Universidade de Aveiro Departamento de Biologia
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Avaliação da toxicidade de contaminantes ambientais em populações naturais de *Pomatoschistus microps* (Krøyer, 1938)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares Avançados, realizada sob a orientação científica do Prof. Doutor Amadeu Soares, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e com a co-orientação da Profª. Doutora Lúcia Guilhermino, Professora Associada do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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resumo

Nas últimas décadas tem-se verificado um aumento do nível de contaminação nos estuários e nas zonas costeiras, como consequência da actividade antropogénica. Assim sendo, o desenvolvimento de métodos de avaliação do grau de exposição e contaminação de populações naturais estuarinas revela-se da maior importância, de forma a minimizar o impacto das actividades humanas.

Os biomarcadores têm sido largamente utilizados em ecotoxicologia na detecção precoce de efeitos químicos, permitindo a antecipação de alterações adversas ao nível da população e uma intervenção protectora atempada. De entre os biomarcadores potencialmente disponíveis, no presente estudo a atenção foi focada na inibição da acetilcolinesterase (ACHE), alterações da lactato desidrogenase (LDH), e indução da glutationa S-transferase (GST) e das monoxigenases associadas ao sistema P450 (P450), que têm sido demonstrados como apropriados numa vasta gama de espécies e cenários reais.

O presente trabalho consistiu no desenvolvimento de uma bateria de biomarcadores em populações naturais de *Pomatochistus microps* (Krøyer, 1838) com o intuito de avaliar o efeito de contaminantes ambientais em estuários da zona costeira atlântica ibérica, contribuindo para esse efeito três estudos com objectivos distintos: (i) caracterização do tipo de colinesterases presentes nos tecidos da cabeça de *P. microps*; (ii) identificar, em *P. microps*, biomarcadores adequados para o contaminante ambiental 3,4-dicloroanilina (DCA); (iii) avaliação da exposição de populações naturais de *P. microps* a diferentes tipos de contaminação ambiental em estuários da costa noroeste portuguesa, utilizando os vários biomarcadores seleccionados.

Importantes alterações verificadas a nível histológico e bioquímico por exposição à DCA indicam-na como substância potencialmente tóxica para populações naturais de *P. microps* de estuários sob influência agrícola. Os resultados obtidos com a avaliação da exposição a diferentes tipos de contaminação ambiental confirmam a utilidade da abordagem de biomarcadores a diferentes níveis de organização biológica e são um contributo para a futura utilização de *P. microps* como biomonitor em estuários da zona costeira ibérica.

abstract

In recent years, the levels of contaminants in estuaries and coastal zones increased as a consequence of anthropogenic activities. For this reason, it is a priority to develop methods to evaluate the degree of exposure and contamination of estuarine natural populations in order to minimise the impact of human activities.
Biomarkers have been widely used in ecotoxicology as early warning signals of chemicals effects, offering the possibility of anticipating severe alterations at a population level and, thus, making possible a protective intervention. Among the biomarkers potentially available for use, in this study attention was focused on the inhibition of acetylcholinesterase (AChE), alterations of lactate dehydrogenase (LDH), and induction of glutathione S-transferase (GST) and the monooxygenase enzymes associated to P450 system (P450), which have been shown to be appropriate for use in a large variety of species and real scenarios.

The aim of the present work was to develop a battery of biomarkers on natural populations of *Pomatoschistus microps* (Krøyer, 1838) in order to assess the effect of environmental contaminants in estuaries from the Atlantic Iberian coast. For this purpose three studies with distinct objectives were carried out: (i) characterization of the cholinesterase forms present in head tissues of *P. microps*; (ii) identification in *P. microps* of suitable biomarkers to assess the environmental contaminant 3,4-dichloroaniline (DCA)*;* (iii) assessment of different contamination impacts in estuaries of the Norwest Portuguese coast using the selected biomarkers.

Exposure to DCA induced important alterations at histological and biochemical levels, that indicate this compound as potentially toxic for natural populations of *P. microps* in estuaries with agricultural influence. The results obtained with the assessment of environmental contamination support the usefulness of the biomarker approach at different levels of biological organization to assess the toxicity of environmental pollution, and provide further support for the use of *P. microps* as a biomonitor in estuaries of the Iberian coast.

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ERRATA

PÁGINA	ONDE SE LÊ	DEVE LER-SE
XXIV(Anexo III)	433	43,3%
XXIV(Anexo III)	0.567	56,7%
XXIV(Anexo III)	0.850	85,0%
XXIV(Anexo III)	0.083	8,3%
XXIV(Anexo III)	0.500	50,0%

INTRODUÇÃO GERAL E OBJECTIVOS

Os ecossistemas estuarinos são habitats naturais de elevada produtividade, onde a biomassa de fitoplâncton suporta uma diversidade de organismos, incluindo espécies costeiras importantes a nível económico, que utilizam estes ambientes como locais para reprodução (Kennish, 1992). O desenvolvimento em larga escala de áreas industriais e urbanas em zonas costeiras conduziu a uma contínua entrada nos ecossistemas estuarinos de vários tipos de poluentes, de entre os quais se salientam hidrocarbonetos policíclicos aromáticos (PAHs), compostos policlorados bifenílicos (PCBs), pesticidas organoclorados e organofosforados e metais pesados (Kennish, 1992). Esta entrada contínua de contaminantes de origem antropogénica resulta num permanente risco ecológico a que estes ecossistemas estão submetidos. No entanto, a presença de um xenobiótico num sistema aquático não indica, por si só, que este sofra efeitos adversos. Como tal, os programas de monitorização, onde se efectuam avaliações de parâmetros ambientais de modo regular, não devem ser somente baseados em análises químicas, uma vez que estas não fornecem indicações sobre eventuais efeitos nefastos sobre o biota (Peakall, 1992; Connell *et al.*, 1999). Como tal, o estabelecimento de relações entre os níveis externos do contaminante e a avaliação dos seus efeitos nos sistemas biológicos revela-se de extrema importância e devem ser parte integrante de programas de monitorização da poluição ambiental (Peakall, 1992; Van der Oost *et al.*, 2003). A biomonitorização é um ramo da monitorização ambiental e refere-se especificamente à utilização de organismos vivos nestes programas (Connell *et al.*, 1999). Alguns grupos de investigação têm vindo a desenvolver programas de biomonitorização em estuários e zonas costeiras da Península Ibérica (Carajaville *et al.*, 2000). No entanto, são ainda poucos os estudos efectuados nesta área em Portugal, sendo de crucial importância o seu rápido desenvolvimento e implementação como forma de possibilitar acções de remediação.

Os efeitos nefastos a nível de populações e comunidades são geralmente de difícil detecção, tendendo a manifestar-se após níveis de organização inferior terem sido afectados e quando eventuais acções de remediação ou de diminuição de risco já não conseguem reverter o processo de toxicidade. Na figura 1 (adaptado de Van der Oost, 2003) está representada a ordem sequencial da resposta dos sistemas biológicos ao stress químico, na qual se baseia o uso de marcadores biológicos de detecção precoce de efeitos adversos causados por xenobióticos, marcadores esses que são denominados de "biomarcadores" (Peakall, 1992).

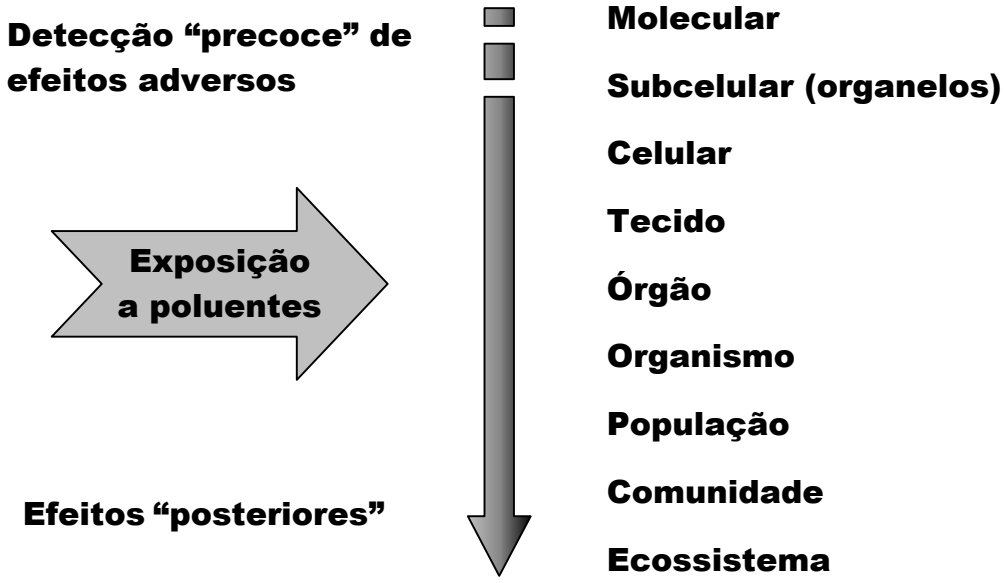

Figura 1 – Representação esquemática da ordem sequencial de resposta à exposição a poluentes num sistema biológico (adaptado de Van der Oost *et al.*, 2003).

Várias definições similares para o termo "biomarcadores" têm sido sugeridas. Em *sensu lato*, um biomarcador pode ser definido como uma resposta quantificável de qualquer nível de organização biológica que pode ser relacionada com a exposição e/ou efeito de contaminantes. No entanto, e para evitar sobreposição com termos e conceitos já existentes e

que se referem a efeitos a nível do organismo (bioindicador) e do ecossistema (indicador ecológico), o termo "biomarcador" é geralmente usado para designar respostas quantificáveis a níveis organizacionais sub-individuais, nomeadamente a nível celular, bioquímico, molecular ou fisiológico em células, fluidos corporais, tecidos ou órgãos de um organismo que possam ser indicativas da exposição e/ou efeito de xenobióticos (Mayer *et al.*, 1992; Peakall, 1992; Walker *et al.*, 2001; Van Gestel & Brummelen, 1996).

O desenvolvimento de uma bateria de biomarcadores a ser utilizada em programas de monitorização requer a adaptação de protocolos às condições e espécies locais. Para tal, é necessário definir os valores basais e as variações sazonais em populações locais. A identificação de biomarcadores adequados que sejam informativos relativamente aos contaminantes potencial ou realmente presentes no ecossistema em estudo revela-se também de máxima importância (Peakall, 1992; Lam & Gray, 2003; Van der Oost *et al.*, 2003).

Vários parâmetros bioquímicos têm sido testados em peixes quanto às suas respostas em relação a diferentes contaminantes e ao seu potencial uso como biomarcadores de exposição e/ou efeito. No presente trabalho, seleccionou-se o peixe estuarino *Pomatochistus microps* (Krøyer, 1838) como organismo indicador e um conjunto de enzimas relevantes em diferentes funções fisiológicas (neurotransmissão, produção de energia e biotransformação de xenobióticos) para serem testadas tendo em vista a sua utilização como biomarcadores em programas de biomonitorização em estuários da zona costeira ibérica. Este trabalho faz parte integrante de um projecto multidisciplinar (Métodos Efectivos para Monitorização da Contaminação Ambiental e Avaliação de Risco na Zona Costeira e em Estuários - CONTROL, financiado pela Fundação para a Ciência e a Tecnologia (FCT), contrato: PDCTM/C/MAR/15266/1999) cujo objectivo central consiste no desenvolvimento e validação de métodos efectivos e económicos e novos testes de toxicidade, baseados em parâmetros a

diferentes níveis de organização biológica, adaptados às condições e espécies locais para uso em zonas costeiras e estuarinas.

A nível do sistema nervoso, a inibição da actividade da acetilcolinesterase (AChE), surge como um dos biomarcadores mais amplamente utilizados no diagnóstico de exposição a compostos anticolinérgicos em ecossistemas aquáticos, tais como pesticidas organofosforados e carbamatos (Peakall, 1992). No entanto, mais recentemente, diversos estudos indicam que a AChE é sensível à presença de outros contaminantes ambientais, incluindo alguns surfactantes, metais pesados, hidrocarbonetos e efluentes de pasta de papel (Gill *et al.*, 1990; Payne *et al.*, 1996; Guilhermino *et al.*, 2000). Esta enzima é responsável pela hidrólise da acetiltiocolina em colina e ácido acético nas sinapses colinérgicas de vertebrados e invertebrados. Quando é inibida ocorre acumulação de acetiltiocolina na fenda sináptica causando disrupção da função nervosa que pode ter consequências letais para o organismo (Mayer *et al.*, 1992; Peakall, 1992). Nos vertebrados, as colinesterases (ChE) dividem-se em duas classes principais, a AChE e a butirilcolinesterase ou pseudocolinesterase (BChE). Estas duas enzimas distinguem-se funcionalmente na sua especificidade em relação a diferentes substratos e na sua susceptibilidade para com inibidores (Kozlovskaya *et al.*, 1993). Estudos efectuados recentemente indicam a presença de mais de um tipo de ChE em tecidos de peixes estuarinos, podendo as formas presentes diferir na sua sensibilidade a agentes anticolinérgicos (Sturm *et al.*, 1999a, 2000), pelo que é essencial proceder à caracterização do tipo de ChE presentes nos tecidos do organismo em estudo, antes da sua utilização como biomarcador (Bocquené *et al.*, 1990; Sturm *et al.*, 1999a; Garcia *et al.*, 2000).

O processo de obtenção de energia é de crucial importância para a realização das diversas actividades metabólicas e normal desenvolvimento do organismo. Em situações onde são requeridas elevadas quantidades de energia, os organismos socorrem-se do processo de glicólise, uma vez que

permite a obtenção rápida de energia metabólica (Mayer *et al.*, 1992; Peakall, 1992). Neste processo, a lactato desidrogenase (LDH) é uma enzima de crucial importância, catalizando a reacção reversível de piruvato a ácido láctico (Mayer *et al.*, 1992). Esta enzima é de crucial importância para a fisiologia dos tecidos musculares, particularmente em condições de stress ou exercício ou hipóxia onde elevadas quantidades de energia são requeridas em curtos períodos de tempo (Wu & Lam, 1997). Este tipo de resposta poderá também verificar-se em situações de stress químico. Diversos autores têm verificado alterações no padrão normal de actividade de LDH após exposição a diferentes contaminantes, incluindo alguns metais, pesticidas e "crude" (Guilhermino *et al.*, 1994; Gagnon & Holdway, 1999; De Coen *et al.*, 2001; Ribeiro *et al.*, 1999; Diamantino *et al.*, 2001).

Enzimas envolvidas nos processos de biotransformação de xenobióticos, estão entre os biomarcadores mais comummente utilizados. De um modo geral, os biomarcadores de efeito mais sensíveis dizem respeito a alterações no nível de actividade de enzimas de biotransformação, que nos peixes, podem ser induzidas ou inibidas pela exposição a xenobióticos (Goksøyr & Förlin, 1992; George, 1994; Bucheli & Fent, 1995). O processo de biotransformação compreende duas fases, fase I e fase II. O sistema composto pelo citocromo P450 (P450) e monooxigenases a ele associadas, sistema também denominado de "*mixed function oxidase*" (MFO), é o sistema enzimático mais importante na fase I (Stegeman & Hahn, 1994). A principal função deste sistema é facilitar a excreção de xenobióticos lipofílicos transformando-os em compostos mais hidrossolúveis (Bucheli & Fent, 1995). O citocromo P450 (P450) representa uma família de enzimas, que desempenham um papel importante no metabolismo de muitos xenobióticos, catalizando tanto as reacções de bioactivação, como as de destoxificação (Stegeman & Hahn, 1994). A indução do P450 e da actividade das suas monooxigenases, nomeadamente da arilhidrocarbono-hidroxilase (AHH) e da 7-etoxiresorufina-0-dietilase (EROD), nos peixes é

provavelmente o biomarcador mais utilizado na monitorização da contaminação ambiental de PAHs e PCBs (Stegeman & Hahn, 1994; Bucheli & Fent, 1995; Van der Oost *et al.*, 2003). Contudo, a indução do P450 é influenciada por outros factores, que incluem temperatura, espécie em questão, peso e sexo do indivíduo (Bucheli & Fent, 1995). Tem-se também vindo a demonstrar que alguns compostos são inibidores do P450 em peixes, incluindo o tributil-estanho (TBT), benzeno, naftaleno e cádmio entre outros (Van der Oost *et al.*, 2003). Sendo assim é necessária uma cuidada interpretação de dados isolados de P450 como biomarcador na avaliação do impacto ambiental (Bucheli & Fent, 1995).

A conjugação de compostos electrofílicos resultantes da fase I de biotransformação com a glutationa (GSH) é catalizada pelas glutationa S-transferases (GST), as quais constituem uma família de enzimas envolvidas no processo de destoxificação de xenobióticos e de compostos endógenos. Para além deste papel essencial, as GST intervêm no transporte intracelular e na defesa contra o stress oxidativo e a peroxidação lipídica (George, 1994). A toxicidade de muitos compostos exógenos pode ser modulada por indução das GST. O efeito de agentes indutores na actividade total desta enzima tem sido observado em diversas espécies de peixes após exposição a PAHs, PCBs, pesticidas organofosforados e carbamatos (Van der Oost *et al.*, 2003). No entanto, outros estudos indicam que não há efeito ou que há inibição das GST por este tipo de compostos.

