their life spans.

Two enzymes play a pivotal role in the osmoregulation of estuarine animals: Na^+–K^+-ATPase and carbonic anhydrase (CA). Na^+–K^+-ATPase is present in high concentrations in salt transporting tissues like intestine and gills, where it maintains ionic and electrical gradients necessary for transepithelial salt movements (Lionetto et al., 2000). It is well known to be directly related to Na^+ and Cl^- exchanges across the tissues (Péquex, 1995). In turn, CA is involved in the hydration of CO_2 to produce H^+ and HCO_3^-, playing a pivotal role in a number of physiological processes like gas exchange, acid–base balance, clearance of waste products from nitrogenous metabolism, and osmoregulation (Lionetto et al., 2000). Both enzymes are present in tissues that are the first interfaces of the organism exposed to the aquatic environment, thus being the primary potential target for the action of environmental pollutants (Fig. 1).

The key mechanism of acute metal toxicity has been reported to be an osmoregulatory impairment associated with gill Na^+–K^+-ATPase inhibition (McGeer and Wood, 1998). Copper, silver, cadmium, zinc, and mercury are some examples of metals reported to cause osmoregulatory disturbances related to metal inhibition of Na^+–K^+-ATPase in freshwater, brackish and marine animals (Péquex et al., 1996; Lionetto et al., 1998; Bianchini and Castilho, 1999; Bianchini and Wood, 2003; Bianchini et al., 2004, 2005b). Regarding CA, inhibitory effects of metals like Ag, Cd, Cu and Zn have been also reported in euryhaline crabs (Lionetto et al., 1998; Vitale et al., 1999; Skaggs and Henry, 2002).

In general, the toxicity of many trace metals is increased at low salinity, a condition where hyper-osmoregulating animals are coping with the hypo-osmotic medium. Therefore, it can be hypothesized that trace metal uptake may be reduced as salinity approaches the isosmotic point of a species because, at this point, there is reduced activity of the ionic exchange pumps (Roast et al., 2002). In light of the above, it could be suggested that metal-induced inhibitory effects on osmoregulatory enzymes would be useful as exposure/effect biomarkers in aquatic animals, including those living in estuarine areas. However, it is also clear that salinity affects osmoregulation processes, as well as metal bioavailability and uptake from solution in euryhaline animals (Roast et al., 2002). Thus, the use of both Na^+–K^+-ATPase and CA as biomarkers in monitoring programs for metals should consider the salinity interaction with both the animal's physiology and metal bioavailability, a feature that, as mentioned in Section 2.1, also affects the compensatory response to metal entry in terms of MT induction.

5. The employment of new biomarkers: genotoxic and immune responses

5.1. Genotoxic responses

The assessment of aquatic species exposure to environmental genotoxicants is a complex issue because of the diversity of potential pollutants that are often found in aquatic systems as complex mixtures (Mitchelmore and Chipman, 1998; Shailaja and D′Silva, 2003). There is a sequence of events between the first interaction of a xenobiotic with DNA and consequent mutation (Van der Oost et al., 2003). The first stage is the formation of adducts of DNA with toxic molecules (Gil and Pla, 2001). Adduct formation can occur directly by chemical covalent binding to specific sites of DNA or after chemical metabolic activation to electrophilic forms, which are highly reactive with nucleophilic centers in DNA. The next stage may be secondary modifications of DNA, such as single and double strand breakage or an increase in the rate of DNA repair; base oxidation and DNA–protein cross-links (Zwart et al., 1999). Secondary modification may be induced by indirect effects of chemicals, such as the increase in reactive oxygen species (ROS) formation, which can lead to oxidative stress (Hermes-Lima, 2004) (Fig. 1). The third stage is reached when the structural perturbations in the DNA become fixed and the affected cells often show altered function, which can lead to uncontrolled cell proliferation and, consequently, to carcinogenesis (Mitchelmore and Chipman, 1998). Finally, when cells divide the damage caused by xenobiotics can lead to DNA mutation and consequent alterations in the following generation (Gil and Pla, 2001).

The detection and quantification of some of these damages have been employed as biomarkers of effect in aquatic organisms environmentally exposed to genotoxic substances (Van der Oost et al., 2003). Ohe et al. (2004) analyzed 128 publications in a review including mutagenic/genotoxic bioassay data and concluded that the kinds of damage most assessed for genotoxicity determination in aquatic organisms (mainly marine species) are mutations at chromosomal level (micronucleus test), DNA adducts (^{32}P-postlabeling) and DNA strand breaks (comet assay), respectively. Publications about DNA damage specifically in estuarine organisms are less abundant, but can demonstrate the sensitiveness of this kind of biomarkers in the characterization of a study area and/or in the understanding of an organism response to environmental toxicants. Both invertebrates and fish have been used as biomonitors to assess the biological effects of aquatic pollutants. Nigro et al. (2006) evaluated, among other biomarkers, the DNA damage measured by comet assay and micronucleus test in native and transplanted mussels in the estuary of River Cecina, Italy. They concluded that results from the comet assay were similar between native and transplanted mussels while the micronucleus frequency was higher in native than in transplanted mussels. This finding demonstrates the use of different DNA damage biomarkers in assessing early and cumulative effects of pollutants, leading to an improvement of the characterization of the studied area. Amado et al. (2006b) also evaluated DNA damage through the comet assay and micronucleus test in native estuarine flounders (*Paralichthys orbignyanus*) seasonally collected at polluted and reference sites in the Patos Lagoon estuary (Southern Brazil). DNA damage quantified by comet assay was similar in flounders from both sites while the micronucleus test demonstrated higher DNA damage in flounders from the polluted site than in flounder from the reference site in almost all seasons. The authors suggested that flounders from the polluted site were subjected to a level of clastogenic agents enough to overwhelm the DNA repair mechanisms, generating irreversible genetic damages (mutations).

On the other hand, Amado et al. (2006a) found higher DNA damage assessed both by the comet assay and micronucleus test in winter croakers (*M. furnieri*) from a polluted site in the Patos Lagoon estuary. In the same study, summer croakers from polluted site had only higher levels of DNA strand breaks. The higher DNA damage values in summer croakers did not lead to a higher micronucleus frequency, probably because breaks detected by comet assay in this season were repairing in nature. It is known that breaks detected by the comet assay can be transiently present when cells repair lesions via base or nucleotide excision. Thus, a high level of breaks in the comet assay may indicate either high damage or an efficient repair process (Collins et al., 1997). In summary, results from these studies have suggested the usefulness of DNA damage assessment using the comet assay in combination with the micronucleus test to investigate the possible mechanisms of genotoxicity in fish.

Overall, biomarkers of DNA damage are valuable tools to assess effects of acute and chronic exposure of aquatic organisms to genotoxic substances. Moreover, as genotoxins may induce changes in DNA that are passed on to future generations, this kind of biomarker can be used in a predictive way, avoiding irreversible ecological consequences.

5.2. Immune responses

There is a general consensus that toxicants may cause detrimental effects on embryonic development and impact the physiological function of endocrine, reproductive, nervous and immune systems (Holsapple et al., 2004; Lathers, 2004; Ladics et al., 2005). Exposure of animals to toxic compounds may cause immunosuppression by direct mechanisms or through neuroendocrine interaction (indirect mechanisms) (Friedman and Lawrence, 2002). Therefore, biomarkers focused on innate immune functions in invertebrates and lower vertebrates are important targets for immunotoxicity research programs. The non-specific immune system is non-dependent of previous exposition to foreign antigens and is the first line of defense involved in inflammatory response. This process was first studied by Metchinikoff in 1882 (Vaughan, 1965), who observed the similarities between the inflammatory response in starfish and higher vertebrates, conceptualizing it as a conserved mechanism. Fish leukocytes are involved in nonspecific cellular defense, such as phagocytosis and phagocyte killing, through oxidative and non-oxidative mechanisms (Secombes, 1996). During phagocytosis, stimulated leukocytes produce reactive oxygen (ROS) and nitrogen intermediates with potent cytotoxic

activities (Neumann et al., 2001). The large increase in O_2 consumption during the ROS production is called "respiratory burst" (Bols et al., 2001), and has been employed as biomarker of toxicant exposure in aquatic environments (Fournier et al., 2000). Phagocyte attachment, ingestion and digestion or killing functions can be altered by several changes on the inside or outside milieu of metazoan organisms. The non-specific immune activity can also be influenced by other factors that are following discussed, as sex, age, sampling stress and parasitism, factors to be considered when immunotoxicology studies are being conducted.

Some studies showed that short-term stress can increase the non-specific immune response but chronic stress can impair this function (Forner et al., 1995; Ortega et al., 2005). Organisms that are exposed to aquatic contaminants shows impairment of oxidative burst when compared to animals not exposed (Amado et al., 2006a). Although it has been stated immunological injury in consequence of pollution exposure, there are yet questions about the sensitivity of oxidative burst and phagocytes as biomarkers.

Models of innate immunity against parasites in teleosts are particularly interesting because of the life history, biochemical and genetic complexity of the eukaryotic parasite compared with viral or bacterial pathogens, and also because of the potential for greater diversity in anti-parasitic mechanisms (Jones, 2001). The prevalence and abundance of parasites appears to be correlated with phagocytic activity and may provide an evaluation of animal health (Mustafa et al., 2000; Chaves et al., 2006). However, it must be noted that the first stages of parasite invasion are likely to evoke a stronger activation of macrophages than well-established or terminal infections (Muñoz et al., 1998). The potential use of parasites as bioindicators for pollution monitoring is widely and controversially discussed (Poulin, 1992; Kennedy, 1997) because many natural factors influence prevalence, infection intensity and biodiversity of parasites. Parasites may reflect the host environmental situations in numerous ways. For example, infection with monoxenous parasites can be use as a short-term bioindicators (Skinner, 1992), or under certain circumstances, reflect long-term immunosuppression effects. On the other hand, parasite species with heteroxenous life cycle represents indicators for long-term effect monitoring. The gradual reduction of biodiversity due to pollution may lead to a decrease in intermediate host species and can result in the extinction of one or more parasite species (Overstreet, 1997). The decrease in heteroxenous species diversity, prevalence and infection intensity may reflect a reduction in biodiversity to a wide extent. Therefore, their use for a long-term biomonitoring can reflect the processes in a known habitat over a longer time scale. In the last years, a marked increase in the use of marine parasites as potential monitors has been observed (MacKenzie et al., 1995). This is associated with the fact that they have a variety of life-cycle patterns with delicate developmental stages that, depending on the species, can infect most groups of animals previously used as indicators (Khan and Thulin, 1991; MacKenzie et al., 1995), and also because many ectoparasites species are sensible to pollutants present in the environment.