Espécie em estudo

A espécie seleccionada para este estudo foi *Pomatoschistus microps* (Kroyer, 1938), pertencente aos Gobiidae, uma família de peixes teleósteos que engloba espécies pertencentes a ecossistemas estuarinos e de água doce de zonas tropicais e temperadas (Miller *et al.*, 1986). *P. microps* tem uma ampla distribuição geográfica que vai desde a costa Atlântica (costa

sudoeste da Noruega, Mar Báltico e costa Europeia até ao sul de Portugal) à costa Mediterrânica noroeste (Miller *et al.*, 1986). Esta espécie tem uma longevidade de 1,6 a 2 anos, sendo a sua época de reprodução de Abril a Agosto (Miller *et al.*, 1986; Arruda *et al.*, 1993). Devido à sua grande abundância e fecundidade e à sua posição como predador intermediário na cadeia trófica, conectando o microbentos com espécies de peixes de maiores dimensões e aves marinhas, *P. microps* tem um papel ecológico de crucial importância nos ecossistemas aquáticos (Cattrijsse *et al.*, 1994). Este peixe epibêntico e eurihalino, cujos habitats preferenciais são as águas salobras de estuários, zonas costeiras e lagoas, encontra-se preferencialmente em substratos arenosos com alguma vegetação (Miller *et al.*, 1986). Este tipo de locais encontra-se muitas vezes poluído por contaminantes resultantes de actividades antropogénicas. Como tal, e devido às suas características ecológicas, *P. microps* é tido como uma espécie relevante em testes ecotoxicológicos (Christiansen *et al.*, 1998). Esta espécie foi seleccionada para o presente estudo uma vez que ocorre abundantemente durante todo o ano mesmo em zonas consideradas contaminadas e, em termos de ordem prática, as amostragens não requerem muitos meios logísticos, reunindo assim as condições adequadas para ser utilizada em programas de biomonitorização e de avaliação do impacto de contaminantes para ecossistemas costeiros e estuarinos.

Locais de estudo

No presente trabalho seleccionou-se um conjunto de estações de amostragem em zonas estuarinas consideradas de referência e outras com diferentes tipos de contaminação, de forma a testar em campo os biomarcadores em estudo. O programa de biomonitorização realizado incluiu amostragens sazonais, durante um ano nos estuários dos rios Minho e Douro e na ria de Aveiro.

O estuário do rio Minho compreende uma área total de 3,4 km^2, que inclui também a embocadura do rio Coura. Este estuário, de características mesotidais e maré semi-diurna, constitui uma zona húmida de grande valor ecológico que alberga importantes manchas de vegetação de elevada biodiversidade (IST/INAG, 2001). No estuário do Rio Minho foi seleccionada uma zona aparentemente não contaminada que foi considerada como estação de referência, a qual se denominou "*Reference 1"* (ver figura 2), uma vez que este estuário é caracterizado pela ausência de grandes áreas metropolitanas, industriais ou agrícolas, embora apresente focos localizados de contaminação sobretudo como resultado do tráfego de pequenas embarcações.

O estuário do Rio Douro é um estuário mesotidal semi-diurno, que se estende até 3 km da "boca" com uma largura de 645 m varrendo uma área total de 1,9 km^2, tendo uma profundidade média de 7,8 m (Vieira & Bordalo, 2000). O desenvolvimento industrial e urbano da região metropolitana do Porto, que circunda a bacia hidrográfica do estuário do Douro, constitui uma ameaça permanente para a qualidade da água deste estuário. No presente trabalho seleccionou-se uma estação neste estuário denominada "*Urban and Industrial effluents"* (ver figura 2).

A laguna de Aveiro, correntemente denominada Ria de Aveiro (figura 2), é um sistema lagunar marinho, situado na costa Atlântica noroeste de Portugal, constituído por três canais principais: Canal de Mira, Canal de São Jacinto e Canal de Ílhavo. Estes canais abrem para o mar através de uma abertura artificial reduzida, a qual é mantida através de dragagens frequentes. A sua área total varia de 42 km^2 na baixa-mar e 47 km^2 na preia-mar, sendo a sua profundidade de 1 m na maior parte da laguna (Morgado *et al.*, 1997). A emissão para a Ria de Aveiro de efluentes de origem doméstica, industrial e agropecuária, e ainda de lixiviados e escorrências de lamas industriais, têm levado à contaminação de algumas

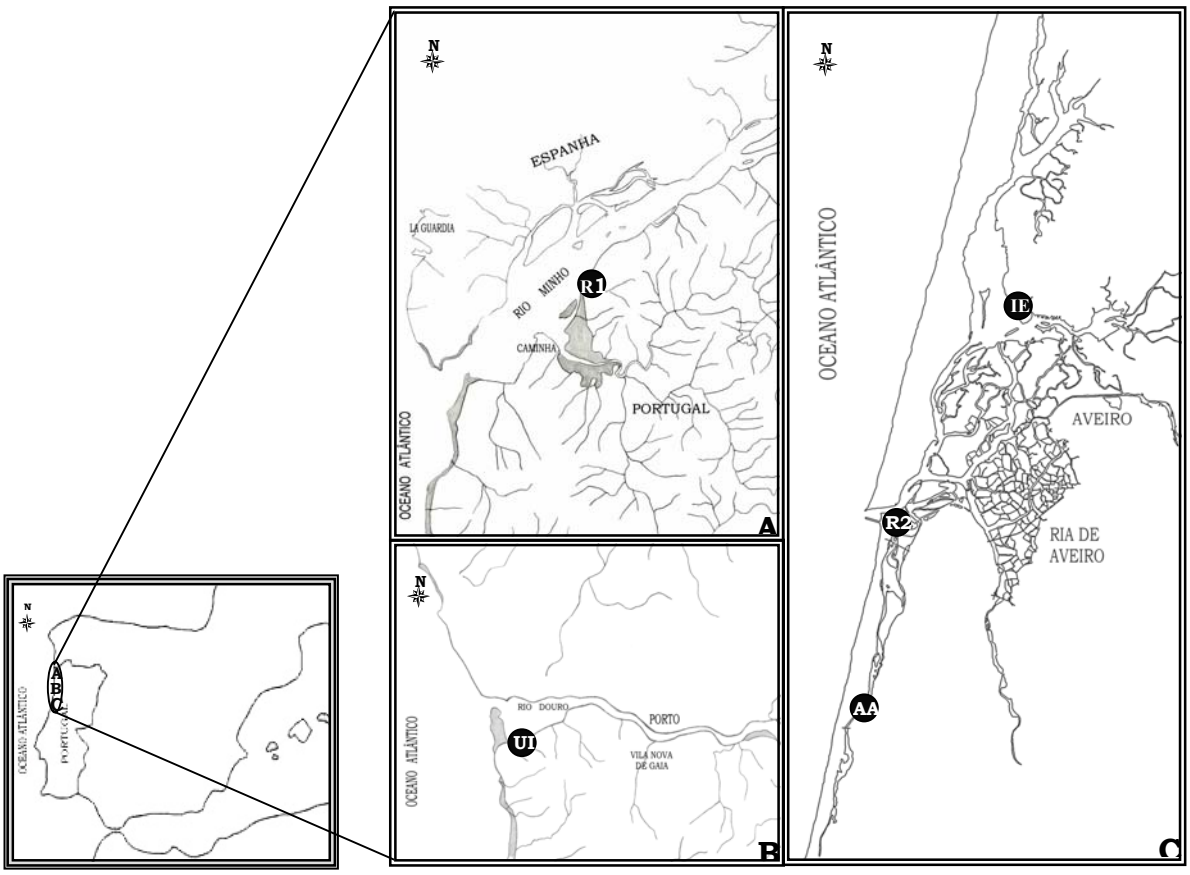

Figura 2 – Mapa demonstrativo da localização geográfica das estações de amostragem seleccionadas na costa noroeste portuguesa. R1, *"Reference 1"*; R2, *"Reference 2"*; UI, *"Urban and Industrial effluents"*; IE, *"Industrial effluents"*; AA, *Agricultural area.*

zonas específicas da laguna com diferentes tipos de poluentes como metais pesados, incluindo mercúrio (Ramalhosa *et al.*, 2000; Silva *et al.*, 2002). No estudo de biomonitorização de estuários realizado no presente trabalho foram seleccionadas três estações de amostragem na Ria de Aveiro: a estação denominada de "R*eference 2*", está situada junto à abertura artificial da Ria (Barra) e sob grande influência marinha; a estação "*Agricultural areas*", situada no canal de Mira em cujas margens proliferam campos agrícolas; e a estação *"Industrial effluents"*, situada no Largo do Laranjo, zona esta que está referenciada como fortemente contaminada por mercúrio (Ramalhosa *et al.*, 2000).

O objectivo central deste trabalho consistiu no desenvolvimento de uma bateria de biomarcadores para avaliação da toxicidade de contaminantes ambientais em populações estuarinas de *P. microps*. Para atingir tal desiderato foram efectuados três trabalhos específicos (Anexos I, II e III) aos quais estão subjacentes os seguintes objectivos:

i) padronizar as condições para a utilização das ChE de *P. microps* como biomarcador em ecossistemas estuarinos;
ii) identificar, em *P. microps*, biomarcadores adequados para o composto 3,4-dicloroanilina (DCA) comummente encontrado em sistemas aquáticos portugueses;
iii) testar em campo uma bateria de biomarcadores em populações naturais de *P. microps* para avaliação de diferentes tipos de contaminação ambiental.

O primeiro objectivo conduziu à caracterização do tipo de colinesterases presentes em diferentes tecidos da cabeça de *P. microps*, utilizando quatro substratos e três inibidores selectivos. Este estudo resultou na elaboração do artigo "*Characterisation of the cholinesterases present in head tissues of the estuarine fish* Pomatochistus microps*: application to biomonitoring*" a submeter (Anexo I).

Tendo em vista o cumprimento do segundo objectivo, estudaram-se os efeitos agudos da DCA, produto de degradação de pesticidas ordinariamente utilizados em Portugal, nos biomarcadores AChE, LDH e GST e na histologia do baço, após 96 horas de exposição de juvenis *de P. microps* a diferentes concentrações deste composto. Este trabalho deu origem ao artigo "*Acute effects of 3,4-dichloroaniline on biomarkers and spleen histology of the common goby* Pomatoschistus microps" a submeter (Anexo II).

Com a finalidade de responder ao terceiro objectivo proposto, efectuou-se um estudo de biomonitorização durante um ano, utilizando amostragens

sazonais, em locais de referência e em locais com diferentes tipos de contaminação, utilizando as enzimas AChE, LDH, P450 e GST em *P. microps*. Este estudo levou à elaboração do artigo "*Impact of chemical exposure on natural populations of* Pomatochistus microps *in estuaries of the Portuguese Northwestern coast*" a submeter (Anexo III).

MATERIAL E MÉTODOS GERAIS

O trabalho experimental realizado no âmbito desta tese pode subdividir-se em três partes principais (Parte I, II e III), tendo cada uma delas subjacente um conjunto de metodologias que se encontram descritas de forma mais detalhada em cada um dos anexos.

Parte I - Caracterização das colinesterases presentes em tecidos da cabeça de *P. microps*

Juvenis de *P. microps* foram capturados num local aparentemente com baixa influência antropogénica no estuário do rio Minho e transportados vivos para o laboratório onde se procedeu de imediato ao seu sacrifício por decapitação. A cabeça inteira ou diferentes partes da cabeça, nomeadamente o cérebro, o olho e os restantes tecidos (incluindo pele e músculo da cabeça) foram isolados e usados para determinação da actividade das ChE. De forma a caracterizar o(s) tipo(s) de ChE presente(s) nos diferentes tecidos da cabeça foram realizadas experiências independentes nas quais foram utilizados diferentes substratos (acetiltiocolina, acetil-β-metiltiocolina, propioniltiocolina e butiriltiocolina) e inibidores específicos (eserina, BW284C51 e iso-OMPA), conforme descrito detalhadamente no anexo I.

Parte II – Efeitos da DCA em biomarcadores e na histologia do baço de *P. microps*

No local do estuário do rio Minho acima referido, foram capturados juvenis de *P. microps*. Estes foram transportados vivos para o laboratório,

onde foram aclimatizados durante um período de 12 dias. Os organismos foram depois expostos individualmente, durante 96 horas, a uma gama crescente de concentrações de DCA (0; 0,50; 0,60; 0,72; 0,86; 1,04; 1,24; 1,49 mg/l). Após este período os organismos foram sacrificados por decapitação, separando-se o baço para análise histológica e a cabeça, músculo e brânquias para determinação da actividade das enzimas AChE, LDH e GST, respectivamente (ver anexo II).

Parte III – Impacto da exposição química em populações naturais de *P. microps*

Juvenis de *P. microps* foram capturados sazonalmente desde o Outono de 2001 ao Verão de 2002 em cinco estações de amostragem correspondentes a dois locais de referência (estuário do rio Minho e Barra na ria de Aveiro) e em áreas sob influência de efluentes urbanos e industriais (estuário do Douro), agrícolas (Canal de Mira na ria de Aveiro) e industriais (Largo do Laranjo na ria de Aveiro). Conforme já referido esses locais foram designados por "*Reference 1*", "*Reference 2*", "*Urban and Industrial effluents*", "*Agricultural areas*" e "*Industrial effluents*", respectivamente. Os organismos foram transportados vivos para o laboratório e sacrificados de imediato por decapitação separando-se a cabeça, músculo, brânquias e fígado para determinações da actividade enzimática de AChE, LDH, GST e P450, respectivamente, de acordo com os métodos descritos no anexo III.

RESULTADOS DISCUSSÃO E CONCLUSÕES

A potencial integração de *P. microps* em programas de biomonitorização de estuários da zona costeira ibérica levou à realização do presente trabalho, cujo objectivo central consistiu no desenvolvimento de uma bateria de biomarcadores para avaliação da exposição de populações naturais de *P. microps* a contaminantes ambientais.

A utilização da AChE como biomarcador requer o conhecimento prévio das formas de ChE presentes nos tecidos da espécie em estudo, dado que estas podem variar na sua sensibilidade a diferentes agentes anti-colinérgicos (Garcia *et al.*, 2000; Sturm *et al.*, 1999a e 2000). A forma de ChE presente em tecidos da cabeça de *P. microps* foi determinada recorrendo a diferentes substratos e inibidores selectivos (Anexo I). Os resultados obtidos sugerem a presença de dois tipos de ChE em homogeneizados da cabeça deste peixe. Os substratos preferencialmente hidrolisados foram a acetiltiocolina e acetil-β-metiltiocolina, sendo a actividade enzimática inibida por elevadas concentrações de substrato e pelo inibidor específico da AChE, BW284C51, comportamento típico da AChE de mamíferos. No entanto, verificou-se também hidrólise de butiriltiocolina, embora a uma taxa menor do que a de acetiltiocolina, e inibição da actividade por iso-OMPA, considerado inibidor selectivo da BChE de mamíferos. No entanto, esta enzima apresenta propriedades atípicas ao registar inibição com o BW284C51 no substrato preferencialmente hidrolisado pela BChE, butiriltiocolina.
Com a finalidade de esclarecer a proveniência em termos tecidulares da BChE detectada procedeu-se a um estudo de caracterização de tecidos da cabeça de *P. microps*, nomeadamente tecidos do cérebro, olho e remanescentes tecidos da cabeça. Os tecidos do cérebro e do olho revelaram apenas a presença de AChE. Os resultados relativos aos remanescentes tecidos da cabeça, que incluem tecido muscular, sugerem também a presença de AChE. No entanto registou-se nestes tecidos uma ligeira inibição da actividade da ChE, com o Iso-OMPA, que, embora não

significativa, provavelmente devido aos elevados erros padrão registados, indicia este homogeneizado de tecidos como provável origem da forma de BChE presente na cabeça de *P. microps*. Vários autores demonstraram a presença de BChE com propriedades atípicas em músculo de espécies marinhas de peixes (Leibel *et al.*, 1988; Stegiel *et al.*, 1989; Sturm *et al.*, 1999a), enquanto que no cérebro e olho de peixes a única forma de ChE encontrada é, na sua generalidade, AChE (Kozlovskaya *et al.*,1993).
Face aos resultados obtidos neste estudo, o biomarcador AChE em *P. microps* deve ser preferencialmente quantificado em tecidos do cérebro e não na cabeça inteira, uma vez que é no cérebro que se regista apenas actividade da AChE e onde esta enzima apresenta maiores níveis de actividade. No entanto, o isolamento do cérebro em espécimes juvenis de *P. microps* (cujo comprimento vai até cerca de 3,0 cm) é bastante moroso e em estudos de biomonitorização e testes laboratoriais de toxicidade, onde é necessário o processamento de um largo número de amostras num curto espaço de tempo, não é de todo comportável. Como tal, e equiponderando os diferentes pontos, nos estudos posteriores realizados com esta enzima (Anexo II e Anexo III) utilizou-se a cabeça inteira.