Changes in both parasite diversity and host metabolic parameters are probably a sudden reaction to a deterioration environmental factors, such as following a chronic exposure to anthropogenic xenobiotics. Williams and MacKenzie (2003) suggested the use of parasites together with a combined monitoring approach including the analysis of sediment contamination, infaunal community composition and laboratory bioassays, including immune parameters. Thus, a combination of metabolic and parasitological data may serve as a sensitive tool for pollution monitoring. Stressors may cause either increased or decreased intensity if infection depending on the host–parasite system under investigation (Williams and MacKenzie, 2003). Such effects on the dynamics of host–parasite system and disease are important complex factors that can only be safely interpreted by specialists on community ecology. Further work is required on the variable effects of pollutants on fish immune responses and their consequential effects on parasite populations.

6. Future perspectives: a Brazilian view

In the present review, several studies in environmental toxicology from Latin American researchers are cited, including the employment of ChE activity as an specific biomarker (Cunha Bastos et al., 1991; Monserrat and Bianchini, 2000, Monserrat et al., 2001, 2002; De La Torre et al., 2002; Barros et al., 2004; Rodríguez-Fuentes and Gold-Bouchot, 2004; Tortelli et al., 2006), the measurement of oxidative stress damage (Bainy et al., 1996; Geracitano et al., 2004a,b; Rosa et al., 2005a,b; Moraes et al., 2006), the effects of toxic metals on osmoregulatory responses (Bianchini and Castilho, 1999; Vitale et al., 1999; Bianchini and Gilles, 2000; Bianchini and Wood, 2003; Bianchini et al., 2002, 2004, 2005a,b) and even the determination of genotoxic and immune responses to monitor environmental pollution (Amado et al., 2006a,b). In Brazil, a large-scale environmental study was conducted under the umbrella of the RECOS project ("Uso e Apropriação de Recursos Costeiros") in the scope of the "Millenium Institute Program" (www.mileniodomar.org.br) from the Brazilian Ministry of Science and Technology. One of the objectives of this project was the standardization of sampling protocols, quantitative and qualitative evaluations of biochemical, physiological and histological biomarkers in different animal species collected from polluted and non-polluted sites. In this way, it can be concluded that there is a growing interest and necessity to conduct environmental studies.

From a scientific point of view, some studies carried out by Latinoamerican researchers fit with international paradigms. As stated in Section 1, the definition of biomarkers can also involve the measurement of behavioural parameters. The ecological relevance of parameters measured at the molecular, biochemical or cellular level is now considered an extremely important feature in environmental studies. Brazilian authors like Leal et al. (2006) have analyzed the effects of lead on mitogen-activated kinases (MAPKs) in the cerebellum of the catfish *Rhamdia quelen*, observing augmented levels of the phosphorylated forms of the extracellular signal-regulated protein kinases (ERK1/2) and $p38^{MAPK}$. As MAPKs drive several biological functions, including synaptic plasticity, the observed effects can be relevant at

higher organization levels, since it has been reported that lead can alter several behavioral parameters (Scott and Sloman, 2004; Leal et al., 2006).

Regarding the possibility of modeling the effect of pollutants in estuaries, it should be pointed out the effort that has been put into validating and calibrating the BLM for metals such as copper and silver using estuarine and marine animals. This model aims to perform risk assessments involving several metals and was originally developed with data available for freshwater animals. Recent studies, including those developed with the participation of Latin American researchers (Bianchini et al., 2004, 2005b), have focused on the possible application of the BLM using euryhaline animals as an attempt to extend this model for brackish and marine waters. Other issues of scientific relevance are related with particular environmental problems that have been recognized in several Latin America countries. The toxicity of arsenic (As) is especially relevant because of the scarcity of information about its molecular, biochemical and cellular effects on aquatic organisms. Furthermore, it should be stressed that some regions of Latin America like the Patos Lagoon (Southern Brazil) have areas with sediments enriched with arsenic (Mirlean et al., 2003). Arsenic bioavailability and toxicity depend on its chemical speciation, the inorganic forms (As^V and As^{III}) being more toxic, whereas methylated ones like methylarsenate (MMA), dimethylarsenate (DMA) and tetramethylarsonium (TETRA) are considered moderately toxic. Finally, chemical species like arsenobetaine (AsB), arsenocholine (AsC) and a family of arsenic-containing carbohydrates (arsenosugars) are considered non-toxic (Geiszinger et al., 2002; Fattorini et al., 2004). Interestingly, there are conspicuous differences in the chemical species accumulated by benthic organisms like polychaetes, as shown in Table 3. For example, the worm *Sabella spallanzanii* accumulates high amounts of moderately toxic compounds, DMA being the predominant form (almost 80% of the total accumulated As) (Fattorini et al., 2004). However, other polychaete species like Arenicola marina showed an unusual feature, accumulating more toxics forms such as As^{III} and As^V (Geiszinger et al., 2002). Finally, a typical estuarine species from the Patos Lagoon estuary, the polychaete *L. acuta* also showed an expressive accumulation of inorganic compounds (Table 3). When exposed to a concentration previously considered safe by the Brazilian regulations (50 μg of As/l), this worm showed increased SOD activity and higher levels of LPO, suggesting that *L. acuta* is under oxidative stress after As exposure (Ventura-Lima et al., in press).

Table 3
Concentration and percentage contribution of arsenic compounds in different polychaete species

Chemical form	Species		
	Sabella spallanzanii[a]	*Arenicola marina*[b]	*Laeonereis acuta*[c]
DMA	83.0%	4.0%	29.6%
AsB	8.7%	6.0%	28.0%
AsC	4.6%	<1.0%	6.7%
TETRA	4.0%	1.5%	7.0%
iAs	ND	As^{III}: 16.0% As^V: 58.0%	22.5%

Concentrations are expressed as a percentage of total As content.
iAs: inorganic arsenic (both As^{III} and As^V); MMA: methylarsonate; DMA: dimethylarsinate; AsB: arsenobetaine; AsC: arsenocholine; TETRA: tetramethylarsonium. ND: not determined.
[a] Data from Fattorini et al. (2004).
[b] Data from Geiszinger et al. (2002).
[c] Data from Ventura-Lima et al. (in press).

As mentioned in the Introduction, the variability in abiotic parameters in estuarine regions can affect in multiple ways several biochemical and physiological responses. Fig. 1 shows some of the possible interactions between abiotic and biotic factors. For example, note the need for osmoregulation should augment the demand for ATP, triggering ROS production at the mitochondrial level and this could synergize the effect of metals like copper that can affect key enzymes for osmoregulation and also promotes oxidative stress (Fig. 1). Also, the occurrence of cyanobacteria blooms can: (1) release toxins, including organosphosphorus-like molecules (Monserrat et al., 2001), (2) induce hypoxia/hyperoxia cycles (Rosa et al., 2005a) and, (3) produce and release ROS ($O_2^{\cdot-}$ and H_2O_2), as reported for some phytoplanktonic organisms (Kim et al., 2002) (Fig. 1). In this way, the complexity of responses and interactions deserves more holistic approaches, including the use of proteomic techniques in toxicological sciences, *i.e.* "toxicoproteomics" (Kennedy, 2002) and environmental sciences, *i.e.* "environmental proteomics" (López-Barea and Gómez-Ariza, 2006). Its employment in environmental toxicology began 6 years ago using invertebrate (*Mytilus edulis*) and fish (*Oncorhynchus mykiss*) species, allowing the identification of toxic responses to nonylphenol under field conditions (Shepard and Bradley, 2000; Shepard et al., 2000). *Chamaelea gallina*, another mollusc species, has been employed as model organism to evaluate the protein expression signatures after experimental exposure to several environmental pollutants (Rodríguez-Ortega et al., 2003). Also, oxidative stress responses (protein oxidation) were employed in environmental studies that revealed higher levels of carbonylated proteins in gill and digestive gland of *M. edulis* sampled at polluted areas (McDonagh et al., 2005). Note that, as stressed Fig. 1, pollutants – and their interaction with environmental variables – can alter the balance between pro-oxidants and antioxidants through several pathways, leading to oxidative stress generation. The examples mentioned above and others not discussed here point to the need for major efforts in the next years to analyze environment quality in different regions of Latin America, where economic development is not always paralleled to environment protection (Krüger, 2001).

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THE CROAKER *MICROPOGONIAS FURNIERI* AS BIOINDICATOR OF TRACE METAL POLLUTION IN THE PATOS LAGOON ESTUARY (SOUTHERN BRAZIL). PART I: TISSUE METAL ACCUMULATION

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ABSTRACT

Seasonal concentrations of Cu, Cd, Zn and Pb were analyzed in liver and gills of juvenile *Micropogonias furnieri* from different sites of the Patos Lagoon estuary (Southern Brazil). In each season, no significant difference was observed between sites of collection or sex. However, significant seasonal differences were observed when data for males and females were taken together. In both tissues, the higher metal concentrations were observed in winter and spring, reflecting the clear seasonal changes in metal concentrations observed in previous studies on water and sediments of the Patos Lagoon estuary. This finding combined with those from biomarker responses to metal accumulation reported in a companion paper, strongly suggest the use of juvenile white croakers *M. furnieri* as a potential bioindicator of trace metal pollution for future biomonitoring programs in the Patos Lagoon estuary (Southern Brazil).

Key words: *Micropogonias furnieri*; croaker; Patos Lagoon; estuary; trace metals.

INTRODUCTION

The majority of aquatic pollutants originated from anthropogenic activities is directly or indirectly transported to coastal areas. Therefore, estuaries are sites of critical contamination, because they receive discharge of a great variety of compounds from rivers that compose the drainage basin (Nipper, 2000; Corsi *et al*., 2003). Also, toxic compounds originated from harbor activities are usually released into estuarine waters (Baumgarten and Niencheski, 1998). Moreover, many pollutants are transferred and accumulated along the food chains, indirectly threatening the health of their consumers that can be as much aquatic organisms as human beings (Rodriguez-Ariza *et al*., 1999).

The Patos Lagoon estuary (32º01'S - 52º05'W, Southern Brazil) stands not only for its ecological importance associated with productivity and biodiversity, but also for its socio-economical relevance related to intense industrial, harbor, agricultural, fishing, and tourist activities (Asmus and Tagliani, 1998). However, its ecological equilibrium has been menaced by the increasing organic and inorganic pollution caused by population growth and expansion of the industrial area associated with the Rio Grande City (Santos *et al*., 1997). Although non-polluted areas can be found (Baumgarten and Niencheski, 1990; 1998), some sites are presenting several ecotoxicological problems (Baumgarten *et al*., 1998; Geracitano *et al*., 2004; Amado *et al*., 2006a; 2006b).

Previous studies on metal contamination in the Patos Lagoon estuary generally considered the chemical analysis of abiotic compartments (Baumgarten *et al*., 1998; Windom *et al*., 1999; Niencheski and Baumgarten, 2000) separated from the toxicological responses (Geracitano *et al*., 2004; Amado *et al*., 2006a; 2006b). However, it is well known that the use of aquatic animals as monitors of metal pollution brings several advantages compared to

chemical analyses of abiotic compartments. Animals accumulate only the biologically available forms of metals over the whole time of exposure, making possible the continuous monitoring of the presence of these contaminants.

In the present study, concentration of metals (Cu, Cd, Zn and Pb) was determined in tissues (liver and gills) of the white croaker *Micropogonias furnieri* (Desmarest, 1823) collected at different sites of the Patos Lagoon estuary. Possible spatial, biometric, sexual and seasonal variations in metal tissue concentrations were considered. The use of the white croaker as a biomonitor of metal contamination in estuaries is then discussed considering the results obtained. Results from the present study were correlated with the response of several biochemical, genetic and immunological biomarkers reported in a companion paper (Chaves *et al.*, 2008).

The white croaker *M. furnieri* was selected for the present study and that reported in the companion paper (Chaves *et al.*, 2008), considering its wide distribution, ecological role, and economical importance in coastal fisheries of Brazil, Uruguay and Argentina. In the Patos Lagoon estuary, *M. furnieri* is the most abundant species caught by artisanal fishery (Reis and Pawson, 1999), being classified as estuarine dependent (Vieira *et al*., 1998).

MATERIAL AND METHODS

Fish collection

Juvenile white croakers were seasonally collected (2006-2007) at five different sites of the Patos Lagoon estuary (Fig. 1), which are historically characterized by the presence of different types and levels of pollutant (Baumgarten and Niencheski, 1998; Barbosa, 2007). A minimum of 8 croakers (8-15 cm) was collected in each site and season, totalizing 277 fish.

Croaker collection was performed for 5 min in shallow waters of each selected site using a trawl-net. Croakers were caught and immediately anesthetized with 10% benzocaine. Blood samples were collected by puncture of the caudal artery using a 1-ml disposable heparinised syringe with hypodermic needle 27/7 and stored in plastic tubes on ice, until analysis of DNA damage and immunological responses analyses (see companion paper; Chaves *et al.*, 2008). Croakers were transferred to the laboratory in plastic bags on ice, measured (total body length), and had their liver and gills dissected, labeled, and stored at -80ºC, until metal concentration (present study) and biomarkers analyses (see companion paper; Chaves *et al.*, 2008).

At the time of fish collection, water chemistry parameters (salinity and temperature) were measured in triplicate at each season and site of collection.

INSERT FIGURE 1

Metal concentration analysis

Gills and liver samples of a minimum of 5 croakers collected at each sampling site in each season were randomly selected for metal concentration analysis, totalizing 118 fish. Tissue samples were individually weighed, dried at 70ºC, and weighed (dry weight). Dried tissues were digested with nitric acid (Suprapur, Merck, USA) for two days, followed by a dilution with MilliQ water. Metal concentration (Cu, Cd, Zn and Pb) was determined in triplicate by atomic absorption spectrophotometry (GBC-AAS 932 Plus, IL, USA), as previously described (Pedroso *et al.*, 2007; Pinho *et al.*, 2007). Metal concentration in tissues was expressed as μg/g dry weight.

Statistical Analyses

Data were expressed as mean ± standard error. Significant differences between treatments (sex, sites of collection, seasons) were assessed by analysis of variance (ANOVA) followed by the Tukey's test ($\alpha = 0.05$). ANOVA assumptions (normality and variance homogeneity) were previously checked. Possible correlations between croaker total body length and tissue metal concentrations were checked using the Spearman correlation index ($\alpha = 0.05$).

RESULTS

Table 1 summarizes mean temperature and salinity data for each season of fish collection. The highest mean temperature and salinity values were observed in summer, while the lowest ones in winter.

INSERT TABLE 1

When all data were analyzed by sex, no significant correlation was observed between metal tissue concentration (liver and gills) and the total body length of male (6.7-15.5 cm; 10.9 ± 0.29 cm; n = 51) or female croaker (7.3-15.1 cm; 11.39 ± 0.24 cm; n = 67). When data for males and females were taken together, no significant difference was observed in metal tissue concentrations (liver and gills) between sites of collection at each season (data not shown). Therefore, data for males or females were grouped by season considering the five different sites of collection as a unique area of the Patos Lagoon estuary (Table 2). Also, no significant difference in the tissue (liver and gills) metal concentration was observed between male and female croakers collected in the same season. Therefore, data for male and females were grouped by season. In this case, significant and marked seasonal variations in metal concentrations were observed in both liver (Fig.2) and gills (Fig. 3).

INSERT TABLE 2

INSERT FIGURE 2

INSERT FIGURE 3

In liver, significantly higher mean Cu, Cd and Zn concentrations were observed in croakers collected in spring. Also, a similar mean higher value for Cu was found in winter. Lower mean values of Cu and Zn were observed in croakers collected in summer. No significant seasonal variation was observed in liver Pb concentration. Overall mean values along the year (n = 118) for Cu, Cd, Zn and Pb were 68.18 ± 13.88; 1.03 ± 0.18; 103.89 ± 10.51; and 22.90 ± 4.93 μg/g dry weight, respectively (Fig.2).

In gills, higher mean Cu and Pb concentrations were observed in croakers collected in winter, while a higher mean Cd concentration was observed in those collected in spring. For Zn, higher mean values were found in croakers collected in autumn and spring. Overall mean values along the year (n=118) for Cu, Cd, Zn and Pb were 4.04 ± 0.34; 0.57 ± 0.04; 63.72 ± 4.74; 22.31 ± 2.24 μg/g dry weight, respectively (Fig. 3).

In general, croakers collected in summer always showed lower metal burdens in both liver (Fig. 2) and gills (Fig. 3).

DISCUSSION

Salinity and temperature values measured in the present study at the different croaker sampling sites are quite similar to those previously reported in other studies performed in the Patos Lagoon estuary (Niencheski and Baumgarten, 2000; Barbosa, 2007). Seasonal variations in these parameters are typical from temperate estuaries, with high and low mean salinity and temperature values in summer and winter, respectively. Despite these marked

seasonal variations, the white croaker *M. furnieri* uses, along the year, the totality of the estuarine area, showing a wide adaptability to this environment (Castello, 1986).

Juveniles and adults *M. furnieri* are benthonic consumers in mud and sand bottoms, with preferences for small invertebrates and, in smaller proportion, for small fish, conferring to this species a great importance in the food chain of the Patos Lagoon estuary (Vieira *et al.*, 1998). Therefore, data observed in the present study for metal tissue burden could be interpreted as an integrated response of higher ecological relevance.

Previous studies suggested that juvenile *M. furnieri* shows preference in exploring shallow waters of the Patos Lagoon estuary (Castello, 1986). In the present study, juvenile croakers were always collected in shallow waters, but at different sites of the Patos Lagoon estuary showing different historic of metal contamination in water and sediments (Niencheski and Baumgarten, 2000; Barbosa, 2007). The fact that no significant differences in liver or gill metal accumulation were observed in fish collected at the different sites suggests that juvenile croakers *M. furnieri* show a marked displacement around shallow waters from different areas of the Patos Lagoon estuary.

Concentrations of trace metals observed in gills and liver of *M. furnieri* from the Patos Lagoon estuary were relatively low, as compared to those reported by other studies with related species in different ecosystems of the world (Nesto *et al.*, 2007; Fernandes *et al*., 2008). However, these values were quite similar to those reported for *Mugil dussumerii* from estuaries of Thailand (Menasveta and Cheevaparanapiwat, 1981), for *Mugil* sp. and *Micropogonias* sp. from Sepetiba Bay (Brazil) (Pfeiffer *et al*., 1985), and *M. furnieri* from the La Plata river estuary (Marcovecchio, 2004).

Considering the mean value over the year, data from the present study shows that liver accumulated more Cu (16.8-fold), Cd (1.8-fold) and Zn (1.6-fold) than gills. On the other

hand, no significant difference was observed for Pb. These findings are in agreement with the fact that fish exposed to metal shows a pattern of accumulation in one tissue varying from metal to metal (Huang *et al*., 2007) and from organ to organ for the same metal (Hollis *et al.*, 2001; Elia *et al.,* 2003). It is also important to consider that the amount of each metal accumulated in a particular tissue could be a result of fish co-exposure to more than one metal (De Conto Cinier *et al*., 1997), as analyzed in the present study using field collected croakers.

Aquatic contamination with trace metals is of utmost environmental significance, since many studies report the occurrence of bioaccumulation of these contaminants, especially in polluted areas associated with urban activities (Marcovecchio *et al.,* 1986; Fernandes *et al*., 2008). The Patos Lagoon estuary is not an exception, showing increased levels of metal fraction potentially available for bioaccumulation in some euthrophized or polluted areas with urban residues (Baumgarten *et al*., 1998). The estuarine area, where croakers employed in the present study were sampled, receives larger contribution of domestic, pluvial and mixed effluents (Almeida *et al.*, 1993; Baumgarten *et al*., 1998), together with the significant influence of harbor activities. It shows a smaller rate of water renewal than that closest to the entrance of the channel communicating the lagoon to the sea. This indicates a higher contribution of freshwater and its low mixture with seawater. Furthermore, the larger residence time of waters causes a higher retention of elements in the studied area, including metals (Barbosa, 2007).

Zinc is one of the most abundant elements in domestic sewers, which also contribute as source of Cd for the aquatic environment (Baumgarten *et al*., 1998; Barbosa, 2007). According to Baumgarten *et al*. (1998), potential sources of Zn to the Patos Lagoon estuary are domestic effluents, lixiviation of the drainage basin, and harbor activities. Industries of fertilizers are also contributing through discharges of mineral residues incrementing the level

of metals in the estuarine environment. According to Baisch *et al*. (1988), bottom sediments from the south area of the Patos Lagoon estuary revealed a larger contamination for metals. High values of Cd were observed along the industrial and harbor areas, while high values of Zn, Cu and Pb were associated with the urban and industrial sources. The fact that these metals are clearly accumulated in both liver and gills of white croakers, as observed in the present study, is in complete agreement with results from the chemical studies mentioned above for abiotic compartments.

Previous studies on the quality of water and sediments of the Patos Lagoon have shown clear evidences that higher concentration of metallic elements prevails in winter, when the level of the lagoon is usually higher, hindering the entrance of the coastal water, and resulting in the predominance of low salinity waters (Möller *et al.*, 2001). According to Barbosa (2007), a larger decrease in Cu, Cd and Pb concentration occurs in summer, as a consequence of the largest dispersion of the pollutant favored by the entrance of sea water that occurs in this season and can extends to autumn. The fact that the croakers collected in summer always showed lower levels of these metals in both liver and gills is in complete agreement with the seasonal pattern of the metal distribution described in surface waters from the Patos Lagoon estuary (Barbosa, 2007).