Numa segunda fase do trabalho foram estudados os efeitos do composto 3,4-dicloroanilina (DCA), contaminante ambiental resultante da degradação de pesticidas comummente utilizados em Portugal, em parâmetros bioquímicos (AChE, LDH e GST) e na histologia do baço, após 96 horas de exposição de juvenis de *P. microps* a diferentes concentrações deste composto (Anexo II). A actividade da AChE não foi significativamente afectada pela exposição à DCA, o que está de acordo com os resultados publicados na literatura (Guilhermino *et al.*, 1996; Sturm *et al.*, 1999b). Foi observado um aumento da actividade da LDH em peixes expostos à concentração de 1,2 mg/l de DCA. Induções a nível da actividade da LDH em algumas espécies têm sido referidas em condições de stress químico (De Coen *et al.*, 2001), nomeadamente em crustáceos expostos durante 21 dias à DCA (Guilhermino *et al.*, 1994). A actividade das GST foi inibida significativamente pelas

concentrações mais baixas de DCA testadas (0,50; 0,60 e 0,72 mg/l de DCA), resultado que carece de futura investigação.

Com os resultados obtidos para as três enzimas em estudo pode concluir-se que a AChE não deve ser utilizada como instrumento de análise para a exposição e/ou efeitos da DCA em *P. microps*. A LDH, quando utilizada como biomarcador em estudos de campo, deverá fornecer uma informação limitante em relação à exposição a DCA, uma vez que as elevadas concentrações a que esta enzima registou inibições raramente são detectadas no ambiente. A inibição apresentada pelas GST na exposição a baixas concentrações é indicativa de resposta desta enzima à presença de DCA, no entanto o seu eventual uso como biomarcador em *P. microps* requer estudos adicionais, uma vez que nas concentrações mais altas testadas esta enzima não apresentou quaisquer alterações.

Importantes alterações histológicas a nível do baço foram registadas após as 96h de exposição à DCA. Essas alterações ocorreram de uma forma gradual em relação à concentração utilizada e incluiram a expansão da polpa vermelha, congestão vascular e deposição de grânulos de hemosiderina. Resultados similares foram também encontrados no baço de mamíferos como resultado da exposição a diferentes tipos de anilinas incluindo a DCA (Khan *et al.*, 1995; Guilhermino *et al.*, 1998). Os resultados obtidos, no seu conjunto, sugerem que os efeitos em peixes são semelhantes aos observados em mamíferos, pelo que esta substância, produto de degradação de herbicidas muito utilizados em Portugal, levanta problemas ambientais em estuários sob influência agrícola. As alterações registadas sugerem ainda a histologia do baço como um potencial instrumento na avaliação da exposição a DCA.

Com o propósito de testar em campo uma bateria de biomarcadores em populações naturais de *P. microps* para avaliação de diferentes tipos de contaminação ambiental, procedeu-se a um estudo de biomonitorização anual de amostragens sazonais, em locais de referência (*Reference 1* e *Reference 2*) e locais com diferentes tipos de contaminação (*Urban and industrial effluents*, *Agricultural areas*, *Industrial effluents*), utilizando os

biomarcadores AChE, LDH, P450 e GST. Os resultados obtidos neste estudo indicam que, tanto diferentes tipos de contaminação ambiental como a sazonalidade parecem ter um efeito sobre os biomarcadores estudados.

A estação *Reference 1* é caracterizada pela ausência de grandes áreas industriais, agrícolas ou metropolitanas. Contudo, durante a primavera, e principalmente no verão esta área torna-se um ponto de turismo efectivo para a prática de desportos náuticos e pesca desportiva. Este facto pode ter contribuído para as actividades relativamente baixas das enzimas AChE e LDH registadas nos espécimes recolhidos nesta estação. Alguns autores demonstraram inibição de AChE por misturas complexas de óleos usados em motores (Payne *et al.* 1996) e PAHs (Kang & Fang, 1997). Gagnon & Holdway (1999) demonstraram também a inibição de LDH por exposição a baixas concentrações deste tipo de compostos, nomeadamente de crude. Atendendo aos resultados obtidos pelos autores supra-citados e dadas as características do local em estudo, as inibições conjuntas da AChE e da LDH obtidas indiciam alguma contaminação por este tipo de compostos.

A actividade enzimática da EROD de espécimes de *P. microps* recolhidos na estação *Urban and industrial effluents* apresentou valores significativamente mais altos que as restantes estações de amostragem, sugerindo a presença no meio aquático de indutores do P4501A1, tais como PAHs e/ou PCBs, como tem sido demonstrado por diversos autores (Goksøyr & Förlin, 1992; Stegeman & Hahn, 1994).

As GST, enzimas da fase II de biotransformação, apresentaram valores significativamente mais elevados em peixes recolhidos na estação *Industrial effluents* quando comparados com os valores registados nos peixes das restantes estações de amostragem, enquanto que os da estação *Agricultural area* registaram valores significativamente mais baixos. O mercúrio é o tipo de contaminação predominante na estação *Industrial effluents* (Ramalhosa *et al.*, 2000), o qual pode estar na origem da indução das GST registada nos espécimes deste local. Alguns autores têm identificado induções das GST como resposta à presença de metais pesados (Lenartova *et al.*, 1997; Chen *et al.*, 1998; Lopes *et al.*, 2001).

Diferentes trabalhos apresentam diferentes respostas das GST à presença de diferentes tipos de pesticidas, nomeadamente indução ou inibição (Pedrajas *et al.*, 1995; Lindström-Seppä *et al.*, 1996). Devido às diferentes respostas das GST a este tipo de compostos, da inibição de GST registada na estação *Agricultural area* apenas se pode extrair que os espécimes recolhidos neste local estão expostos a um determinado tipo de impacto diferente dos restantes locais analisados, e que pode eventualmente ser originado a partir da lixiviação dos campos agrícolas.

Um dos atributos que os biomarcadores de contaminação ambiental devem contemplar é a não-dependência de efeitos advindos de variações sazonais (Connell *et al.*, 1999; Walker *et al.*, 2001). No entanto, este tipo de variações são comummente encontradas numa variedade de biomarcadores em diferentes espécies bioindicadoras, e como tal, são factores importantes a ter em conta em estudos de biomonitorização (Sheehan & Power, 1999). No presente estudo em termos de análise discriminante, a bateria de biomarcadores revelou um índice de discriminação mais elevado em relação aos efeitos da variação sazonal do que em relação aos efeitos da variação de locais de amostragem. Os resultados mais relevantes em termos de variação sazonal referem-se à diminuição da actividade da AChE durante o Inverno e Primavera, e também da diminuição da EROD durante o Inverno. Em relação à AChE, este facto pode ser ocasionado pelas escorrências de compostos anti-colinérgicos dos campos agrícolas, uma vez que é maioritariamente durante esta altura do ano que são aplicados diversos tipos de compostos nas culturas. Resultados similares foram também obtidos por Escartín & Porte (1997). A diminuição da actividade da EROD deve, por outro lado, estar relacionada com a diminuição da temperatura que se regista nas águas temperadas durante o Inverno, a qual pode causar uma atenuação da indução do P4501A1 (Stegeman & Hahn, 1994).

Em conclusão, apesar das flutuações sazonais verificadas, importantes induções ou inibições foram obtidas como resposta da bateria de biomarcadores aos diferentes tipos de contaminação ambiental em estudo, a saber: na estação *Urban and Industrial effluents* foram registadas induções

da actividade da EROD, enquanto na estação *Agricultural areas* se registou inibição de GST e na estação *Industrial effluents* se verificou indução da actividade das GST. Os resultados obtidos neste estudo confirmam a adequação e relevância da utilização de *P. microps* como biomonitor em estuários da zona costeira ibérica. Estes resultados sublinham ainda a utilidade da abordagem de biomarcadores a diferentes níveis de organização biológica na avaliação da toxicidade de contaminantes ambientais. No entanto, uma vez que dois dos biomarcadores seleccionados, AChE e LDH, se revelaram pouco discriminatórios em relação a determinados tipos de contaminação ambiental, a inclusão de novos biomarcadores com potencial capacidade para os distinguir será importante em futuros estudos de biomonitorização utilizando *P. microps*.

No que respeita ao estudo de toxicidade aguda, importantes alterações verificadas a nível histológico e bioquímico por exposição à DCA revelaram-se como eventuais ferramentas preditivas da exposição e/ou efeitos deste composto em *P. microps*, as quais necessitam ser exploradas e viabilizadas por meio de estudos ecotoxicológicos adicionais, de forma a serem utilizadas em programas de monitorização ambiental como indicativos da exposição a este tipo de compostos.

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ANEXO I

Characterisation of the cholinesterases present in head tissues of the estuarine fish *Pomatochistus microps*: application to biomonitoring

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ABSTRACT

In the last years biomarkers have been widely used for the assessment of effects and/or exposure to environment contaminants. One of the most frequently used is the inhibition of cholinesterases (ChE), which is a useful indicator of organophosphate and carbamate exposure. Recent studies indicated that more than one ChE may be present in tissues of fish and that different forms may vary in their sensitivity to anti-cholinesterase agents. Cholinesterase activity for juvenile common gobies (*Pomatoschistus microps*), which is a widespread fish in estuaries of the Atlantic coast of north-western Europe, was determined with four substrates (acetylthiocholine iodide, acetyl-β-metylthiocholine iodide, propionylthiocholine iodide and S-butyrylthiocholine iodide) and three ChE inhibitors (eserine sulphate, BW284C51 and iso-OMPA) in different tissues of the fish head. In addition, the range of ChE activity that may be considered as "normal" for non-exposed fish *P. microps* was determined.

The results obtained suggest the presence of two types of ChE in the entire head homogenate. In the brain, eye and other head tissues, including muscle tissue, AChE seemed to be the predominant form. The range of "normal" activity registered for natural populations of *P. microps* juveniles was 82.0±7.1 nmol.ml^{-1}.mg prot^{-1} in entire head homogenate.

The present study underlies the relevance of ChE characterisation before its use as a biomarker in biomonitoring studies.

Keywords: cholinesterases; AChE; BChE; *Pomatoschistus microps*; wild populations

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INTRODUCTION

Inhibition of AChE activity has been widely used in aquatic animals to diagnose exposure to anticholinesterase compounds, such as organophosphate (OP) and carbamate (CB) pesticides (Peakall, 1992; Fulton & Key, 2001). These widespread pesticides are used to control agricultural pests, but are potentially toxic for non target organisms. The primary toxicity of these substances is due to an irreversible inhibition of acetylcholinesterase (AChE), a key enzyme of the nervous system. The inhibition provokes an accumulation of acetylthiocholine in synapses with disruption of the nerve function that can end in the dead of the organism (Peakall, 1992). More recently, several studies indicate that cholinesterases (ChE) are also sensitive to other type of environmental contaminants, such as metals and complex mixtures of used hydrocarbons (Gill *et al.*, 1990; Payne *et al.*, 1996; Guilhermino *et al.*, 1998).

The development of biomarkers based on the study of biological responses of organisms to pollutants has provided the biochemical tools essential to the implementation of programs for monitoring contaminants effects. Although ChE activity is widely used as specific biomarker to monitor the effects of anticholinesterase pesticides, not only in humans but also in wild life, its use in a particular species requires the characterisation of the enzyme(s) present in the tissues to be analysed and the determination of the activity range that may be considered as “normal” for non-exposed individuals (Bocquené *et al.*, 1990; Garcia *et al.*, 2000).

Vertebrate cholinesterases are traditionally divided into two main classes, AChE (EC 3.1.1.7) and butyrylcholinesterase or pseudocholinesterase (BChE, EC 3.1.1.8). The two enzymes are distinguished functionally, primarily on the basis of substrate specificity and to their susceptibility to diagnostic inhibitors (Kozlovskaya *et al.*, 1993). The main function of AChE is the hydrolysis of acetylcholine, the mediator of cholinergic synapses in the nervous system, but the role of BChE remains to be clarified (Kozlovskaya *et al.*, 1993).

Recent research indicated that more than one ChE may be present in tissues of estuarine fish and the different forms may have distinct sensitivities to anti-cholinesterase agents (Sturm *et al.*, 1999 and 2000;). BChE is mainly found in serum, but may also contribute to ChE activity in muscle and other tissues. Brain tissue contains exclusively AChE in most teleost fish (Kozlovskaya *et al.*, 1993; Sturm *et al.*, 1999). Axial muscle tissue may contain exclusively AChE, as in many freshwater teleosts, or both AChE and BChE, as in most marine species (Kozlovskaya *et al.*, 1993; Chuiko, 2000; Garcia *et al.*, 2000). Therefore, it is essential to perform the characterisation of the ChE present in the tissue and species to be used in biomonitoring programs (Bocquené *et al.*, 1990; Sturm *et al.*, 1999).

In order to standardise the conditions for the use of ChE activity from the common goby (*Pomatochistus microps*) as a biomarker in estuarine environments, the aim of this work was: (i) to characterise the soluble ChE present in the entire head and in different head tissues homogenates using four substrates and three selective ChE diagnostic inhibitors and (ii) to determine the “normal” range of ChE activity of non-exposed individuals from wild populations of *Pomatoschistus microps* (Krøyer, 1838) of the Portuguese north-western coast.

The epibenthic euryhaline fish *Pomatoschistus microps* is a widespread organism in estuaries of the Atlantic coast of north-western Europe. These minute fish are intermediate predators in the food-web, connecting microbenthos with larger predator fish, playing an important

role in estuarine ecosystems (Miller, 1986; Arruda *et al.*, 1993). Therefore, *P. microps* seems to be a relevant species for use as bioindicator of pollutant effects in estuarine environments.

MATERIAL AND METHODS

1. Biological material

Juvenile fish (length ranging from 22 to 30mm) were collected in a non-impacted site in the estuary of the Minho river, using a landing net during low water tide. Fish were transferred live to the laboratory and sacrificed by decapitation, after being measured. The brain tissue of juveniles common gobies is very difficult to remove, thus the entire head seemed to be the most suitable biological material for routine biomonitoring studies. However, for further use of this fraction as a ChE source, a careful characterisation of the different ChE present in different head tissues is convenient to be performed. Thus, the entire head, or different parts of the head, namely the brain, the eye and the remaining tissues of the head (which include head skin and muscle) were isolated and used for ChE determinations with different substrates and selective inhibitors.

2. ChE determinations

The tissues were homogenated in 1 mL of potassium phosphate buffer (0.1M, pH 7.2) and samples were stored at –20ºC until enzymatic analysis. The supernatants obtained after centrifugation of the homogenates (4ºC, 6000 rpm, 3 min) were removed and used as enzyme extract for ChE activity determinations, which was determined in triplicate and carried out according to the Ellman method (Ellman *et al.*, 1961) adapted to microplate reader (Guilhermino *et al.*, 1996). The enzymatic

activity was expressed in Units (U) per mg of protein (1U is a nmol of substrate hydrolysed per minute).

3. Protein assays

Protein content of the samples was determined, in triplicate, by the Bradford method (Bradford, 1976) adapted to microplate, using γ-bovine globulin's as standard and a wavelength of 595 nm. A Labsystem Multiskan EX microplate reader was used.

4. Cholinesterase characterisation

In order to characterise the substrate preferences of *P. microps* ChE present in the homogenated tissues, different substrates were used. In independent experiments, AcSCh, AcβMeSCh, BuSCh and PrSCh were used as substrates at increasing concentrations (from 0.005 to 20.5 mM).

Eserine, 1,5-bis(4-allyldimethyl-ammonimphenyl) pentan-3-one dibromide (BW284C51) and tetraisopropyl pyrophosphoramide (iso-OMPA) were selected as specific inhibitors of ChE, AChE and BuChE, respectively. For each inhibitor, stock solutions were prepared in ultra-pure water or ethanol, as appropriate. From each inhibitor, 5 µl of each stock solution were incubated with 495 µl of enzyme extract for 30 minutes at 25ºC before the addition of the substrate AcSCh. Inhibitor concentrations ranged from 6.25 to 200 µM (eserine and BW284C51) and 0.25 to 8.0 mM (iso-OMPA). Ultra-pure water was added to controls and an additional control with ethanol was used in the experiments with iso-OMPA.

Based on the results obtained with the different substrates and selective inhibitors, ChE classification was accomplished as follows: after 30 minutes incubation of enzyme extracts with BW284C51 and iso-OMPA (same concentration ranges as above) ChE was assayed with different

substrates: AcβMeSCh (specific for AChE), BuSCh and PrSCh. The effects of BW284C51 and iso-OMPA on ChE activity of the entire head were assayed on the four different substrates. In the brain, eye and other head tissues the effects of BW284C51 and iso-OMPA were assayed with AcSCh and AcβMeSCh. Three *P. microps* juveniles were used per treatment in all the different characterisation experiments.

5. *Normal range of ChE activity*

The range of ChE activity that can be considered as "normal" for the *P. microps* was determined in 20 organisms collected in the river Minho estuary, in an area that can be considered as non-contaminated.