The use of fish as biological indicators in programs of pollution monitoring in aquatic environments is widely recognized (Reddy *et al*., 2001; Corsi *et al*., 2003). However, the use of biological indicators of pollution is only efficient when some important aspects are considered when selecting a suitable bioindicator (Markert, 2007). In this context, it is important to note that basic information on biological and ecological aspects of *M. furnieri* are available. Also, considering *M. furnieri* abundance all year round in estuarine waters, its non-migrating nature, easy sampling and identification (Vazzoler, 1991; Vieira *et al.*, 1998;

Reis and Pawson, 1999), capability of bioaccumulating metals, and capability of providing information on the quality of the environment, as well as its changes over seasons, as observed in the present study, we strongly suggest the use of the juvenile white croaker *M. furnieri* as a potential bioindicator of trace metal pollution for future biomonitoring programs in the Patos Lagoon estuary (Southern Brazil). This suggestion is strongly supported by the observed responses in biochemical, genetic and immunological biomarkers reported in the companion paper (Chaves *et al.*, 2008).

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Figure Captions

Figure 1. Sites of collection (A-E) of juvenile white croaker *Micropogonias furnieri* (Teleostei: Scianidae) at the Patos Lagoon estuary. Marinheiros Island (A); Rio Grande Inlet (B); Porto Velho (C); Porto Novo (D); Coroa do Boi (E).

Figure 2. Seasonal variation in Cu (A), Cd (B), Zn (C), and Pb (D) concentrations in liver of *Micropogonias furnieri* from the Patos Lagoon estuary (Southern Brazil). Data are means ± standard error. Different letters indicate significantly different means ($p < 0.05$). n: number of fish analyzed.

Figure 3. Seasonal variation in Cu (A), Cd (B), Zn (C), and Pb (D) concentrations in gills of *Micropogonias furnieri* from the Patos Lagoon estuary (Southern Brazil). Data are means ± standard error. Different letters indicate significantly different means ($p < 0.05$). n: number of fish analyzed.

Table 1. Water temperature and salinity registered during croaker sampling period (2006-2007) in the Patos Lagoon estuary (Southern Brazil). Data are expressed as mean ± standard error. n: number of measurements.

Season	Temperature (ºC)	Salinity	n
Summer	24.25 ± 0.37	15.75 ± 3.27	8
Autumn	20.00 ± 1.03	6.00 ± 1.34	5
Winter	16.33 ± 1.48	1.92 ± 0.27	6
Spring	23.80 ± 0.58	7.00 ± 3.30	5

Table 2. Metal concentration (μg/g dry weight) in liver and gills of *Micropogonias furnieri* from the Patos Lagoon estuary (Southern Brazil). Data are means ± standard error. n: number of fish. For all metals, no significant difference was observed between sex in each season.

Liver						
Season	Sex	Cu	Cd	Zn	Pb	n
Summer	Male	28.52 ± 4.51	0.69 ± 0.29	76.51 ± 5.13	16.60 ± 3.49	12
	Female	31.17 ± 6.07	0.44 ± 0.15	83.64 ± 9.54	19.61 ± 3.65	17
Autumn	Male	50.58 ± 8.53	0.53 ± 0.11	114.29 ± 9.19	18.51 ± 2.35	17
	Female	40.27 ± 8.96	0.28 ± 0.05	102.91 ± 10.77	21.15 ± 3.39	16
Winter	Male	107.41 ± 34.01	0.39 ± 0.14	86.48 ± 15.11	17.30 ± 3.99	9
	Female	121.37 ± 20.64	0.60 ± 0.21	101.32 ± 10.71	21.57 ± 3.26	16
Spring	Male	82.90 ± 15.25	1.99 ± 0.33	134.82 ± 12.16	23.19 ± 4.14	13
	Female	83.22 ± 13.13	3.07 ± 0.56	131.15 ± 11.55	19.31 ± 3.83	18
Gills						
Season	Sex	Cu	Cd	Zn	Pb	n
Summer	Male	3.16 ± 0.41	0.41 ± 0.04	57.75 ± 5.97	20.06 ± 2.22	12
	Female	3.89 ± 0.38	0.45 ± 0.03	57.24 ± 3.44	21.39 ± 1.78	17
Autumn	Male	4.35 ± 0.27	0.57 ± 0.04	71.35 ± 6.93	20.04 ± 1.81	17
	Female	4.12 ± 0.28	0.62 ± 0.07	69.49± 3.65	19.28 ± 2.98	16
Winter	Male	4.92 ± 0.34	0.34 ± 0.05	58.76 ± 3.89	23.90 ± 2.17	9
	Female	5.39 ± 0.52	0.47 ± 0.04	63.70 ± 6.59	27.44 ± 2.58	16
Spring	Male	3.73 ± 0.30	0.94 ± 0.08	73.90 ± 4.40	24.58 ± 0.92	13
	Female	2.82 ± 0.28	0.82 ± 0.04	57.58 ± 3.10	21.80 ± 3.51	18

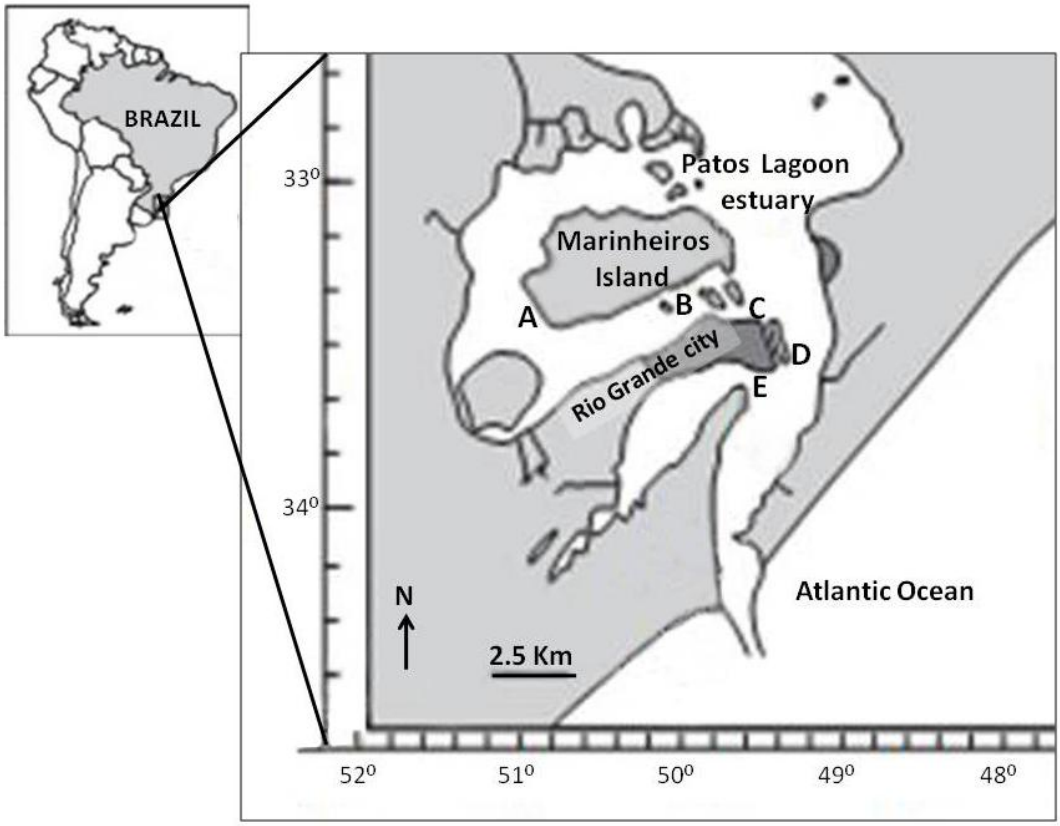
BRAZIL
33º
34º
Patos Lagoon
estuary
Marinheiros
Island
A
B
C
D
E
Rio Grande city
N
2.5 Km
Atlantic Ocean
52º
51º
50º
49º
48º

Figure 1

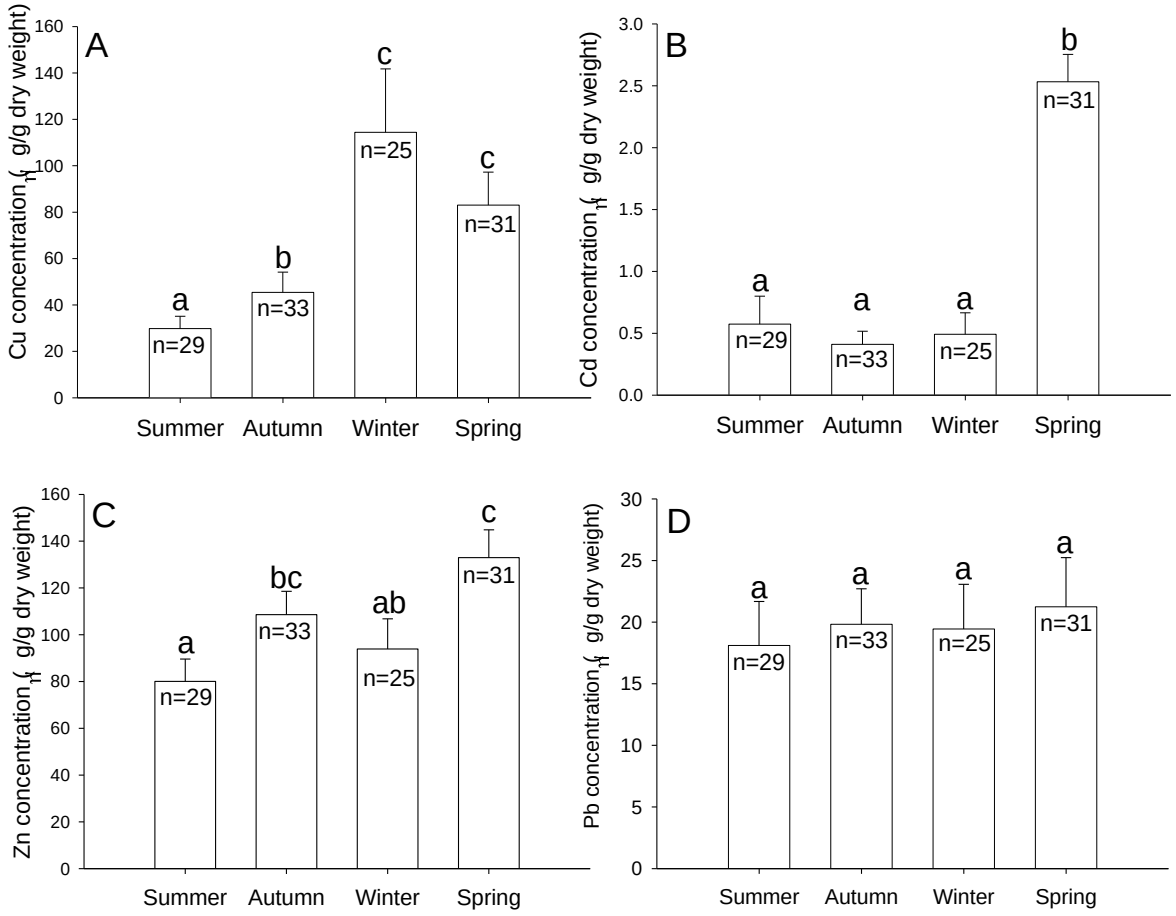
A
Cu concentration (μg/g dry weight)
160
140
120
100
80
60
40
20
0
a
b
c
c
n=29
n=33
n=25
n=31
Summer
Autumn
Winter
Spring
B
Cd concentration (μg/g dry weight)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
b
n=29
n=33
n=25
n=31
Summer
Autumn
Winter
Spring
C
Zn concentration (μg/g dry weight)
160
140
120
100
80
60
40
20
0
a
bc
ab
c
n=29
n=33
n=25
n=31
Summer
Autumn
Winter
Spring
D
Pb concentration (μg/g dry weight)
30
25
20
15
10
5
0
a
a
a
a
n=29
n=33
n=25
n=31
Summer
Autumn
Winter
Spring

Figure 2

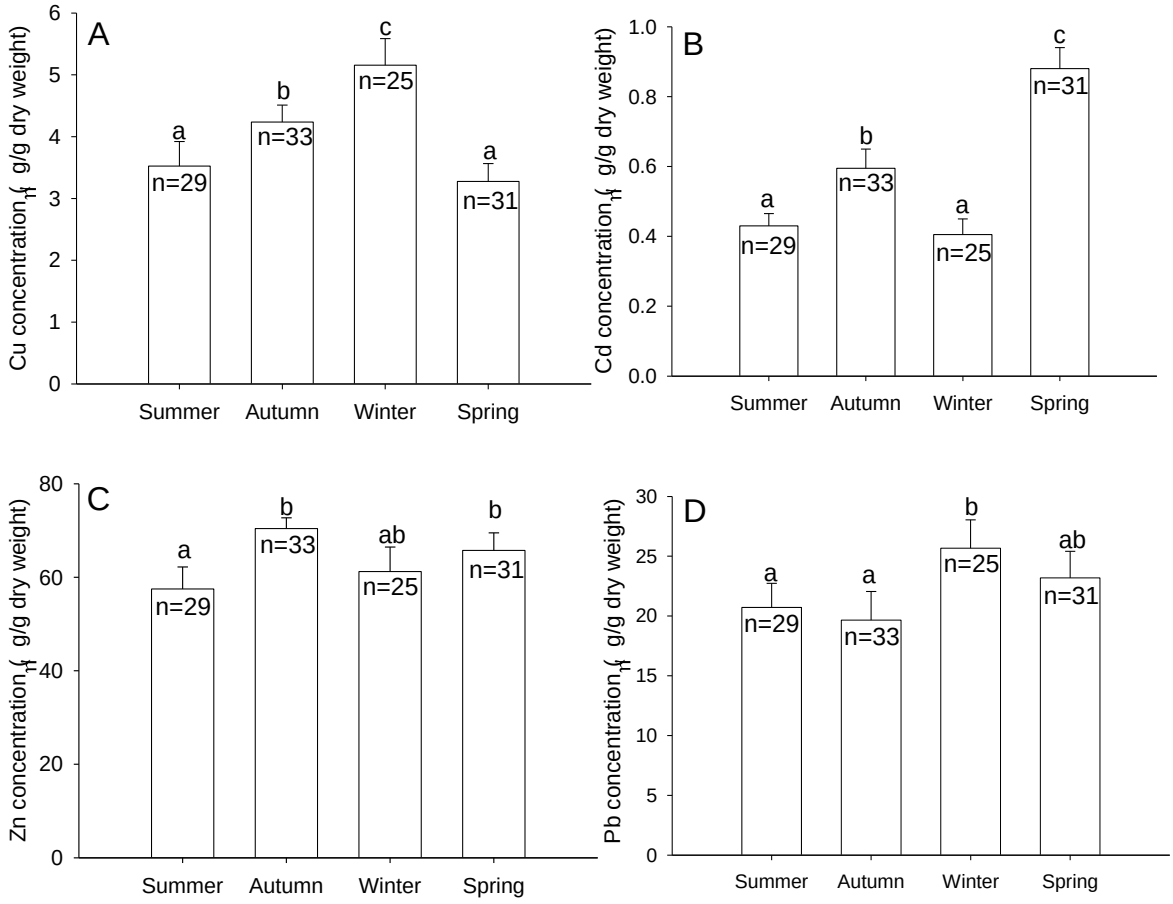
A
Cu concentration (μg/g dry weight)
a
b
c
a
n=29
n=33
n=25
n=31
Summer
Autumn
Winter
Spring
B
Cd concentration (μg/g dry weight)
a
b
a
c
n=29
n=33
n=25
n=31
C
Zn concentration (μg/g dry weight)
a
b
ab
b
n=29
n=33
n=25
n=31
D
Pb concentration (μg/g dry weight)
a
a
b
ab
n=29
n=33
n=25
n=31

Figure 3

THE CROAKER *MICROPOGONIAS FURNIERI* AS BIOINDICATOR OF TRACE METAL POLLUTION IN THE PATOS LAGOON ESTUARY (SOUTHERN BRAZIL). PART II: BIOMARKER RESPONSES

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ABSTRACT

Seasonal variations in biochemical (liver and gill metallothionein-like proteins [MTLP] and lipid peroxidation), genetic (micronucleated erythrocytes) and immunological (leukocytes phagocytosis and respiratory burst) biomarkers were evaluated in juvenile croakers *Micropogonias furnieri* from the Patos Lagoon estuary (Southern Brazil). All biomarkers analyzed showed significant seasonal variations. Present results combined with those on tissue metal accumulation reported in the companion paper indicate that the seasonal change in liver MTLP concentration is positively correlated with liver Cu and Zn accumulation. In turn, seasonal variation in gill MTLP concentration seems to be associated with gill Cd accumulation. Seasonal changes in the other biomarkers indicate that croakers are more susceptible to metal contamination in winter and spring, when their protective capability is significantly reduced and the metal tissue burdens are significantly elevated. Under this situation, generally higher levels of liver and gill lipid peroxidation, erythrocyte DNA damage and immunossupression were observed.

Key words: *Micropogonias furnieri*; biomarkers; metals; metallothionein; DNA damage; immune responses.

INTRODUCTION

Despite the recognized importance of studies considering the concentration of pollutants in abiotic and biotic compartments, it is crucial to detect and evaluate the biological impact of these contaminants on the exposed organisms. This need stimulated the development of biomarkers, which are biological alterations expressing the exposure and toxic effects of pollutants present in the environment (Livingstone, 1993). Actually, several biochemical, genetic and immunological parameters have been used as biomarkers in different fish species (Van der Oost *et al*., 2003; Amado *et al*., 2006; Monserrat *et al*., 2007).

Important alterations in biological responses were observed in white croakers *Micropogonias furnieri* collected at a highly polluted semi-enclosed bay from the Patos Lagoon estuary (Southern Brazil) (Amado *et al*., 2006). The white croaker is an estuarine dependent species exclusively occurring in the Patos Lagoon estuary at the juvenile stage, exploring the totality of the estuarine environment along the year (Vieira *et al.,* 1998).

The estuarine portion of the Patos Lagoon is characterized by different environments, presenting open areas (1 to > 5 m deep) and semi-enclosed bays of shallow waters (< 1 m deep) (Calliari, 1998). Juvenile white croakers clearly show preference in exploring shallow waters of the Patos Lagoon estuary (Castello, 1986). However, semi-enclosed bays show smaller rate of water renewal than the open areas, leading to a lower mixture of seawater with freshwater and a larger residence time of waters, causing a higher retention of elements, including metals **(**Möller *et al*., 2001; Barbosa, 2007). Therefore, croakers living in the shallow waters would be more likely subject to the estuarine impact of aquatic pollutants present in semi-enclosed bays of the Patos Lagoon estuary. In fact, we have described in the companion paper that juvenile white croakers from these areas significantly accumulated metals (Cu, Cd, Zn and Pb) in liver and gills (Chaves and Bianchini, 2008), reflecting the

seasonal pattern of metal distribution in water and sediment of the Patos Lagoon estuary (Chaves and Bianchini, 2008).

In light of the above, the aim of the present study was to investigate the seasonal pattern of some biochemical, genetic and immunological biomarkers in juvenile white croaker *M. furnieri* from different sites of the Patos Lagoon estuary, and check what biomarker(s) could be reflecting the seasonal changes in metal bioaccumulation reported in the companion paper (Chaves and Bianchini, 2008).

MATERIAL AND METHODS

Tissue (liver and gills) samples of juvenile croakers collected as described in the companion paper (Chaves and Bianchini, 2008) were employed for biomarkers analyses.

Metallothionein-like proteins

Metallothionein-like proteins (MTLP) concentration was determined in liver and gill samples using the method described by Viarengo *et al*. (1997). Briefly, samples were homogenized in a cold buffer solution containing sucrose (500 mM), Tris–HCl (20 mM), PMFS (0.5 mM), and β-mercaptoethanol (0.01%) as reducing agent. The pH was adjusted to 8.60. Sample absorbance (412 nm) was determined using 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB; 0.43 mM; from Sigma, USA) in a microplate reader (Bio-Tek ELX-800, USA). Solutions of different glutathione (GSH) concentrations (0 to 500 μM) were employed as standards. MTLP concentration was expressed in terms of GSH equivalents (μmol GSH/g wet tissue).

Lipid peroxidation

Lipid peroxidation (LPO) was determined in liver and gill samples employing the thiobarbituric acid reactive substances (TBARS) assay described by Oakes and Van der Kraak (2003). Briefly, samples were homogenized in a 1.15% KCl solution containing 35 mM butylated hydroxytoluene (BHT). The homogenate was added to the reaction mixture containing 12.4 mM sodium dodecyl sulfate (SDS), 0.8% 2-thiobarbituric acid (TBA), 20% acetic acid adjusted to pH 3.5 with NaOH, and double distilled water (ddH_2O). An additional BHT solution (67 mM) prepared in ethanol was added prior to heating the mixture in a water bath (95ºC) for 60 min. After cooling, Milli-Q water and n-butanol were added under thorough stirring. Fluorescence of the immiscible organic layer was measured (excitation = 515 nm; emission = 553 nm) using a fluorometer (Victor^2, PerkinElmer, USA). TMP (1,1,3,3-tetramethoxypropane) was used as an external standard. LPO was expressed as nmol TMP/mg wet weight.

Micronucleus

Micronucleus test was performed in erythrocytes as described by Hooftman and de Raat (1982). A drop of croaker blood was smeared on microscope slides, air-dried, fixed with methanol for 10 min, stained with 5% Giemsa (Merck, USA) in phosphate buffer (60 mM KH_2PO_4 and 60 mM Na_2HPO; pH 6.8) for 20 min, washed with distilled water, and air-dried. The relative frequency of micronucleated cells was evaluated under light microscope (1000 x magnification) by scoring an average of 2,000 mononucleated erythrocytes per slide.