6. Chemicals

DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), AcSCh (acetylthiocholine iodide), AcβMeSCh (acetyl-β-metylthiocholine iodide), BuSCh (S-butyrylthiocholine iodide), PrSCh (propionylthiocholine iodide), eserine sulfate, BW284C51 (1,5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide), iso-OMPA (tetraisopropyl pyrophosphoramide), were obtained from Sigma (The Netherlands). All the other chemicals were from Merck (Germany) except the Bradford reagent, which was purchased from Bio-Rad (Germany).

7. Statistical analyses

Data were analysed by one way Analysis of Variance (ANOVA) when the criteria of normality and equality of variance were satisfied (after square root transformation if necessary) or using the non-parametric Kruskal-wallis test in the remaining cases. Significant different treatments were determined by Dunnet's test (Zar, 1996).

RESULTS

In order to investigate the substrate preferences of ChE present in the entire head and in different head tissues of *P. microps*, four substrates were assayed: AcSCh, AcβMeSCh, PrSCh and BuSCh. The ChE activity in the different head tissues as a function of increasing concentrations of substrates is presented in figure 1. The substrate preferences of *P. microps* ChE was qualitatively similar in the four different head tissue homogenates tested, namely the entire head, brain, eye and other head tissues. In all tissues, AcSCh was the substrate cleaved at the highest rate and always presented the highest activity at 2.56 mM of AcSCh (130.8 ± 4.3 U/mg protein in head and 43.0 ± 1.0 U/mg protein in eye). A slightly decrease of activity was observed when AcβMeSCh was used as substrate (highest activity: 95.2 ± 6.9 U/mg protein in brain tissue). Less activity was observed when PrSCh was used as substrate (highest activity: 42.8 ± 3.5 U/mg protein in brain tissue) and even less activity was obtained with BuSCh (highest activity: 5.9 ± 0.2 U/mg protein in entire head). Furthermore, a reduction of AChE activity was observed in all tissues at concentrations of substrate higher than 2.56 mM, except when BuSCh was used.

The effects of eserine sulphate on ChE activity in the different tissues are presented in figure 2. An almost full inhibition of activity was found for all the tissues analysed.

In the entire head enzymatic extract, the selective inhibitor of AChE, BW284C51 significantly inhibited ChE activity using both AcSCh and AcβMeSCh ($p<0.001$; Fig. 3A) and on PrSCh ($p<0.001$; Fig. 3B), showing inhibitions of about 98% on AcSCh and AcβMeSCh and 84% on PrSCh at concentrations up to 200 µM. ChE activity of *P. microps* head with BuSCh (Fig. 3B) was also significantly inhibited by BW284C51 ($p<0.05$) at 100 and 200 µM, however, about 25% of the enzymatic activity was not inhibited up to 200 µM. The ChE activity of the brain, eye and the other

head tissues was significantly inhibited by BW284C51 with AcSCh and AcβMeSCh ($p<0.001$; Fig. 5A and 5B). In the brain and in the eye, ChE activity registered inhibitions of about 98-99% with both substrates up to 200 µM of BW284C51. The other head tissues, which include muscle and skin, presented 96.6% of inhibition by using AcSCh and 94.4% with AcβMeSCh up to 200 µM.

The effect of the selective inhibitor of BChE, iso-OMPA, on *P. microps* ChE depended on the tissue and on the assayed substrate. In the entire head enzymatic extracts, using AcβMeSCh as substrate, ChE activity remained unaffected ($p>0.05$) by iso-OMPA at concentrations up to 8 mM, but with both AcSCh and PrSCh significant inhibitions were found ($p<0.05$ and $p<0.01$, respectively) of about 26% and 42%, respectively, at 8 mM of inhibitor concentration (Fig. 4). The head ChE activity with BuSCh was significantly inhibited by iso-OMPA ($p<0.001$) at all concentrations tested, showing complete inhibition (95%) at 8 mM. In the brain, eye and the other head tissues, the ChE activity with AcSCh and with AcβMeSCh were not significantly affected by iso-OMPA ($p>0.05$) at concentrations up to 8 mM.

The "normal" range of head ChE activity measured of non-exposed *P. microps* individuals was 82.0 ± 7.1 U/mg protein.

DISCUSSION

The first aim of this study was to characterize the ChE activity of *P. microps* head tissues. In order to display such characterization ChE must be distinguished from non-specific esterases. This fact is important since tissues may contain significant amounts of non-specific esterases, which contribute to the measured activity and may show different sensitivity towards anticholinesterase agents (Garcia *et al.*, 2000). The contribution of non-specific esterases was estimated using the compound eserine

sulphate, which is considered as a specific inhibitor of ChE at low concentrations, 10^{-6}-10^{-5} M range (Eto, 1974). In the present study, the enzymatic activity measured in the different head tissues was almost completely inhibited by eserine sulphate, within the µM range, which is considered typical for ChE. This result indicates that the enzyme(s) assayed in our experimental conditions is(are) a ChE(s) and not other type of esterases.

The ChE activity measured in all head tissues shows a distinct preference for AcSCh as substrate, over the other three substrates used. In addition, enzymatic inhibition was observed at high concentrations of substrate. These findings suggest the presence of AChE activity since this enzyme hydrolyses AcSCh more rapidly than PrSCh or BuSCh and it is inhibited by high substrate concentrations (Eto, 1974). The response of *P. microps* head ChE to the substrates assayed was similar to the response commonly found in brain tissue of other marine teleost fish. For example, three marine fish studied by Sturm *et al.* (1999) showed a ChE activity with AcβMeSCh ranging from 61% to 73% of the activity obtained with AcSCh (100%), the activity with PrSCh ranged from 19% to 49%, while the activity with BuSCh was less than 3%.

The response of ChE activity in the entire head homogenate to BW284C51, considered as selective AChE inhibitor, measured with four diagnostic substrates (AcSCh, AcβMeSCh, PrSCh and BuSCh) was a strong inhibition with both AcSCh and AcβMeSCh (of about 98% at 200 M) and a lower but significant decrease with PrSCh (of about 84% at 200 µM). These results suggest the presence of AChE, which is strongly inhibited by BW284C51 (Kozlovskaya *et al*, 1993). Furthermore, the activity with BuSCh was significant inhibited with BW284C51 (up to 75% inhibition at 200 µM), which yields some evidence for the presence of an atypical ChE that cleaved substrates diagnostic for BChE but was sensitive to BW284C51, an AChE inhibitor.

The results obtained in the entire head homogenate with iso-OMPA, the BChE inhibitor, yields evidence of the presence of a ChE that cleaves AcβMeSCh, resistant to iso-OMPA, and another ChE that cleaves BuSCh and is inhibited by iso-OMPA. The behaviour of the first enzyme justifies its classification as AChE as it hydrolyses AcβMeSCh, substrate specific for AChE, and it is insensitive to the BChE inhibitor, iso-OMPA. The second type of ChE presents classical characteristics of BChE, presenting sensibility to iso-OMPA with the substrate BuSCh. This is also suggested by the partial inhibition of activity with AcSCh by iso-OMPA, that significantly inhibited the activity at the lowest concentration, 0.25 mM, but did not registered additional inhibition with increasing concentration up to 8.0 mM of iso-OMPA. However, this enzyme presenting BChE characteristics, also registered sensibility to the AChE inhibitor, BW284C51, which is considered an atypical feature for BChE. Similar results obtained by other authors support the present interpretation. Stieger *et al.* (1989) and Sturm *et al.* (1999) have also found sensibility to BW284C51 of BChE of marine teleost fish.

In order to clarify the provenience of the atypical BChE found in the entire head homogenate, experiments with separated head tissues were carried, namely with the brain, eye and the other remaining head tissues (which include muscle and skin).

Brain and eye tissues seem to have only AChE since they hydrolysed AcβMeSCh at a high rate and BuSCh at a very low rate, and almost full inhibition with BW284C51 and insensibility to iso-OMPA were found. These results are in agreement with findings published in the literature for other teleost fish (Szabó *et al.*, 1992; Kozlovskaya *et al.*, 1993).

The other tissues from the head include muscle, which in other fish species showed the presence of an atypical BChE in addition to AChE (Leibel *et al.*, 1988; Stegier *et al.*, 1989; Sturm *et al.*, 1999). In this study, ChE activity with AcSCh and AcβMeSCh was almost completely inhibited

by BW284C51 and was not significantly affected by iso-OMPA. However, the activity on both substrates registered diminution in all the iso-OMPA concentrations tested, when compared to the controls. This diminution may not present significantly differences due to the relatively elevated mean standard errors registered in those experiments. Despite this fact, we can conclude that the atypical BChE found in the whole head homogenate is not present in the brain, nor in the eye, but may be present, at low activity levels when compared to AChE levels, in other head tissues. However, further experiments should be carried, in order to confirm the presence of the atypical BChE in non-specific head tissues and to study the specific properties of this enzyme.

Using AcSCh as substrate, ChE activity of *P. microps* head tissues, determined in non-exposed organisms was 82.0 ± 7.1 U/mg protein. This value is within the range of values reported in the literature for several species of fish. Varó *et al.* (2003) indicate a mean of 43.32 ± 4.42 U/mg protein as the "normal" range of AChE in the brain of the European sea bass juveniles (*Dicentrarchus labrax*), and Sturm *et al.* (1999) refer to AChE activity values of 195.9 ± 56.8, 314 ± 35.5 and 85.8 ± 23.1 U/mg protein in the brain of the three marine teleost fish *Limanda limanda*, *Platichthys flesus* and *Serranus cabrilla*, respectively.

In conclusion, for further use of ChE from *P. microps* juveniles as a biomarker in biomonitoring programs, brain seems to be the most suitable tissue for enzymatic measurements, since it presented the highest ChE activity and only AChE seems to be present. However, due to the small size of *P. microps* juveniles' brain, is not practical the use of brain tissue if a large number of samples need to be processed. Despite the registered presence in the entire head homogenate of two types of ChE, the atypical BChE is a small fraction of the total ChE activity and may not influence in a great proportion eventual inhibitions in field by environmental contaminants. Nonetheless, a careful analysis of field data should be

attained if the entire head and not the brain is selected for AChE measurements.

Acknowledgements

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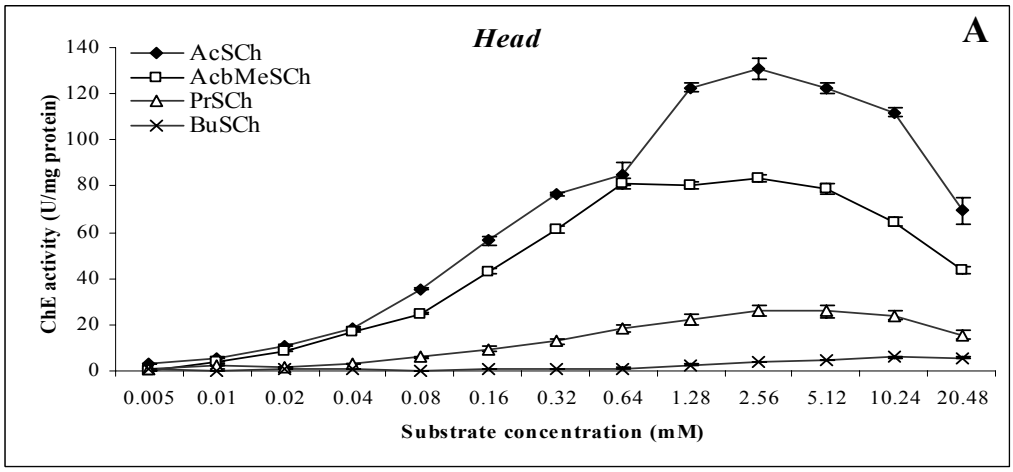
FIGURES LIST


Head
A
AcSCh
AcbMeSCh
PrSCh
BuSCh
ChE activity (U/mg protein)
Substrate concentration (mM)
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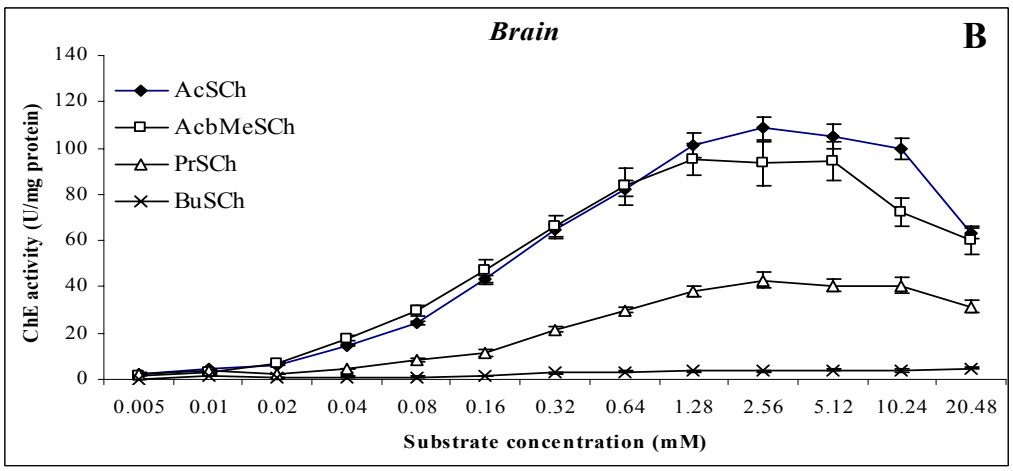
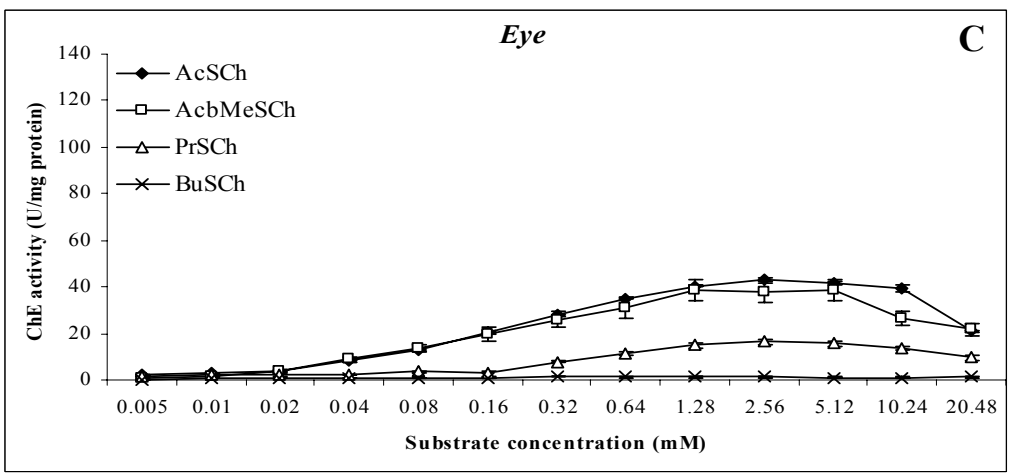

Brain
B
AcSCh
AcbMeSCh
PrSCh
BuSCh
ChE activity (U/mg protein)
Substrate concentration (mM)
0.005 0.01 0.02 0.04 0.08 0.16 0.32 0.64 1.28 2.56 5.12 10.24 20.48


Eye
C
AcSCh
AcbMeSCh
PrSCh
BuSCh
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Substrate concentration (mM)
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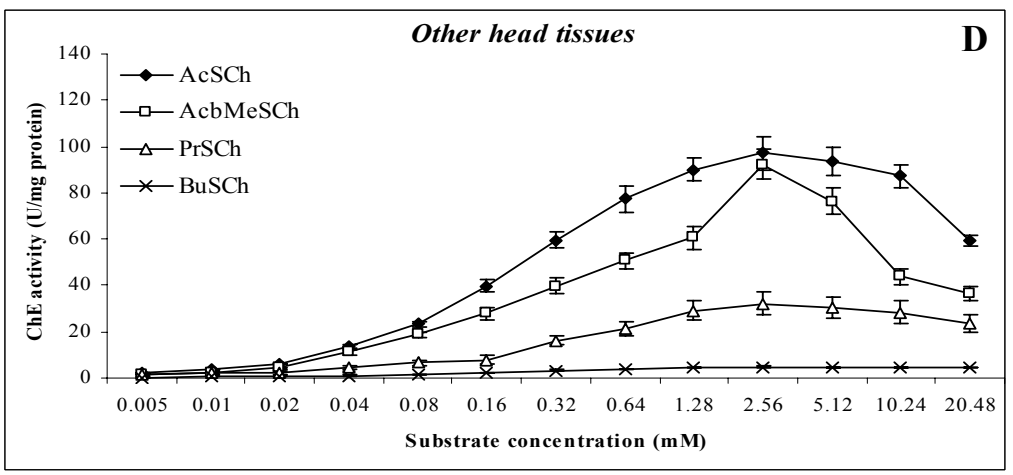

Other head tissues
D
AcSCh
AcbMeSCh
PrSCh
BuSCh
ChE activity (U/mg protein)
Substrate concentration (mM)
0.005 0.01 0.02 0.04 0.08 0.16 0.32 0.64 1.28 2.56 5.12 10.24 20.48

Figure 1

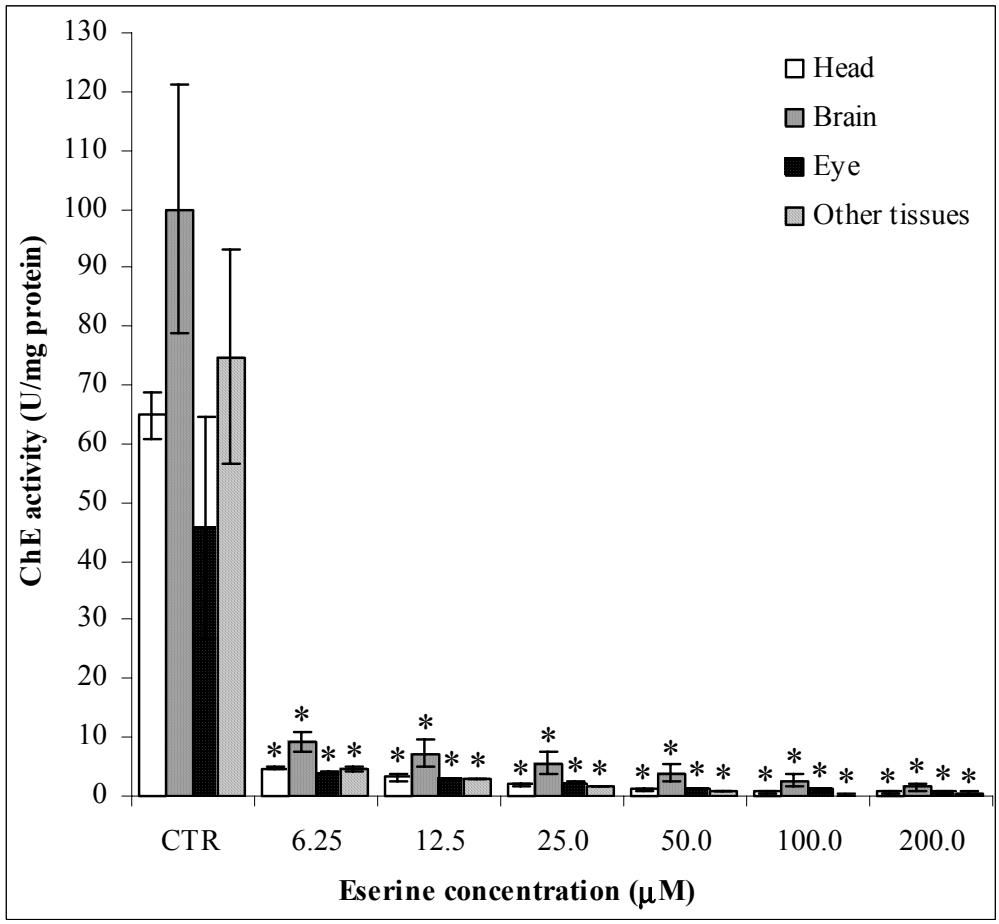

130
120
110
100
90
80
70
60
50
40
30
20
10
0
ChE activity (U/mg protein)
Head
Brain
Eye
Other tissues
CTR
6.25
12.5
25.0
50.0
100.0
200.0
Eserine concentration (μM)

Figure 2

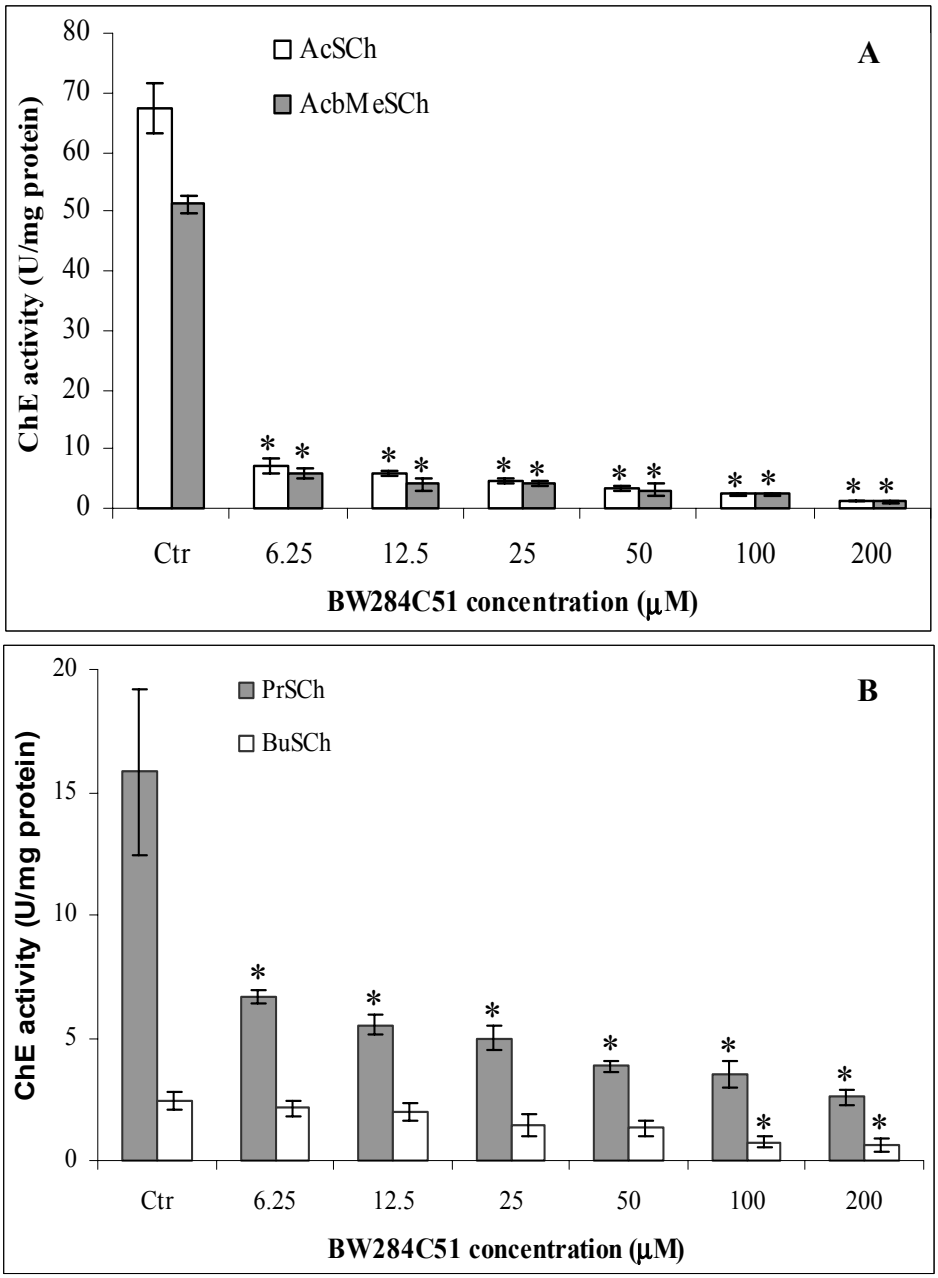

A
AcSCh
AcbMeSCh
ChE activity (U/mg protein)
80
70
60
50
40
30
20
10
0
Ctr
6.25
12.5
25
50
100
200
BW284C51 concentration (μM)
B
PrSCh
BuSCh
ChE activity (U/mg protein)
20
15
10
5
0
Ctr
6.25
12.5
25
50
100
200
BW284C51 concentration (μM)

Figure 3

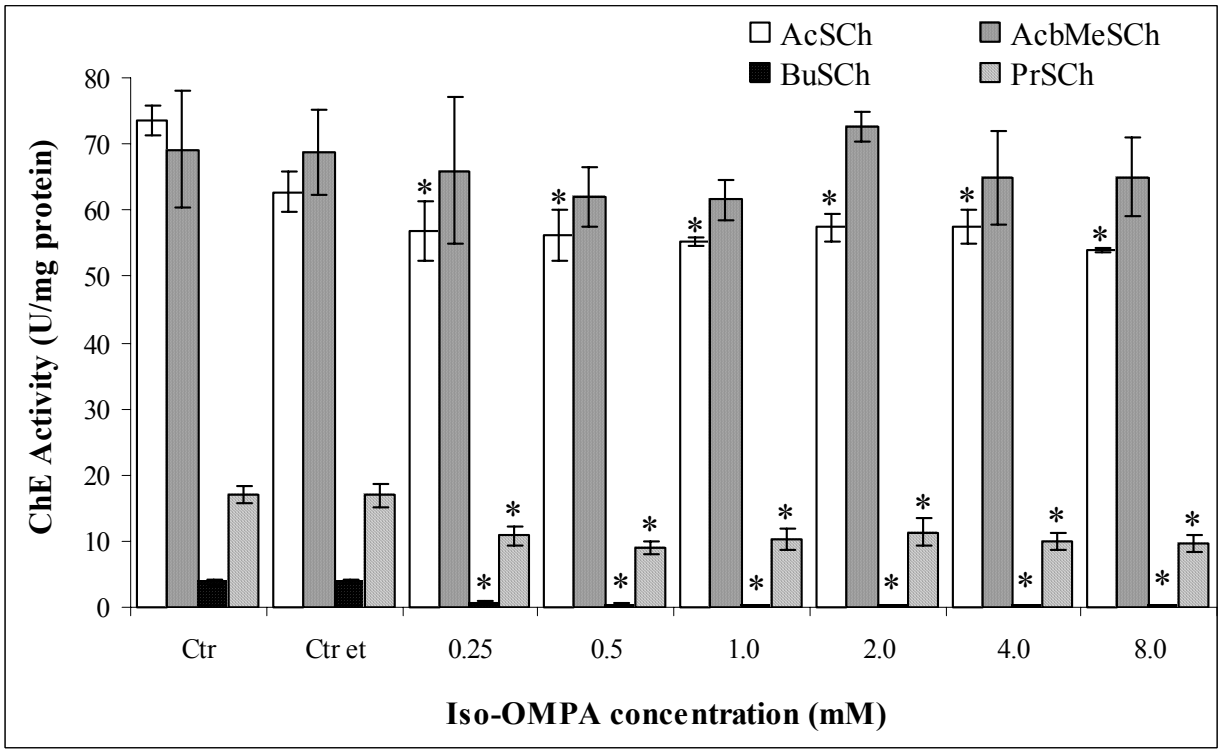
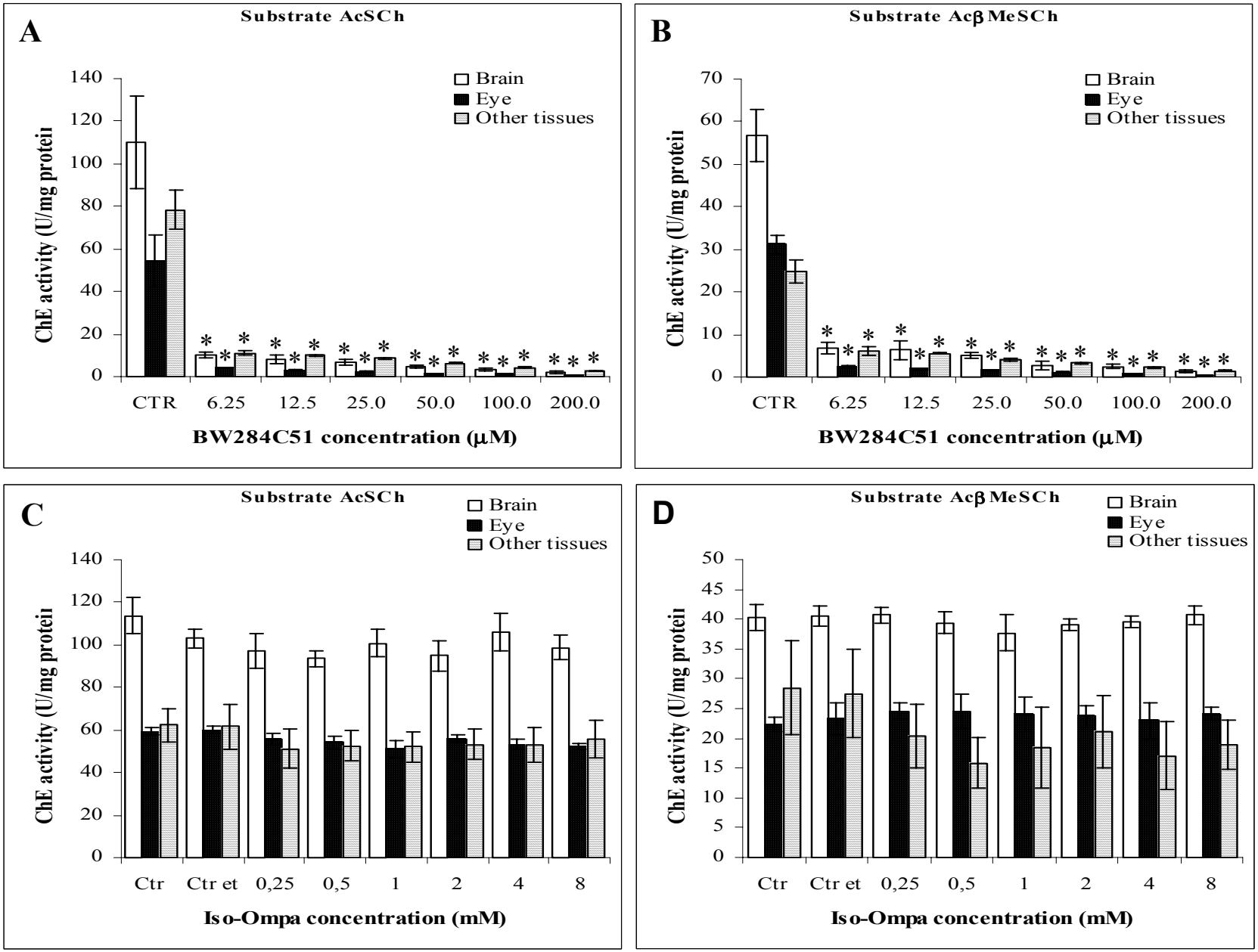

AcSCh
AcbMeSCh
BuSCh
PrSCh
ChE Activity (U/mg protein)
80
70
60
50
40
30
20
10
0
Ctr
Ctr et
0.25
0.5
1.0
2.0
4.0
8.0
Iso-OMPA concentration (mM)

Figure 4


A
Substrate AcSCh
Brain
Eye
Other tissues
ChE activity (U/mg protein)
140
120
100
80
60
40
20
0
CTR
6.25
12.5
25.0
50.0
100.0
200.0
BW284C51 concentration (μM)
B
Substrate AcβMeSCh
Brain
Eye
Other tissues
ChE activity (U/mg protein)
70
60
50
40
30
20
10
0
CTR
6.25
12.5
25.0
50.0
100.0
200.0
BW284C51 concentration (μM)
C
Substrate AcSCh
Brain
Eye
Other tissues
ChE activity (U/mg protein)
140
120
100
80
60
40
20
0
Ctr
Ctr et
0,25
0,5
1
2
4
8
Iso-Ompa concentration (mM)
D
Substrate AcβMeSCh
Brain
Eye
Other tissues
ChE activity (U/mg protein)
50
45
40
35
30
25
20
15
10
5
0
Ctr
Ctr et
0,25
0,5
1
2
4
8
Iso-Ompa concentration (mM)

XX

Figura 5

ANEXO II

Acute effects of 3,4-dichloroaniline on biomarkers and spleen histology of the common goby *Pomatoschistus microps*

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ABSTRACT

The aromatic amine, 3,4-dichloroaniline (DCA) is a model environmental contaminant, precursor for synthesis and degradation product of several herbicides, which is commonly found in European estuarine ecosystems. In this work, the possibility of using biochemical and histological markers to assess sub-lethal effects of DCA in natural populations of *Pomatoschistus microps* juveniles was investigated.

Alterations on the activities of the enzymes acetylcholinesterase (AChE), lactate dehydrogenase (LDH), and glutathione S-transferase (GST) and histological alterations on spleen were investigated after 96 hours of exposure to sublethal concentrations of DCA (0, 0.50, 0.60, 0.72, 0.86, 1.04, 1.24, 1.49 mg/l). At the concentrations tested DCA had no effect on AChE activity, but significant alterations were found in both LDH and GST activities. As already described for mammals, in fish DCA induced important splenic histological alterations, including expansion of red pulp and deposition of hemosiderin granules.

Keywords: 3,4-dichloroaniline; biomarkers; acute toxicity; *Pomatoschistus microps*

INTRODUCTION

The protection of estuarine ecosystems against the adverse effect of contaminants, which reach these areas as a consequence of anthropogenic activities, is of great concern since they are highly valuable areas in terms of economical, genetic and recreational resources (Kennish, 2000).