Immunological responses

Phagocytosis and respiratory burst measurements were carried out on peripheral blood leukocytes. Firstly, erythrocytes were lysed with NH_4Cl (155 mM) and leukocytes were suspended in Hank's Buffered Salt Solution (HBSS) supplemented with Roswell Park

Memorial Institute medium (RPMI 1640), heparin sodium, and heat-inactivated fetal bovine serum. They were counted in a Neubauer hemocytometer and cell viability was always >90%, as determined by the Trypan blue exclusion test.

The phagocytosis test was adapted from a flow citometry method described by Chilmonczyk and Monge (1999). An aliquot of 10^7 cells was incubated for 1 h in a multiwell-plate to allow leukocytes to adhere. After adherence to the plate, the supernatant was removed and HBSS was added. Cells were incubated with fluorescein-labelled opsonized *Escherichia coli* bacteria for 1 h (22ºC) to proceed with phagocytosis. Wells were quickly rinsed with Trypan blue solution as a quenching solution, and immediately washed with HBSS. Fluorescence was read (excitation = 485 nm; emission = 530 nm) using the fluorometer (Victor^2, PerkinElmer, USA).

Respiratory burst measurement was performed using the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay based on Brubacher and Bols (2001). An aliquot of 10^7 cells was incubated for 1 h (22ºC) in a multiwell-plate to allow leukocytes to adhere. After adherence to the plate, the supernatant was removed, and HBSS was added. Cells were incubated with 10 μM H_2DCFDA and 30 nM phorbol-12-myristate-13-acetate (PMA) as a triggering agent. Fluorescence was read (excitation = 485 nm; emission = 530 nm) after 40 min using a fluorometer (Victor^2, PerkinElmer, USA).

Statistical Analysis

All results were expressed as mean ± standard error. Significant differences between croakers collected at different sites and seasons were assessed by analysis of variance (ANOVA) followed by the Tukey's test (α =0.05). ANOVA assumptions (normality and variance homogeneity) were previously checked. Spearman correlation index was used to verify possible correlations between the different parameters analyzed. Data on liver and gill

metal accumulation reported in the companion paper for 118 (Chaves and Bianchini, 2008) out of the 277 croakers analyzed in the present study were also used for correlation analysis. All statistical analysis was performed using the software Statistica 7 (StatSoft 2004, Tulsa, OK, USA).

RESULTS

Water chemistry and fish morphometric data

Higher temperature and salinity values were registered in summer, while the lower ones were observed in winter (see companion paper; Chaves and Bianchini, 2008). No significant difference was observed in mean total body length of juvenile croakers collected in different seasons (Table 1).

Biomarkers

For all biomarkers analyzed, no significant differences were observed between sampling sites at each season. Therefore, data were grouped considering the five sampling sites as a unique environment of the Patos Lagoon estuary.

Significant seasonal variations ($P<0.05$) in liver and gill MTLP concentrations were observed. In liver, lower (1.46 ± 0.07 μmol GSH/g wet tissue) and higher (3.16 ± 0.17) values were observed in spring and autumn, respectively (Fig. 1A). In gills, those values were observed in winter (0.21 ± 0.04 μmol GSH/g wet tissue) and summer (0.80 ± 0.04 μmol GSH/g wet tissue), respectively (Fig. 1B).

A significant ($P<0.05$) seasonal variation was observed in LPO in both liver and gills of juvenile croakers. In liver, lower (0.52 ± 0.06 nmol TMP/mg wet tissue) and higher (1.29 ± 0.12 nmol TMP/mg wet tissue) values were observed in summer and spring, respectively (Fig.

2A). In gills, lower (0.19 ± 0.01 nmol TMP/mg wet tissue) and higher (0.33 ± 0.04 nmol TMP/mg wet tissue) values were observed in spring and autumn, respectively (Fig. 2B). A significant positive correlation (*r=0.44*) was observed between liver and gill LPO values.

The relative frequency of micronucleated erythrocytes was significantly higher (*P<0.05*) in juvenile croakers collected in spring. Mean lower (1.28 ± 0.14) and higher (1.95 ± 0.17) values were observed in autumn and spring, respectively (Fig. 3). This biomarker showed negative and significant correlations with liver MTLP (*r = -0.31*) and respiratory burst (*r = -0.33*) values.

Leukocyte phagocytosis (PG) (Fig. 4A) and respiratory burst (RB) (Fig. 4B) showed significant seasonal variations (*P<0.05*). For both biomarkers, lower values (PG = $6.68x10^3$ ± $0.60x10^3$; RB = $309.95x10^3$ ± $33.25x10^3$) were observed in winter, while the higher ones (PG = $31.05x10^3$ ± $2.86x10^3$; RB = $663.73x10^3$ ± $57.28x10^3$) were registered in autumn.

Significant and negative correlations were observed between phagocytosis values and Zn *(r=-0.48)* or Pb *(r=-0.59)* concentration in liver, and Cu *(r=-0.64),* Zn *(r=-0.55)* or Pb *(r=-0.70)* concentration in gills. Also, negative and significant correlations were observed between respiratory burst values and Cu *(r=-0.54)* or Zn *(r=-0.46)* concentration in liver, and Cu *(r=-0.39)* concentration in gills.

For all biomarkers analyzed, no significant correlation was observed between biomarkers values and the water chemistry or fish biometric data, except for the relative frequency of micronucleated cells that showed a significant a significant negative correlation *(r=-0.48)* with water salinity values.

Discussion

Metallothioneins (MT) are a family of low molecular weight cytosolic, cysteine-rich proteins (Langston, *et al*., 2002; Van der Oost, *et al*., 2003) known to be inducible by heavy

metals, and able to bind with metals in fish tissues (Viarengo, *et al*., 2000). Biological functions of MT include homeostasis of essential metals, detoxification of essential and non-essential metals and antioxidant defense (Viarengo, *et al*., 2000; Amiard, *et al*., 2006). They have been widely used as a biomarker to assess metal toxicity in aquatic environments (Roesijadi, 1996; Gorbi, *et al*., 2005; Fernandes *et al*., 2008).

Despite a previous work has not described a seasonal variation in the liver MTLP concentration in *M. furnieri* collected in a polluted site of the Patos Lagoon estuary (Amado *et al*., 2006), our results showed a significant seasonal variation in MTLP concentration in both liver and gills. Differences observed in these two studies could be associated with the higher number of sites of collection and of fish analyzed in each season in the present study.

The higher hepatic MTLP concentration observed in croakers collected in autumn and winter coincided with the higher mean hepatic accumulation of Zn and Cu described in the companion paper (Chaves and Bianchini, 2008), suggesting that the MTLP synthesis in croakers was due to an excess of accumulation of these metals, as previously reported for other species (Langston *et al*., 1998). In fact, MT expression generally increases with the elevation of tissue concentrations of metallothionein-inducing metals, reflecting the metal bioavailability in the environment (Leung *et al*., 2002; Ross *et al*., 2002). However, the lower values of liver MTLP concentration observed in spring was paralleled by a markedly higher liver Cd burden, suggesting a higher susceptibility of croakers to Cd exposure at this season.

Although the whole enzymatic and non-enzymatic antioxidant system was not evaluated in the present study, the fact that liver LPO levels continuously increased from autumn to spring paralleled by a continuous decrease in liver MTLP concentration during the same period strongly suggest that oxidative damage to lipids are favored in croakers when antioxidant protection is decreased, as previously described for other fish species (Fatima *et al*., 2000). It is also important to note that higher levels of Cu, Zn and Cd were observed in

juvenile croakers collected in spring (see companion paper; Chaves and Bianchini, 2008). Non-essential metals can bind to sensitive intracellular targets if detoxification mechanisms are over-whelmed or damaged by metal influx (Langston and Zhou, 1986; Hogstrand and Haux, 1991). In some situations, Cd can also act inactivating important cellular antioxidants (Achard-Joris *et al*., 2007). Liver Cd effects are widely believed to be associated with its ability to cause inhibition of the overall antioxidant system (Bagchi *et al*., 1996, Asagba and Eriyamremu, 2008). Therefore, the observed oxidative damage to lipids could be a response from the excess of Cu and Zn, but especially Cd in liver of croakers collected in spring.

Different from liver, gills showed a different seasonal pattern in the MTLP concentration, showing higher values in the warmer seasons (summer and spring). Although MT generally respond to metal exposure, studies on seasonal variability of these proteins have provided conflicting results, as they can be influenced by a variety of environmental and physiological factors (Viarengo *et al*., 1997; Regoli *et al*., 2004; Ivankovic *et al*., 2005). Gills are directly exposed to the environment, being more susceptible to both biotic and abiotic stressors. Therefore, the possible influence of abiotic factors, such as temperature and salinity, on the expression of other MT isoforms different from those involved in metal homeostasis cannot be ruled out (Amiard *et al*., 2006). In fact, fluctuations on MTLP concentrations in gills of estuarine animals seems to be more closely correlated to variation on general protein metabolism in response to salinity changes than to variations in metal accumulation (Legras *et al*., 2000). Furthermore, although metals are recognized as being the primary inducers of MT, synthesis of these proteins may also be influenced to some extent by other pollutants, in particular pro-oxidants such as PHAs and PCBs compounds (Bauman *et al*.,1991; Fernandes *et al*., 2007). As metals are generally less bio-available in high salinities, possible organic compounds present in the Patos Lagoon estuary could be associated with the higher MTLP concentration observed in summer, when higher mean salinity and temperature values were

observed. The fact that the highest mean level of gill MTLP concentration was paralleled by a relatively high level of LPO in this tissue, suggest that the induced MTLP isoforms were not able to protect against the oxidative damage induced by these contaminants. In fact, LPO can be stimulated via oxidation of polyunsaturated fatty acids induced by both inorganic (Viarengo *et al*., 1990) and organic contaminants (Shaw *et al*., 2004). In turn, the higher MTLP concentration observed in gills of croakers collected in spring could be related to the significant increased Cd burden registered in this tissue (see companion paper; Chaves and Bianchini, 2008). In fact, gills seem to be the first critical target for Cd in fish (Tjalve *et al*., 1986).

Despite differential seasonal pattern were observed in liver and gill MTLP concentration, significantly higher LPO levels were also observed when lower levels of gill MTLP were registered (autumn and winter), as observed for liver (spring). In agreement with that, a lower gill LPO value was observed in spring paralleled by an increased gill MTLP concentration. These findings strongly support the idea that oxidative damage to lipids is favored in croakers when the antioxidant protection is decreased, as previously discussed.