The aromatic amine 3,4-dichloroaniline (DCA) is an environmental contaminant, precursor for synthesis and a degradation product of several herbicides (*e.g.* diuron, linuron and propanil) (Crossland, 1990). DCA is known to be toxic to mammals (Khan et al., 1997; Guilhermino et al., 1998) and fish (Adema & Vink, 1981; Crossland, 1990; Schäfers et al., 1991; Valentovic et al., 1997).

The hematopoetic system is the primary target of aniline insult in mammals. Methemoglobinemia, haemolytic anemia and the development of splenic toxicity have been found after exposure to this group of compounds. Many of the characteristics of splenotoxicity in mammals, such as hyperplasia, hyperpigmentation are not restricted to aniline exposure, but also occur when animals are exposed to substituted anilines such as chloroanilines (Guilhermino et al., 1998). Despite the well known toxicity of DCA to fish, causing erythrocyte damage and methaemoglobinaemia (Crossland, 1990), little attention has been paid to DCA toxicity effects on fish spleen.

At sub-individual levels, biochemical markers have been suggested as indicators of chemical exposure and/or sublethal effects (Peakall, 1992). Among the biomarkers potencially available for use, inhibition of acetylcholinesterase (AChE) alterations of lactate dehydrogenase (LDH) and induction of glutathione S-transferase (GST) have been widely used as biomarkers due to their important role in neurotransmission, obtention of energy and biotransformation.

The activity of AChE, a neurotransmission enzyme, has been used as an indicator of exposure to anticholinesterase agents, such as organophosphates and carbamates, in a variety of species, including marine organisms (Peakall, 1992). More recently, several studies indicate that this biomarker is also sensitive to other environmental contaminants including some metals and complex mixtures of combustion hydrocarbons (Gill et al., 1990; Payne et al., 1996). DCA is not known to exert a specific effect in AChE. LDH is the terminal enzyme of anaerobic glycolysis, therefore, being of crucial importance to the muscular physiology, particularly in conditions of chemical stress when high levels of energy may be required in a short period of time (De Coen et al., 2001). Alterations of the normal LDH activity pattern were found after exposure to a variety of different types of compounds (Ribeiro et al., 1999; Gagnon & Holdway, 1999), including DCA (Guilhermino et al., 1994). GST are a family of enzymes important in the biotransformation of xenobiotics, being of crucial importance in the detoxification of a large number of substances, such as PAHs, PCBs, polychlorinated dibenzodioxins (PCDDs) and phenobarbital (George, 1994).

The test organism chosen was *Pomatoschistus microps* (Krøyer, 1838). This epibenthic and euryhaline fish is a relevant test organism due to its important role in the food-web of estuarine ecosystems and its wide geographical distribution. In addition, *P. microps* inhabits estuaries and shallow near-shore waters, which are potentially, polluted environments (Miller, 1986; Arruda et al.,1993).

The central objective of this study was to investigate the acute affects of DCA on AChE, LDH, GST and spleen histology of *P. microps*.

MATERIAL AND METHODS

1. Chemicals

Techical grade 3,4-DCA (98% purity) was purchased from Sigma-Aldrich (Germany). DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), AcSCh (acetylthiocholine iodide), piruvate, NADH (β-nicotinamide adenine dinucleotide reduced form), CDNB (1-chloro-2,4-dinitrobenzene), GSH (glutathione), were obtained from Sigma (The Netherlands). All the other chemicals were from Merck (Germany) except Bradford reagent, which was purchased from Bio-Rad (Germany).

2. Test conditions

Juvenile fish of *P. microps* (length ranging from 22 to 30mm) were collected in the estuary of Minho river, which may be considered as a reference estuary, using a landing net during low water tide. Fish were acclimatized to laboratory conditions for two weeks before the start of the toxicity test, in 60-l glass tanks in well-aerated saltwater (salinity 6‰). During acclimatization fish were fed every 24 h with Tetramin® and the test chamber was kept at 20ºC with a controled photoperiod (14 h light:10 h dark).

3. Acute toxicity test

The toxicity tests were conducted for 96 hours after the acclimatisation period. DCA stock solution was prepared by dissolving 3,4-DCA in ethanol. Test solutions were prepared by dilution of stock solutions in saltwater (salinity 6‰). Nine treatments were used: control, ethanol control and 3,4-DCA at nominal concentrations of 0.5, 0.6, 0.72, 0.86, 1.04, 1.24, or 1.49 mg/l) Groups of nine *P. microps* were exposed per treatment in individual 1-L glass recipients. Saltwater was used in the

control recipients, and the ethanol control was prepared with saltwater and ethanol at the highest concentration used in the 3,4-DCA treatments. Fish were not fed during the test period and the medium was changed every second day to maintain water quality and DCA concentration. The water temperature, pH, oxygen concentration and conductivity were measured daily and dead fish were recorded each 12 h.

At the end of the test, the number of dead fish was recorded and the live fish were sacrificed by decapitation. Head, muscle and gills were isolated and used for AChE, LDH, and GST determinations, respectively. Spleen was isolated for histological analysis.

4. Biomarker analysis

Head was homogenated in 1 mL of cold potassium phosphate buffer (0,1M, pH 7.2) on ice. Samples were stored at –20ºC until enzymatic analysis. The supernatant obtained after centrifugation of the homogenate (4ºC, 6000 rpm, 3 min) was removed and used as enzyme extract for ChE activity determination. The determination of ChE activity was carried out according to the Ellman method (Ellman, et al., 1961) adapted to microplate (Guilhermino et al., 1996). The enzymatic activity was expressed in Units (U) per mg of protein (1U is a nmol of substrate hydrolysed per minute).

For LDH determinations, the dorsal muscle was homogenised in 1 mL of cold Tris-NaCl buffer (0,1M, pH 7.2) on ice. Samples were stored at –20ºC for enzymatic analysis. Samples were centrifuged (4ºC, 6000 rpm, 3 min) and the supernatant was removed and used to determine LDH activity. LDH activity was measured following the methodology described by in Vassault (1983) with the modifications introduced by Diamantino et al. (2001). Enzymatic activity was expressed in Units (U) per mg of protein (1U is a µmol of substrate hydrolysed per minute).

For GST determinations, a pair of gills was homogenised in 0,5 mL of cold potassium phosphate buffer (0.1M, pH 6.5) and stored at –80ºC.

Samples were centrifuged (4ºC, 9000 g, 30 min) and the supernatant was used to determine GST activity. GST activity was measured following the general methodology described by Habig & Jacoby (1981) adapted to microplate reader (Frasco & Guilhermino, accepted). Enzymatic activity was expressed in Units (U) per mg of protein (1U is a nmol of substrate hydrolysed per minute).

5. Protein assays

Protein content of the samples was determined, in triplicate, by the Bradford method (Bradford, 1976) adapted to microplate, using γ-bovine globulin's as standard and a wavelength of 595 nm. A Labsystem Multiskan EX microplate reader was used.

6. Spleen histological analysis

The entire spleen was fixed by immersion in Bouin's fluid just after being collected from fish. Then they were dehydrated and embedding in paraffin, sectioned in 5 µm sections, stained with haematoxylin-eosin, mounted in Canada balsam and observed by light microscopy (Kiernan, 1990).

7. Statistical analyses

For each biomarker, data from different treatments were compared by one way Analysis of Variance (ANOVA) and statistical different treatments were identified by Dunnett's test (Zar, 1996). All statistical analysis were performed using Sigma Stat for Windows, version 2.03.

RESULTS

Water dissolved oxygen was always above 80% during the test and the variation of pH was less than 1 pH Unit.

The survival of the animals at the end of the test was 100% for all the treatments until 1.03 mg/l of DCA, whereas in the group treated with 1.49 mg/l of DCA the survival was about 78% (mortality 2/9).

The *in vivo* effects of DCA on head ACHE, muscle LDH and gill GST are presented in figure 1 (A, B, C). In all the experiments no statistical differences were found between the control and the ethanol control.

At the concentrations tested, DCA did not induced significant effects on AChE (one-way ANOVA: $F_{8,70}$= 1.041; $p>0.05$) (Fig. 1A).

Significant differences in LDH activity were found at 1.2 mg/l of 3,4-DCA (one-way ANOVA: $F_{8,70}$= 6.998; $p<0.001$), while no differences were found for the other concentrations tested (Fig. 1B).

GST activity was significantly altered by exposure to low concentrations of DCA (0.50, 0.60 and 0.72 mg/l) when compared to the controls (one-way ANOVA: $F_{8,70}$= 12.809; $p<0.001$), while no significant differences relatively to control were found in the highest concentrations tested (Fig. 1C).

Histological analysis indicated important alterations in the spleen. As evident from figure 2 (A, B, C, D and E), dose-related expansion of splenic red pulp was characterised by prominent vascular congestion and increased red pulp cellularity at 0.5 and 1.0 mg/l of DCA when compared to the control (figure 2). These changes were closely associated with the deposition of hemosiderin granules (arrowheads in figure 2) in the red pulp of spleen in a dose-dependent manner, dramatically increasing at concentrations equal or higher than 0.5 mg/l of DCA.

DISCUSSION

The main objective of this study was to investigate the acute effects of 96 h exposition to DCA on the biomarkers AChE, LDH and GST and on the spleen histology of the fish *P. microps.* No significant effects of DCA on AChE were found at the concentrations tested, in agreement with the results obtained to other species by Guilhermino et al. (1996) and Sturm et al. (1999).

The biomarker AChE did not registered inhibition of enzymatic activity after exposition to the different DCA treatments. This result is in agreement with other reported studies, in which DCA as no effect on AChE activity (Guilhermino et al. 1996; Sturm et al., 1999) and underlies the specificity of AChE response in *P. microps*, since it was not affected by exposure to this type of pesticides metabolites.

Increased LDH activity levels have been registered in conditions of chemical stress when high levels of energy may be required in a short period of time (De Coen et al., 2001). Increased LDH activity levels were found in *Daphnia magna* Straus exposed during 21 days to DCA (Guilhermino et al., 1994). In the present study, LDH was significantly induced at 1.2 mg/l of DCA. However at the highest concentration tested, 1.49 mg/l of DCA, no induction was found, possibly due to the high debility of the organisms and their consequent inability to respond at this high DCA exposition, since the mortality registered was 22%. Nevertheless, high concentrations of DCA (1.2 mg/l of DCA) seem to inhibit LDH *in vivo*. Since these concentrations of DCA are unlikely to be found in the environment, this result should be taken in account as it may indicate a low response of LDH towards DCA exposure.

Liver GST are involved in the biotransformation of several pollutants, therefore an induction of GST activity has been widely used as environmental biomarker. However this enzyme may also be inhibited by

exposure to environmental contaminants such as metals, PAHs and organochlorine and organophosphate pesticides (Tuvikene et al., 1999; Lemaire et al. 1996, Pedrajas et al., 1995). In this study, inhibition of gill GST at the three lowest concentrations tested was found, while no significantly alterations were registered at the highest concentrations of DCA. This is a result of diffcult interpretation, so more studies are required to clarify this mechanism that may be responsible for this pattern of inhibition.

Aniline and several substituted anilines are known to cause splenic toxicity in mammals (Bus & Popp, 1987; Khan et al. 1997). An association between erythrocyte damage and the severity of the spleen toxicity is well established. Since one of the major functions of the spleen is to remove damaged erythrocytes, the deposition and subsequent breakdown of damaged-aniline erythrocytes in this organ will result in deposition of iron-containing proteins, such as hemosiderin and ferritin in the spleen. In addition, an accumulation of the parent compound and/or toxic metabolites in this organ may also occur, inducing further splenic damage (Bus & Popp, 1987; Khan et al. 1995). However, splenic damage due to aniline exposition is not so well established in aquatic animals, including fish, as in mammals. The present study indicates important histological alterations in the spleen, namely expansion of red pulp with vascular congestion and a notable deposition of hemosiderin granules in a dose-dependent manner. These results seem to be similar to the effects that have been described for mammals (Khan et al. 1995; Guilhermino et al., 1998). The deposition of hemosiderin granules observed may result from the scavenged of DCA-damaged erythrocytes in the spleen as commonly established for mammals. These histological alterations could be potential useful in biomonitoring programs of aquatic systems being a complement to biomarkers approach in the assessment of exposition and/or effects on *P. microps* of DCA, product of degradation of several pesticides.

Acknowledgements

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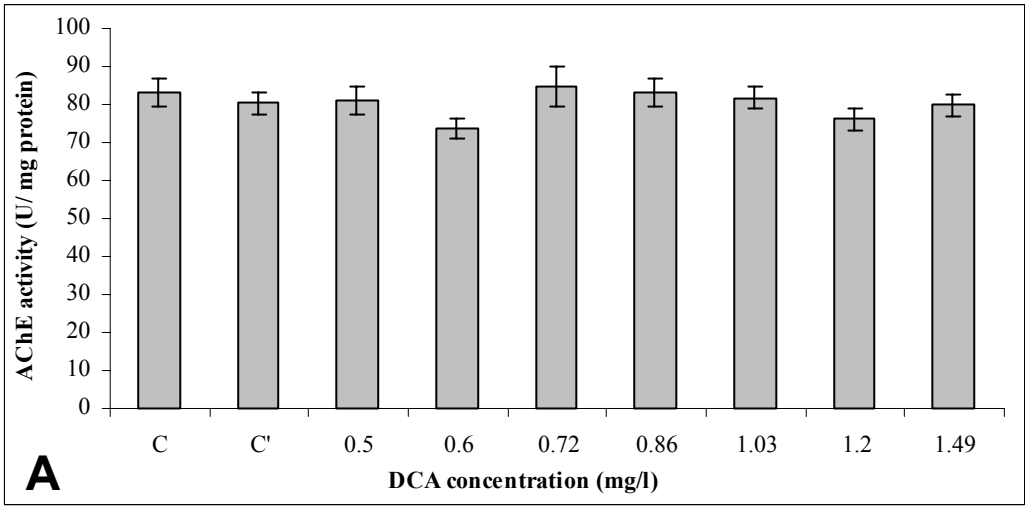
LIST OF FIGURES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90
80
70
60
50
40
30
20
10
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C
C'
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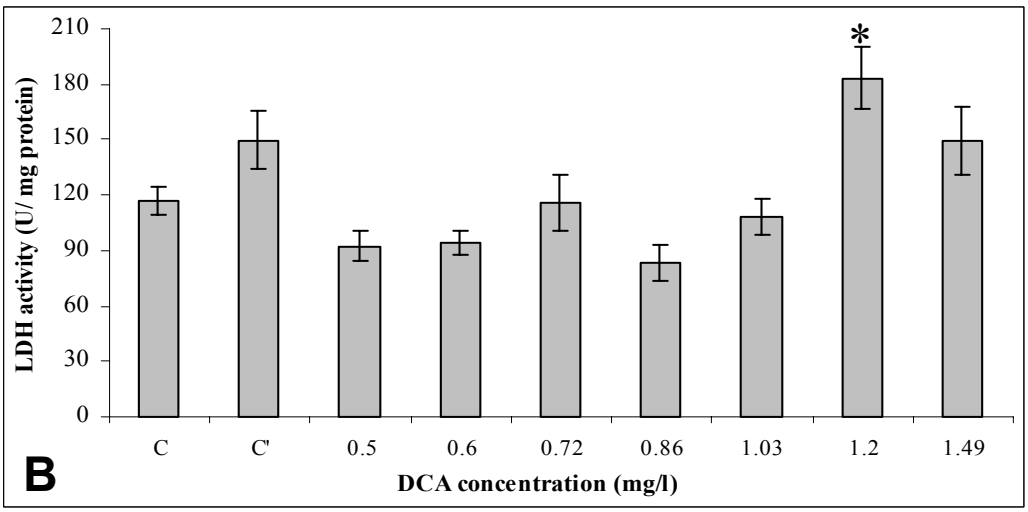
0.6
0.72
0.86
1.03
1.2
1.49
A
DCA concentration (mg/l)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150
120
90
60
30
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*
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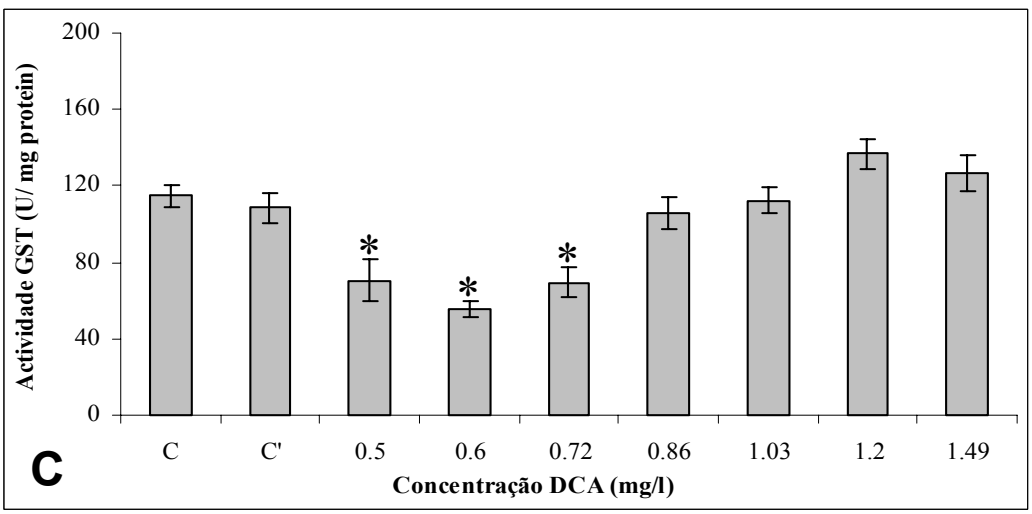
C'
0.5
0.6
0.72
0.86
1.03
1.2
1.49
B
DCA concentration (mg/l)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200
160
120
80
40
0
*
*
*
C
C'
0.5
0.6
0.72
0.86
1.03
1.2
1.49
C
Concentração DCA (mg/l)