An important role in toxicity of several pollutants is assumed by the enhancement of intracellular reactive oxygen species (ROS) and perturbation of antioxidant efficiency which often prelude the onset of damage of DNA, proteins and lipid (Winston and Di Giulio, 1991). The overall enhancement or reduction of pollutant-induced toxicity greatly depends on the imbalance between pro-oxidant and antioxidant status, as a result of xenobiotic interferences. Hence, enhanced pollutant oxygenation rates may increase toxicity via oxidative stress, rendering fish antioxidants less effective.

Changes in environmental parameters, such as salinity and temperature, or metabolic changes can modulate the bioavailability of pollutants and the efficiency of the cellular defense mechanisms, thus stimulating or compensating for the pollution effect. This is

probably the reason why some studies have detected different seasonal trends in micronucleus frequency (Bolognesi *et al*., 2004; Barsiene *et al*., 2006; Magni *et al*., 2006), while other studies did not verify the reliability of micronucleus test (Dailianis *et al*., 2003). In the present study, a seasonal variation in this biomarker was observed, showing higher and lower mean values in spring and autumn, respectively.

The higher levels of mutation, assessed through micronuclei expression, in juvenile croakers collected in spring could be related to a decrease in their antioxidant status in this season, as observed for liver MTLP concentration, paralleled by a significant increase in metal (Cu, Cd and Zn) accumulation in both liver and gills (see companion paper; Chaves and Bianchini, 2008). This statement is in agreement with the fact that DNA damage induced by Cd (Marrazzini *et al*., 1994; Sanchez-Galan *et al*., 1999), Cu and Pb (Pytharopoulou *et al*., 2006) has being reported in other species.

Because of the pollution load, tissue damage in aquatic organisms is commonplace. It includes minor lesions, ulceration and necrosis and is mainly ascribed to the non-specific disturbance of cell membrane caused by lipophilic xenobiotics (Wester and Canton, 1992; Banerjee and Bhattacharya, 1994; Noga *et al*., 1998). Such tissue aberrations facilitate invasion and growth of infectious agents (Bucke, 1993; Vethaak *et al*., 1996). In this context, innate (natural or non-specific) immunity is the collection of defense mechanisms that protect an organism against infection, without depending upon prior exposure to any particular microorganism.

Phagocytic cells play a key role in the fish immune system. They secrete reactive oxygen species (ROS) involved in their bactericidal activity. These cells are highly sensitive to pollution by organic and inorganic pollutants (Bols *et al*., 2001). In fact, previous studies have reported suppression of phagocytic function by environmental contaminants (Duffy *et al*., 2002; Zelikoff *et al*, 2002; Amado *et al.*, 2006). Results from the present study on

phagocytosis and respiratory burst measurements clearly indicate that phagocytes from croakers collected in winter and spring are showing a significant immunossupression. Since immunotoxicity has been demonstrated to be related to Cu, Zn and Pb, exerting inhibitory effects on immune response and increasing the severity of infections in fish (Baker and Knittel, 1983; Anderson *et al*., 1989, Rougier *et al*., 1994; 1996), the immunossupression observed in phagocytes of croakers collected in winter and spring could be associated with an exposure to sublethal concentration of these metals in the Patos Lagoon estuary. The significant negative correlation observed between the concentration of Cu, Zn and/or Pb in liver or gills and the immunological biomarkers (phagocytosis and respiratory burst) strongly support this idea. These lower non-specific immune responses can lead to opportunistic diseases, such as viral infections and parasite infestation (Neumann *et al*., 2001) in white croakers from the Patos Lagoon estuary, in both winter and spring.

Results from the presented study suggest that not just a single chemical in the Patos Lagoon estuary would be causing the physiological alterations observed in white croakers. A combination of several chemicals could be involved in the response of biochemical, genetic and immunological biomarkers analyzed. The fact that the Patos Lagoon estuary is characterized by high levels of contaminants, including metals (Baisch *et al*., 1988; Baumgarten and Niencheski 1990; Baumgarten *et al*., 1998) and hydrocarbons (Medeiros *et al*., 2005), gives support to this hypothesis. It is important to note that the present results, combined with those reported in the companion paper (Chaves and Bianchini, 2008), clearly point to a significantly higher susceptibility of juvenile white croakers *M. furnieri* from the Patos Lagoon estuary to aquatic contamination in winter and spring when their protective capability, measured through MTLP concentration in liver and gills, is markedly reduced.

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Table 1. Morphometric data of juvenile croakers *Micropogonias furnieri* collected at the Patos Lagoon estuary (Southern Brazil). No significant difference was observed in total body length of croakers collected at different seasons. n: number of fish analyzed.

Season	Total body length (cm)	n
Summer	12.07 ± 0.26	74
Autumn	11.11 ± 0.28	60
Winter	10.83 ± 0.20	68
Spring	12.28 ± 0.44	75

Figure Captions

Figure 1. Metallothionein-like proteins (MTLP) concentration in liver (A) and gills (B) of juvenile croakers (*Micropogonias furnieri*) collected at the Patos Lagoon estuary. Different letters indicate seasonal significant differences ($P < 0.05$). Values are expressed as means ± standard error. n: number of fish analyzed.

Figure 2. Lipid peroxidation content in liver (A) and gills (B) of juvenile croakers *Micropogonias furnieri* collected at the Patos Lagoon estuary. Different letters indicate significant seasonal differences ($P < 0.05$). Values are expressed as means ± standard error. n: number of fish analyzed.

Figure 3. Relative frequency of micronucleated erythrocytes of juvenile croakers *Micropogonias furnieri* collected at the Patos Lagoon estuary. Different letters indicate significant seasonal differences ($P < 0.05$). Values are expressed as means ± standard error. n: number of fish analyzed.

Figure 4. Phagocytosis (A) and respiratory burst (B) in leukocytes of juvenile croakers *Micropogonias furnieri* collected at the Patos Lagoon estuary. Different letters indicate significant seasonal differences ($P < 0.05$). Values are expressed as means ± standard error. n: number of fish analyzed.

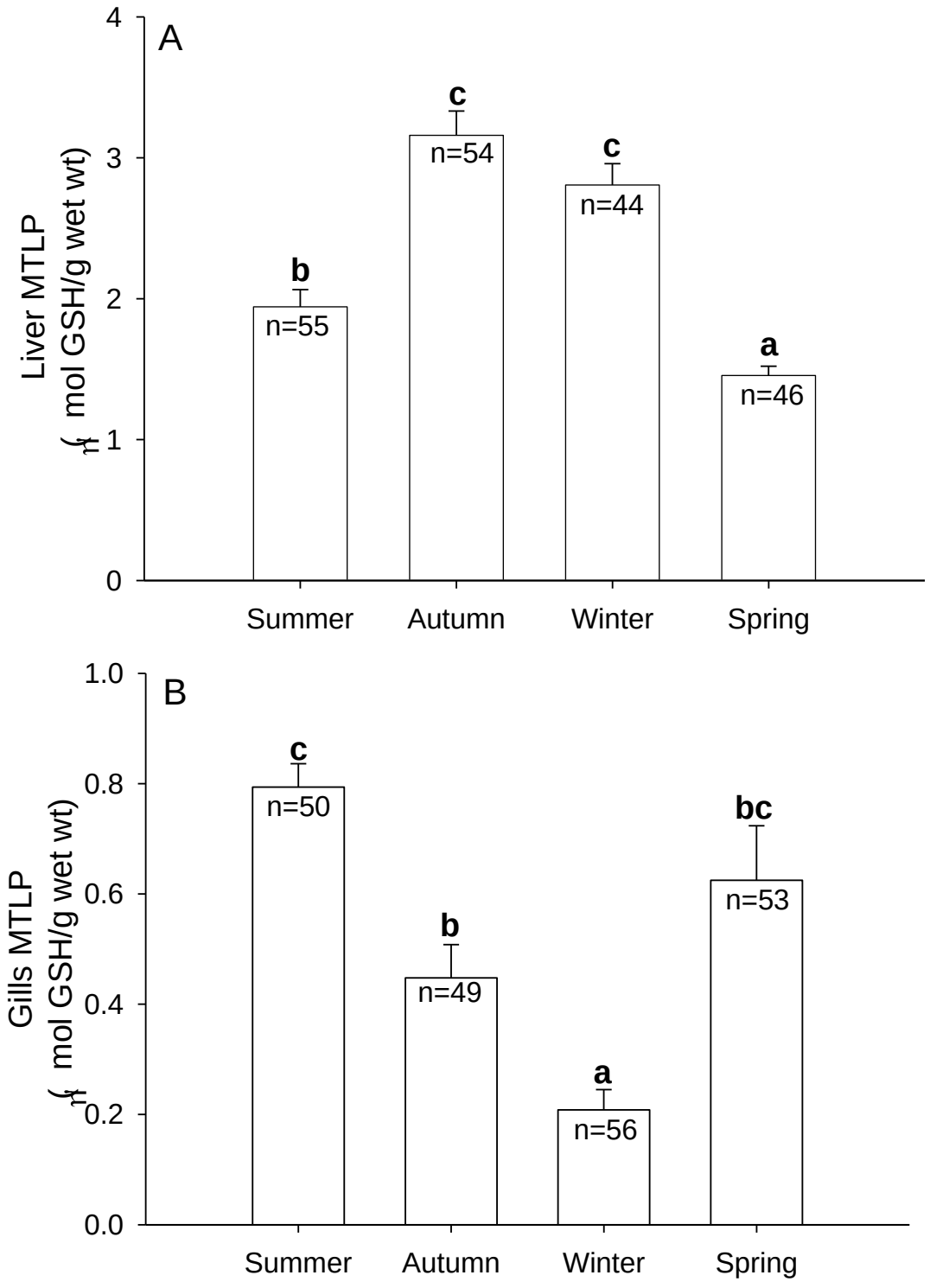
A
Liver MTLP
(mol GSH/g wet wt)
4
3
2
1
0
b
n=55
c
n=54
c
n=44
a
n=46
Summer
Autumn
Winter
Spring
B
Gills MTLP
(mol GSH/g wet wt)
1.0
0.8
0.6
0.4
0.2
0.0
c
n=50
b
n=49
a
n=56
bc
n=53
Summer
Autumn
Winter
Spring

Figure 1

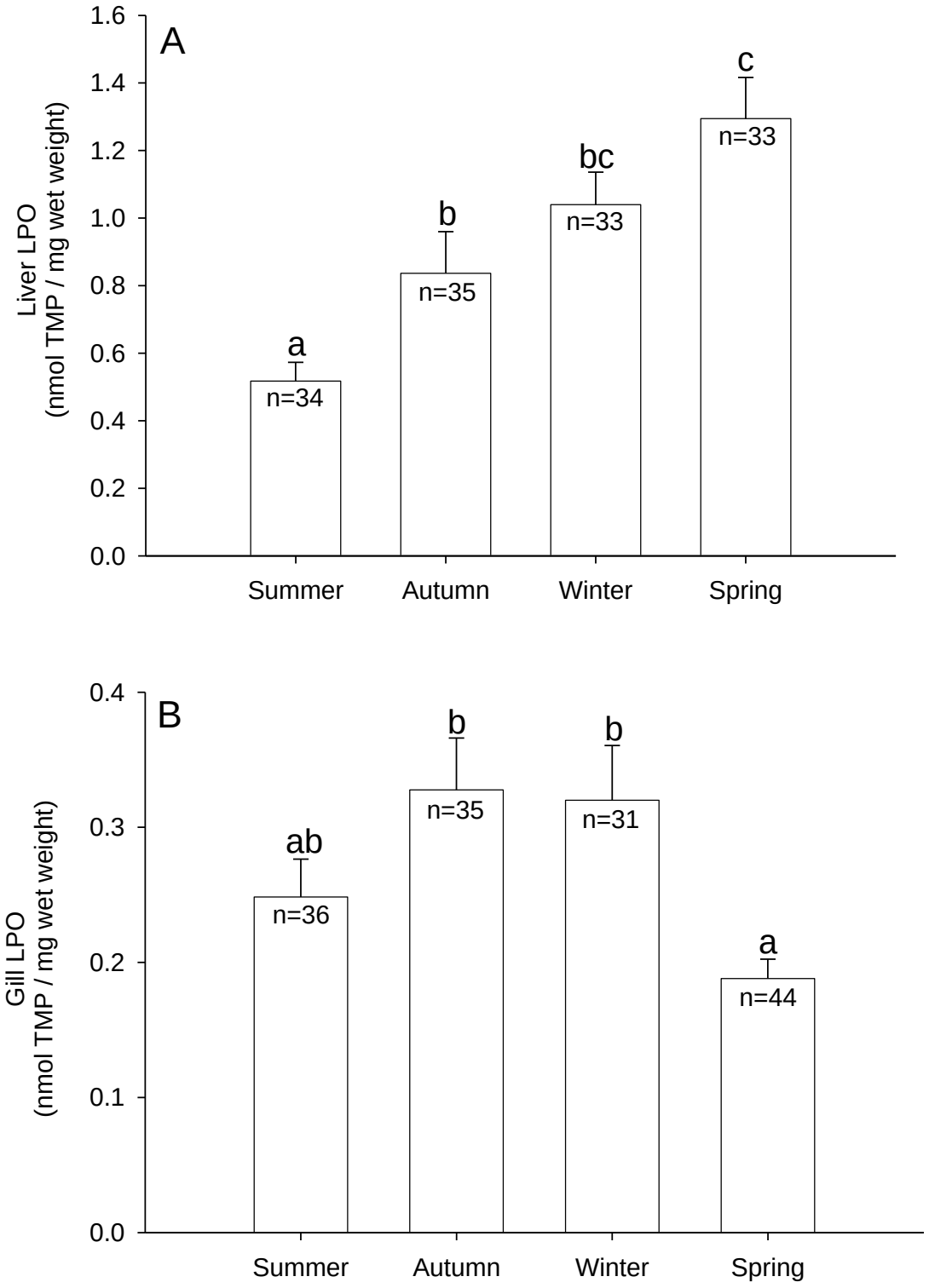
A
Liver LPO
(nmol TMP / mg wet weight)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
bc
c
n=34
n=35
n=33
n=33
Summer
Autumn
Winter
Spring
B
Gill LPO
(nmol TMP / mg wet weight)
0.4
0.3
0.2
0.1
0.0
ab
b
b
a
n=36
n=35
n=31
n=44
Summer
Autumn
Winter
Spring

Figure 2

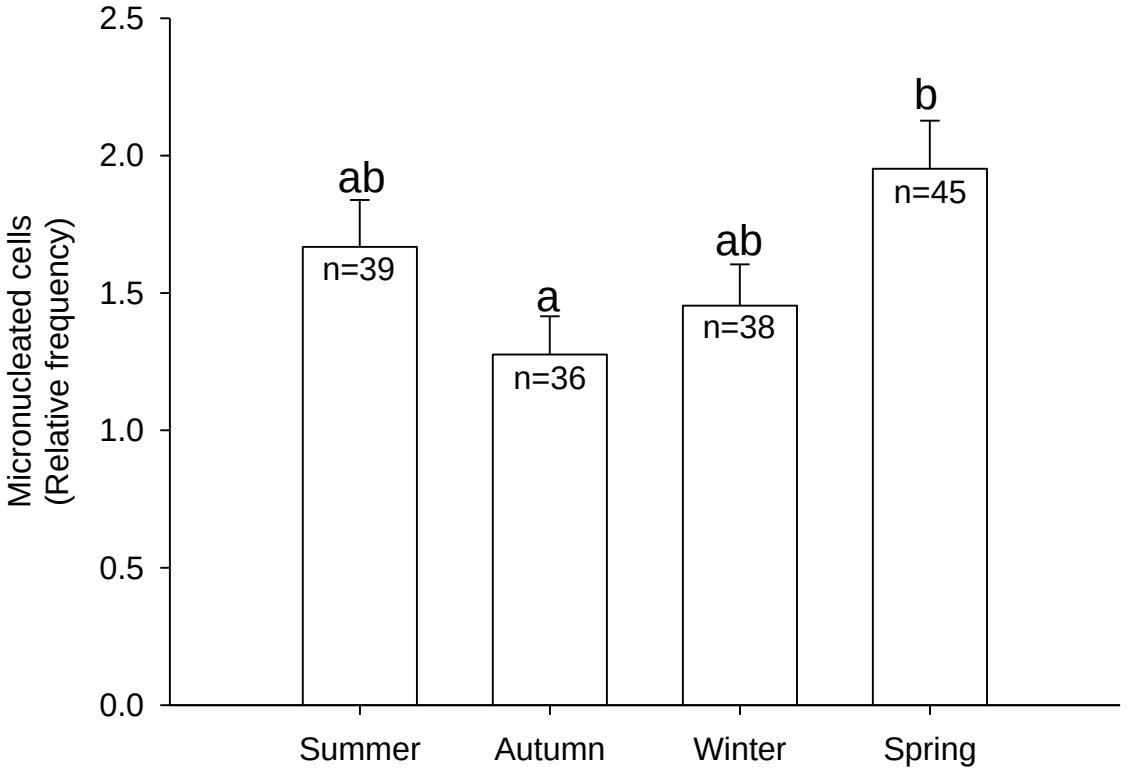
Micronucleated cells
(Relative frequency)
2.5
2.0
1.5
1.0
0.5
0.0
ab
n=39
a
n=36
ab
n=38
b
n=45
Summer
Autumn
Winter
Spring

Figure 3

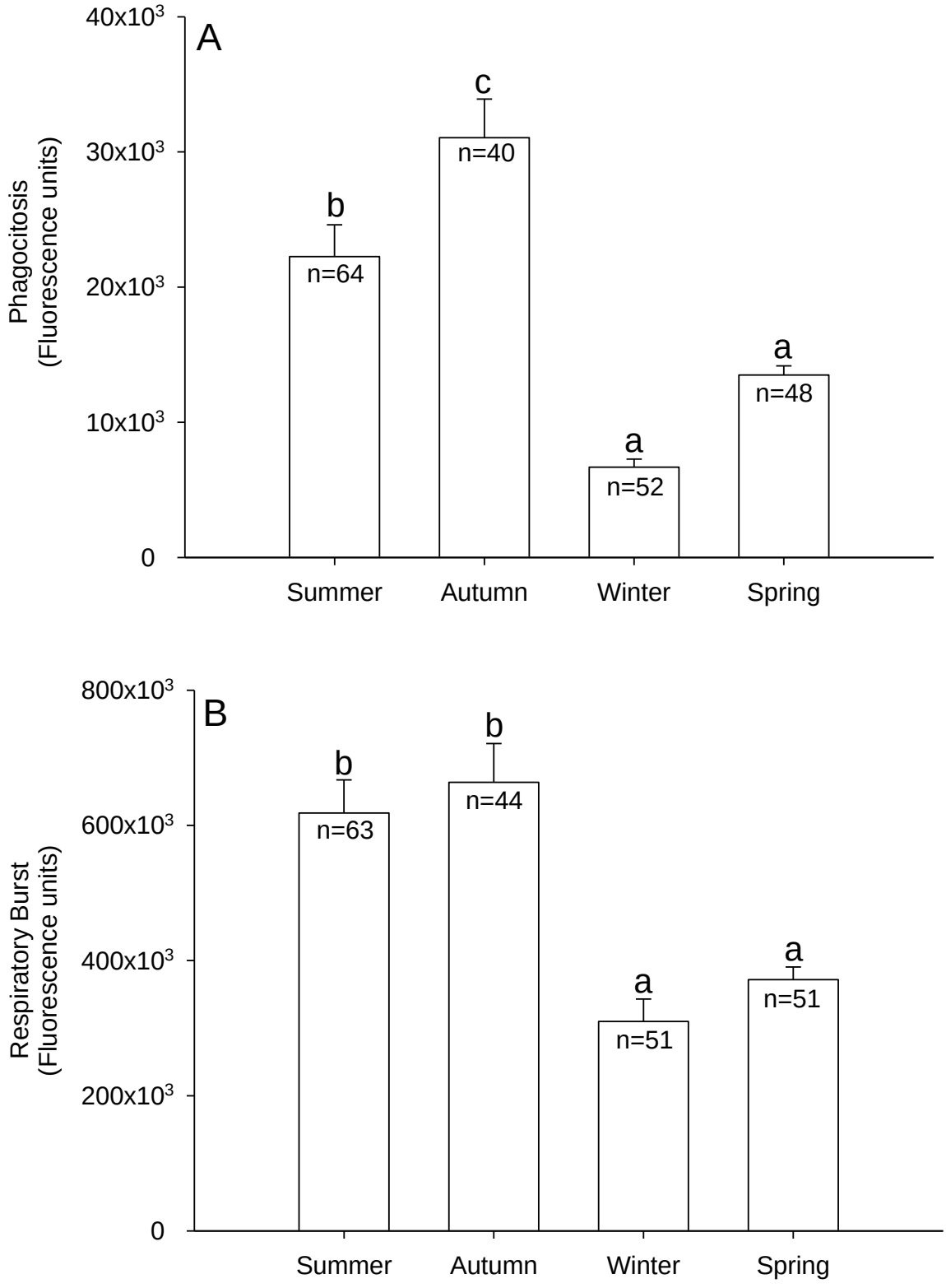
A
Phagocitosis
(Fluorescence units)
40x10³
30x10³
20x10³
10x10³
0
b
n=64
c
n=40
a
n=52
a
n=48
Summer
Autumn
Winter
Spring
B
Respiratory Burst
(Fluorescence units)
800x10³
600x10³
400x10³
200x10³
0
b
n=63
b
n=44
a
n=51
a
n=51
Summer
Autumn
Winter
Spring

Figure 4