Figure 1

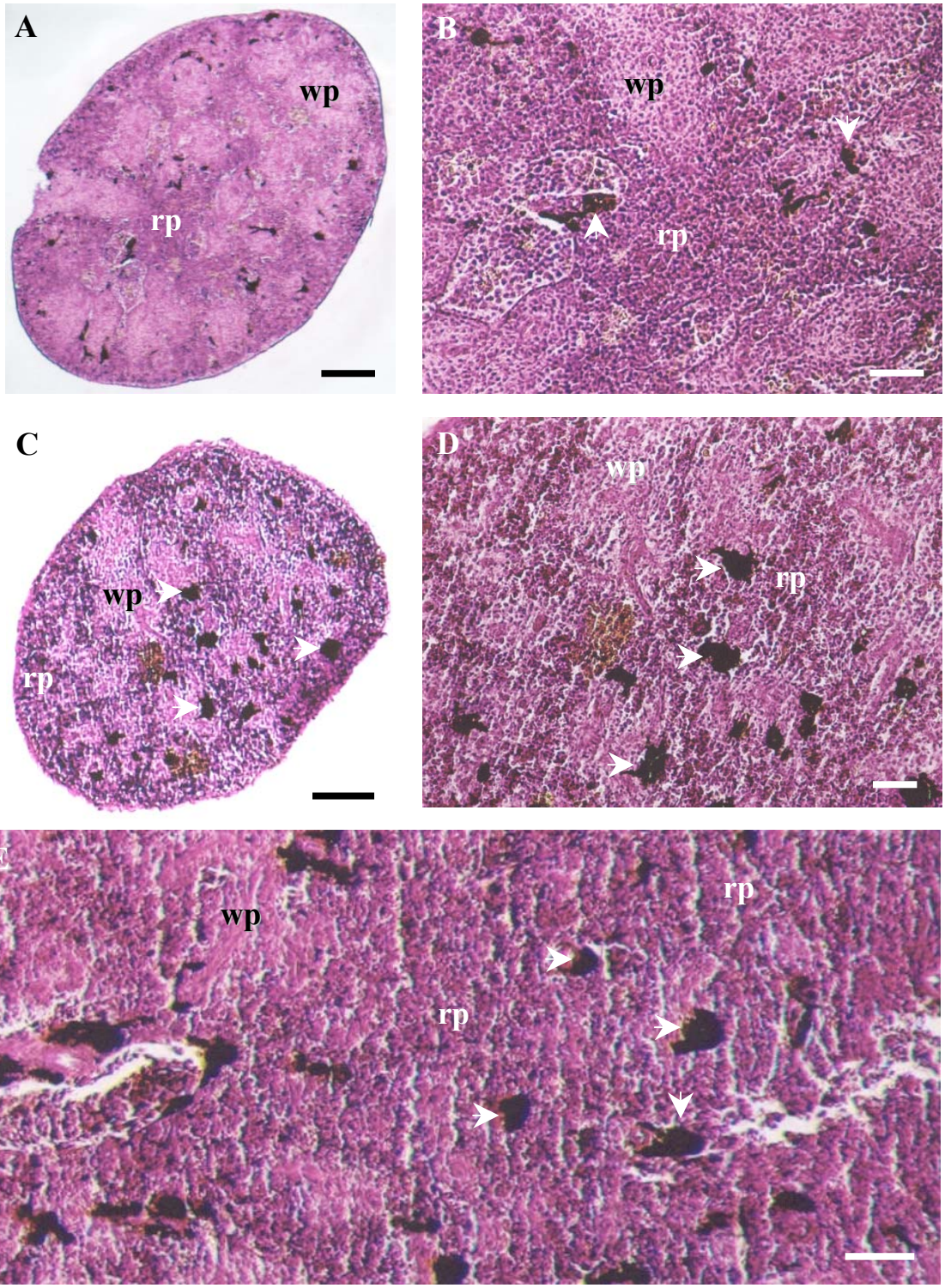

Figure 2

ANEXO III

Impact of chemical exposure on natural populations of *Pomatochistus microps* Krøyer (1838) in estuaries of the Portuguese Northwest coast

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Abstract

Juveniles of the estuarine fish *Pomatoschistus microps* were collected from autumn 2001 to summer 2002 in five stations along the Portuguese North western coast, in reference sites and in areas receiving urban, industrial and agricultural effluents. The activity of the enzymes acetylcholinesterase (AChE), lactate dehydrogenase (LDH), 7-ethoxyresorufin *O*-deethylase (EROD) and glutathione S-transferases (GST) were used as environmental biomarkers. On the basis of statistical analysis, fish collected in different stations were shown to have distinct patterns of biomarker responses. *P. microps* from the Minho Estuary showed significantly lower levels of both AChE and LDH activity. Fish from Douro Estuary, under influence of urban and industrial effluents, presented the highest levels of EROD activity. GST activity registered a significantly higher level in the station

from the Aveiro lagoon under influence of industrial effluents and described as heavily polluted, and a lower level in the other station from Aveiro lagoon which is closer to agricultural areas. Furthermore, the response of all biomarkers presented a seasonal variation along the sampling period. The results indicated that the battery of biomarkers used in this study seems to be a useful tool to distinguish different types of environmental contamination in estuarine systems, and *P. microps* a suitable species to be used as bioindicator.

Keywords: Estuaries; *Pomatoschistus microps*; Seasonal variation; AChE; LDH; EROD; GST; Different sources of environmental contamination

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1. Introduction

In recent years, the levels of contaminants in estuaries and coastal zones increased as a consequence of anthropogenic activities (Kennish, 1992). Estuaries are productive natural habitats, where large phytoplankton populations support a variety of other organisms, including many commercially important marine fish and crustacean species that use them as nursery grounds (Cattrijsse *et al.* 1994). For this reason, it is a priority to develop methods to evaluate the degree of exposure and contamination of natural populations in order to minimise the impact of human activities.

Biomarkers have been widely used in Ecotoxicology as early warning signals of chemicals effects, offering the possibility of anticipating severe alterations potentially induced at a population level (Peakall *et al.*, 1992). They are particularly important in the case of contamination by complex mixtures of chemicals where determinations of the presence of individual agents by chemical analysis provide limited information regarding the effects induced on organisms. Among the biomarkers potentially available

for use, inhibition of acetylcholinesterase (AChE), alterations of lactate dehydrogenase activity (LDH), as well as the induction of glutathione S-transferases (GST) and monooxygenase enzymes of the P450 system (P450) have been shown to be appropriate for use in a large variety of species and real scenarios (Galgani *et al.*, 1992; Wu & Lam, 1997; Sanchez-Hernandez *et al.*, 1998; Doyotte *et al.*, 2001; Niyogi *et al.*, 2001; Porte *et al.*, 2001).

Inhibition of AChE activity has been widely used to diagnose the exposure to anticholinesterase compounds, such as organophosphorus (OP) and carbamates (CB) pesticides (Fulton & Key, 2001). More recently, several studies indicate that this biomarker is also sensitive to other compounds, including some metals and surfactants (Gill *et al.*, 1990; Labrot *et al.*, 1996; Guilhermino *et al.*, 1998). Furthermore, field studies have been showing the suitability of this biomarker for use in freshwater (Payne *et al.* 1996) and marine environments apparently not contaminated by pesticides (Galgani *et al.*, 1992; Payne *et al.* 1996).

Lactate dehydrogenase (LDH) is the terminal enzyme of anaerobic glycolysis, therefore, being of crucial importance to the muscular physiology, particularly in conditions of chemical stress when high levels of energy may be required in a short period of time (De Coen *et al.*, 2001). Alterations of normal LDH activity pattern were found after exposure to sodium bromide and 3,4-dichloroaniline (Guilhermino *et al.*, 1994), zinc chloride (Diamantino *et al.*, 2001), parathion-ethyl and endosulfan (Ribeiro *et al.*, 1999), crude oil (Gagnon & Holdway, 1999) or under hypoxia conditions (Wu & Lam, 1997; Cooper *et al.*, 2002).

EROD (7-ethoxyresorufin *O*-deethylase) activity may be used as indicative of the cytochrome P4501A1 enzyme system function, which is responsible for the Phase I biotransformation of ubiquitous environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) and several congeners of polychlorobyphenyls (PCBs) (Stegeman & Hahn, 1994). EROD activity in fish liver has been recognized as an useful

biomarker in various species of fish exposed to these compounds (Goksøyr & Förlin, 1992; Bucheli & Fent, 1995; Sanchez-Hernandez *et al.*, 1998).

Glutathione S-transferases (GST) are a family of enzymes that catalyse the conjugation of reduced glutathione (GSH) with a variety of both endogenic and xenobiotic compounds, being of crucial importance in the detoxification of a large number of substances (George, 1994). It has been demonstrated that this enzyme is induced by exposure to PAHs, PCBs, OP and CB pesticides, polychlorinated dibenzodioxins (PCDDs) and by phenobarbital compounds (George, 1994; Niyogi *et al.*2001; Stephensen *et al.*, 2002).

The main objective of this study was to investigate the annual variation of activity of AChE, LDH, GST and P450 in wild *Pomatoschistus microps* collected in five sites of three estuaries in the norwest atlantic coast of europe. The first sampling site was located in Minho river estuary, and has been used as a reference site due to its relatively low contamination. The second site was in the Douro river estuary under influence of both industrial and urban effluents. The third site was in the mouth of the Aveiro lagoon and due to its low contamination was used as reference 2. Fourth and fifth sites were inside the Aveiro lagoon in areas receiving considerable agricultural runoff and industrial effluents, respectively.

Pomatoschistus microps (Krøyer, 1838), an epibenthic and euryhaline fish, was selected as bioindicator organism in this study due to its position as an intermediate predator in estuarine food-web, wide geographical distribution, and abundance in estuaries and near-shore waters, which are potentially contaminated environments (Miller, 1986; Arruda *et al.*, 1993).

2. Material and Methods

2.1. Study area

Five sampling sites were selected for this study (Fig. 1): one site in the Minho river estuary (41°52' N latitude and 8°50' E longitude) chosen as *Reference 1* (R1) due to its low contamination; one site in the Douro river estuary, near Porto city (41°11' N latitude and 8°36' E longitude) and under the influence of urban and industrial effluents (*Urban and Industrial effluents*, UI) three sites in the Aveiro lagoon (40°39' N latitude and 8°44' E longitude) with different contamination types, namely a non-impacted site near the artificial connection of the lagoon to the sea (*Reference 2*, R2), a site under intensive agriculture run-off in the Mira channel (*Agricultural area*, AA) and an industrial impacted site in the Laranjo bay (*Industrial effluents*, IE). The reference sites are located in different environments: the reference 1 is located in a river estuary, while the second reference site is located in a lagoon and close to the sea.

2.2. Biological material

Twenty juvenile fish (length ranging from 22 to 30mm) were collected in each sampling site, using a landing net during low water tide, four times during the year (Autumn 2001, Winter 2002, Spring 2002 and Summer 2002). Fish were transported alive to the laboratory and sacrificed by decapitation, after being measured. Head, muscle, gills, and liver were isolated and used for AChE, LDH, GST and P450 determinations, respectively.

Head was homogenated in 1 mL of cold potassium phosphate buffer (0,1M, pH 7.2) on ice and homogenates were stored at –20°C until being used for enzymatic analysis. The supernatant obtained after centrifugation of the homogenate (4°C, 6,000 rpm, 3 min) was removed and used as an enzyme extract for ChE activity determination.

LDH was extracted by homogenisation of the dorsal muscle tissue in 1 mL of cold Tris-NaCl buffer (0,1M, pH 7.2) on ice, and were stored at –20ºC until enzymatic analysis. Samples were centrifuged (4ºC, 6,000 rpm, 3 min) and the resultant supernatant was removed and used to determine LDH activity.

CYP1A1 samples were prepared by homogenisation of 9 fish livers in 0,5 mL of buffer (50 mM Tris, 1 mM, ditiotreitol, 1 mM EDTA, 20% v/v glicerol, pH 7.4). Homogenates were centrifuged (4ºC, 500 g, 15 min followed by 4ºC, 10,000 g, 45 min) and the resultant supernatant was stored at –80ºC until analysis for the enzyme.

GST were prepared by homogenization of a pair of gills in 0,5 mL of potassium phosphate buffer (0,1M, pH 6.5) and stored at –80ºC until analysis. Samples were centrifuged (4ºC, 9,000 g, 30 min) and the supernatant was used to determine GST activity.

2.3 Enzymatic determinations

The determination of AChE activity was carried out according to the Ellman method (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996). The enzymatic activity was expressed in Units (U) per mg of protein (1U is a nmol of substrate hydrolysed per minute).

LDH activity was measured according to the technique described by Vassault (1983) adapted to microplate as described in Diamantino *et al.* (2001). Enzymatic activity was expressed in Units (U) per mg of protein (1U is a µmol of substrate hydrolysed per minute).

EROD activity was determined according to the spectrophotometric method described by Hodson *et al.* (1991) with the modifications described in Frasco & Guilhermino (accepted). The enzymatic activity was expressed in Units (U) per mg of protein (1U is a pmol of substrate hydrolysed per minute).

GST activity was measured following the general methodology described by Habig & Jacoby (1981) adapted to microplate (Frasco &

Guilhermino, accepted). The enzymatic activities was expressed in Units (U) per mg of protein (1U is a nmol of substrate hydrolysed per minute).

2.4 Protein determinations

Protein content of the samples was determined, in triplicate, by the Bradford method (Bradford, 1976) adapted to microplate, using γ-bovine globulin's as standard and a wavelength of 595 nm. A Labsystem Multiskan EX microplate reader was used.

2.5 Chemicals

DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), AcSCh (acetylthiocholine iodide), CDNB (1-chloro-2,4-dinitrobenzene), GSH (glutathione), piruvate, NADH (β-nicotinamide adenine dinucleotide reduced form) 7-ER (7-ethoxyresorufin), NADPH, (β-nicotinamide adenine dinucleotide phosphate reduced form) were obtained from Sigma (The Netherlands). All the other chemicals were from Merck (Germany) except the Bradford reagent, which was purchased from Bio-Rad (Germany).

2.6 Statistical analyses

In order to minimise deviations from the normality data transformations were performed: log transformations to AChE and GST data, and square root transformations to LDH. EROD data presented a normal distribution, thus were not transformed. Data were analysed using two-way Analysis of Variance (ANOVA) without interaction between the two factors (local and season) and statistical different treatments were identified by Tukey post hoc test ($p<0.05$) (Zar, 1996). All statistical analysis, including discriminant analysis was performed using Minitab for windows, version 13.0 (Minitab, Pennsylvania, USA).

3. Results

The local and seasonal variation of AChE, LDH, EROD and GST activities in the head, muscle, liver, and gills, respectively, of *P. microps* are presented in figure 2. Attending to the local effect, AChE activity of fish from (63.79 U/mg protein) were significantly lower than the levels from all the other stations (Table I; two-way ANOVA: $F_{4,392}$=4.75; $p<0.05$). In respect to the seasonality effect on AChE activity, and without taking in account the spatial variations, all seasons presented significant differences among them (Table I; two-way ANOVA: $F_{3,392}$=76.90; $p<0.05$). AChE activity showed lower levels in winter and spring (62.62 and 57.15 U/mg protein, respectively) than in the other seasons.

The results of LDH activity in *P. microps* muscle are shown in figure 2 (C and D). Considering the several sampling stations, LDH activity levels in *Reference 1* (154.81 U/mg protein) were significantly lower when compared with the other stations (Table I; two-way ANOVA: $F_{4,392}$=33.88; $p<0.05$), which did not show significant differences among them. When comparing the different seasons, LDH activity in autumn was lower (159.96 U/mg protein) than in the other seasons (Table I; two-way ANOVA: $F_{3,392}$=51.11; $p<0.05$).

EROD activity in fish liver (Fig. 2E and 2F) from *Urban and industrial effluents* station was significantly different from all the other stations (Table I; two-way ANOVA: $F_{4,51}$=12.63; $p<0.05$). This station presented higher levels (11.99 U/mg protein) than in the other sampling stations, during all the year. EROD activity showed a similar pattern in all stations, during the sampling year (Fig. 2B). The enzyme activity is significantly lower in winter (1.96 U/mg protein), when compared to autumn and summer (7.35 and 6.95 U/mg protein, respectively) (Table I; two-way ANOVA: $F_{2,51}$=12.36; $p<0.05$), exhibiting low activity levels, even at undetectable levels.

Taking into account the different sampling stations, the *Agricultural areas* and the *Industrial effluents* presented significantly different GST levels when compared to the other stations (Fig. 2G and 2H) (Table I; two-way ANOVA: $F_{4,392}$=9.24; p<0.05). GST activity in *Industrial effluents* exhibited the highest levels (141.99 U/mg protein) and in the other hand *Agricultural areas* registered the lowest levels (120.25 U/mg protein). Attending to the seasonality effect, GST activity levels, in autumn and winter, are significantly different from the other stations (Table I; two-way ANOVA: $F_{3,392}$=23.84; p<0.05). In winter, the lowest GST activity levels (110.70 U/mg protein) were found, and in autumn the highest level (144.99 U/mg protein) were recorded.

The results of discriminant analysis for the response of the biomarkers battery to local and seasonal effect are presented in table II. It was registered an higher discriminant power for seasonal effect (72.0%) than for local effects (50.0%) with AChE, LDH, EROD and GST biomarkers. Attending to the local effects, *Reference 1* had the higher discriminant power (85.0%) followed by both *Urban and Industrial effluents* and *Agricultral area* stations (56.7%), which included an elevated number of observations in the own station group (51 and 34, respectively) (Table II). The others stations, *Reference 2*, and *Industrial effluents*, presented lower discriminant power (8.3% and 43.3%, respectively) due to the higher distribution of observations through the different stations groups (Table II).

4. Discussion

The results presented in this study indicate significant differences in AChE, LDH, EROD and GST activities among fish from the five stations studied. Furthermore, significant differences in enzymatic activities among seasons were found.

River Minho estuary, where *Reference 1* is located, is characterised by the absence of any major industrial, agricultural or metropolitan areas. However, during spring and mostly in summer it becomes a point of tourism for fishing and aquatic sports which may result in an increase of contaminants in the estuary. This may contribute to the relatively low levels of AChE activity in this sampling site. Payne *et al.* (1996) provided evidence that complexe mixtures of combustion hydrocarbons could be responsible for depressed AChE levels registered in field studies. Kang and Fang (1997) have also registered inhibition of AChE, purified from the electric eel, in the presence of PAHs.

The *Industrial effluents* station is located in the Laranjo bay, a region in the Aveiro lagoon that has been described as highly contaminated by mercury (Ramalhosa *et al.*, 2000) and other pollutants from the adjacent industrial areas. Therefore, an inhibition of AChE was expected since several authors registered an inhibition of AChE by heavy metals (Gill *et al.*, 1990; Labrot *et al.*, 1996). However in the *Industrial effluents* station such inhibition was not observed. Payne *et al.* (1996) support the idea that heavy metal background levels in field have little potential for AChE inhibition, despite the fact that *in vivo* and *in vitro* experiments, using higher concentrations of heavy metals, have demonstrated potential for AChE inhibition.

LDH activity levels in *Reference 1* were significantly lower when compared with the other stations Suppression of LDH activity has been previously reported following exposure to hydrocarbons. Gagnon & Holdway (1999) have registered a significant LDH activity inhibition in the gills of the Atlantic salmon (*Salmo salar*) during exposure to low concentrations of crude oil. Attending to the results obtained by the supra cited authors, the inhibitions registered in *Reference 1* of both AChE and LDH may support the idea of the presence of this type of contaminants in this station.

Fish from *Urban and Industrial effluents* station had higher EROD activity than the other stations suggesting the presence of P4501A1 inducers, such as PAHs and PCBs, as registered by other authors in other environments (Stegeman & Hahn, 1994). Fish from reference stations, *Agricultural areas* and *Industrial effluents* stations had lower EROD levels, particularly in winter. Low EROD activity, even at undetectable levels, is normally found in individuals from uncontaminated areas (Goksøyr, & Förlin, 1992). However, even with low EROD activity, an elevated content of P4501A1 protein may be present. Therefore the measure of EROD catalytic activity could lead to underestimation of the inducing contaminants in the environment. To overcome this inconvenient, determination of P4501A1 protein content or hybridization studies with CYP1A1 mRNA may detect eventual inductive responses not seen with EROD activity (Bucheli & Fent, 1995).

Fish from *Industrial effluents* station showed significantly higher GST activity than the fish from all the other stations indicating the presence of inducers of this enzyme. The mercury present in the environment (Ramalhosa *et al.*, 2000) was probably inducing GST. Other authors have identified a remarkable induction of GST by heavy metal pollution (Lenartova *et al.*, 1997; Chen *et al.*, 1998; Lopes *et al.*, 2001). However other authors have registered inhibition of GST with this type of compounds (Tuvikene *et al.*, 1999; Bagnasco *et al.*, 1991)

Fish from *Agricultural effluents* station presented lower GST activity levels than the fish from all other stations. Induction of GST activity in fish has been observed following exposure to organochlorine and organophosphate pesticides (Pedrajas *et al.*, 1995). However, Lindström-Seppä *et al.* (1996) reported an inhibition of GST activity in fish exposed to the organochlorine hexachlorobenzene.

Independence from seasonal variation is a desirable feature for biomarkers of xenobiotic pollution (Peakall, 1992). However such variation is commonly found in a variety of biomarkers in different bioindicator species (Bucheli & Fent, 1995; Escartín & Porte, 1997; Ronisz *et al.*, 1999; Nyogi *et al.*, 2001). Seasonal variations in pollutant input and in biochemical processes involving specific biomarkers are important factors that need to be taken in account in biomonitoring studies (Sheehan & Power, 1999).

AChE has been widely used in aquatic organisms as a biomarker of exposure to organophosphate and carbamate pesticides (Galgani *et al.*, 1992), which drain from the agricultural areas. These types of compounds are commonly used in several crops in Portugal. In this study, statistically significant inhibitions of AChE activity were observed in fish collected in winter and spring relatively to fish collected in summer and autumn. These results are suggesting that this type of compounds may be present in aquatic systems during winter and spring due to the agricultural run-off. Escartín and Porte (1997) observed important seasonal variations in AChE activity of mussels collected from the Ebro Delta (Spain) in relation to pesticide concentration, however, they have also noted that an increase in AChE activity could be anticipated in a non-contaminated environment with temperature increase. This type of seasonal pattern with AChE was also registered in the station *Agricultural areas* (data not shown).

In the present study, seasonal alterations of LDH activity were observed. Further studies are necessary to confirm the registered decrease of LDH activity in autumn as a seasonal trend for this enzyme and its origin.

In this study, significantly lower EROD activities were detected in winter. This type of result has been related to environmental factors (*e.g.*, temperature changes and photoperiod) or biotic factors (*e.g.*, sex, reproduction and developmental status) (Stegeman & Hahn, 1994). In this regard, in the present study only juvenile fishes were collected, allowing one to eliminate variations due to sex and reproductive cycle when

measuring enzymatic activities. The lower temperature registered in temperate waters in winter, which can cause attenuation of the P4501A1 induction response (Stegeman & Hahn, 1994) and the higher river run-off during this season may be the principal factors to underlie such diminution in EROD activity in winter.

Comparing the periods of sampling without taking into account the spatial variations, GST activity was lower in winter. Seasonal variations in GST activity appeared to be similar to EROD activity. Nyogi *et al.* (2001), in barnacle, *B. balanoides*, and Ronisz *et al.* (1999) in eelpout, *Z. viviparous*, have also reported lower GST activities during winter conditions.

In this study, an important seasonal effect was found on the studied biomarkers, which may be due to variations in climatic conditions and/or to the enzyme activities own seasonal fluctuation (Peakall *et al.*, 1992). Nevertheless important inductions and/or inhibitions were detected by the battery of biomarkers for the different study sites in relation to the contamination present. Attending to the discriminant analysis of local *versus* the biomarkers AChE, LDH, EROD and GST, the higher discriminated locals were *Reference 1*, *Urban and Industrial effluents* and *Agricultural area*, which presented significant biomarkers alterations that suggested the presence of contamination.

In summary, these findings indicate significant responses to the different contamination locals: the *Urban and Industrial effluents* station registered an important EROD induction; the *Agricultural areas* station presented GST inhibition and in the *Industrial effluents* station GST was inducted. Furthermore, factors other than pollutants, such as seasonal variations, might have an influence on biomarkers variation. This suggests that the use of the biomarker approach in the common goby makes this widespread organism a suitable object for monitoring biological effects in estuarine systems. Nevertheless, field validation studies should be carried,

correlating the measured biomarkers with the xenobiotics that may be inducing these biomarkers, to confirm the validity of this procedure.

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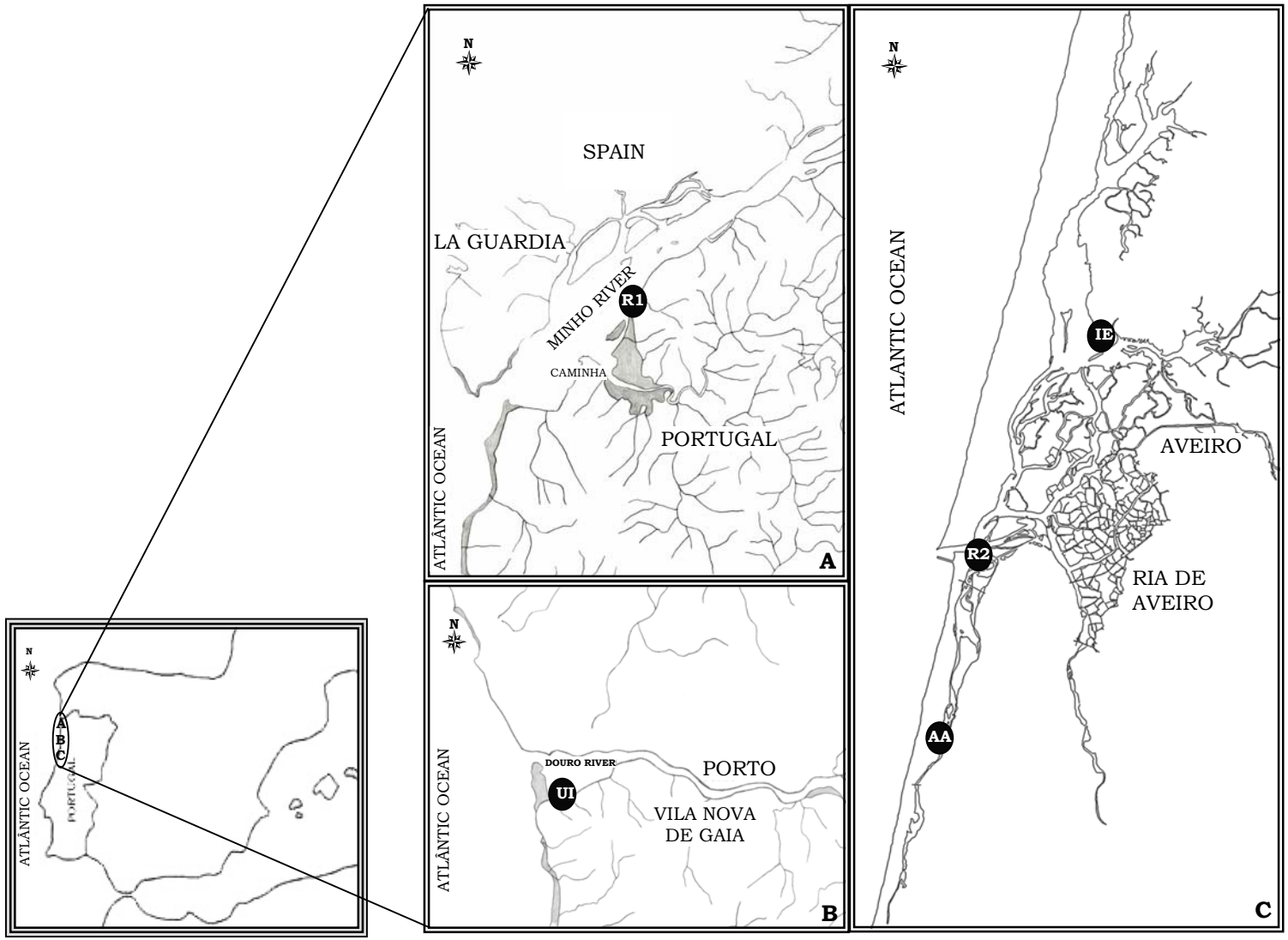
N
ATLÂNTIC OCEAN
A
B
C
SPAIN
LA GUARDIA
MINHO RIVER
R1
CAMINHA
PORTUGAL
DOURO RIVER
PORTO
UI
VILA NOVA
DE GAIA
ATLANTIC OCEAN
IE
AVEIRO
R2
RIA DE
AVEIRO
AA

Figure 1

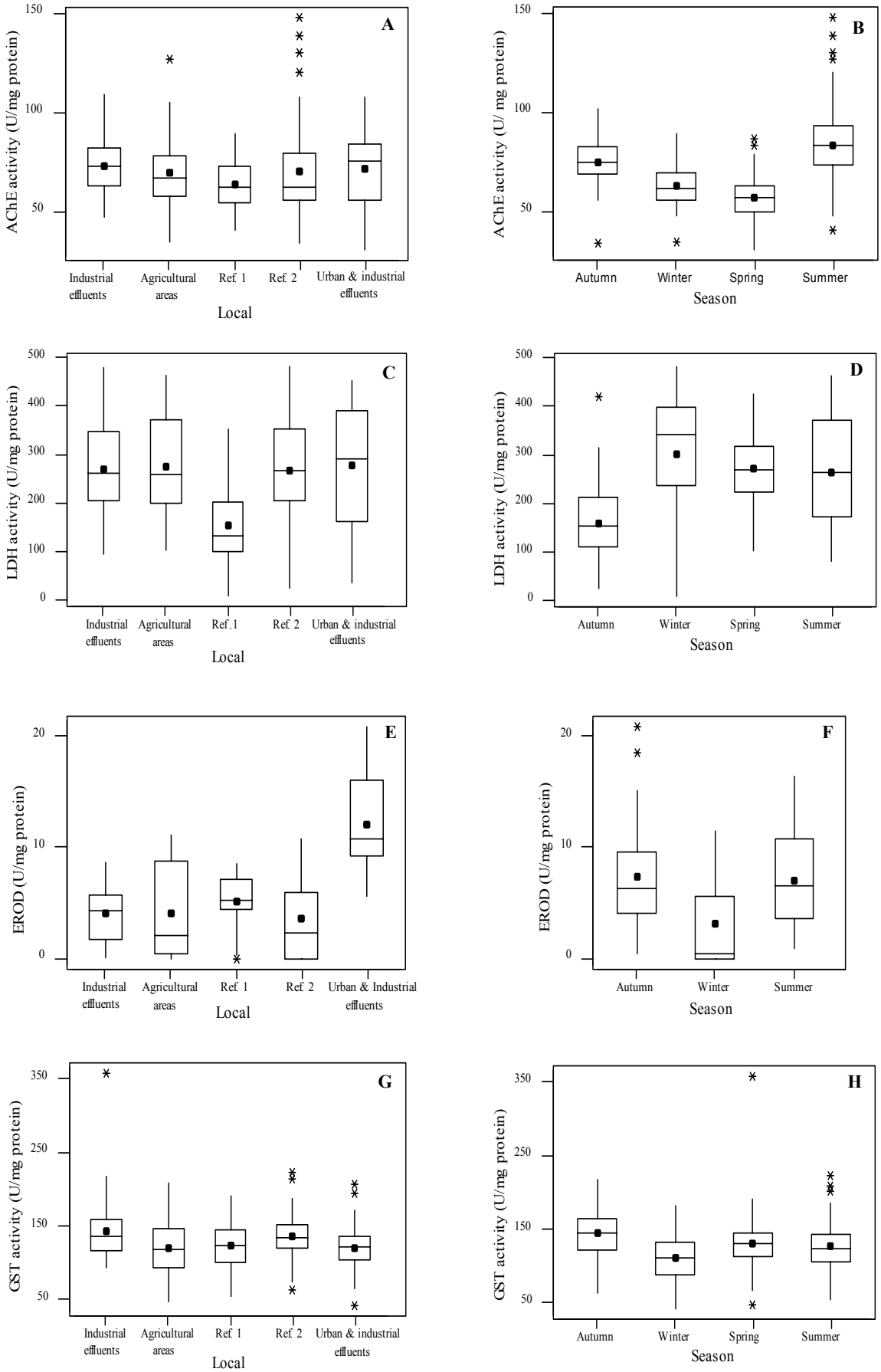
A
AChE activity (U/mg protein)
150
100
50
Industrial effluents
Agricultural areas
Ref. 1
Ref. 2
Urban & industrial effluents
Local
B
AChE activity (U/ mg protein)
Autumn
Winter
Spring
Summer
Season
C
LDH activity (U/mg protein)
500
400
300
200
100
0
Ref.1
Local
D
LDH activity (U/mg protein)
Season
E
EROD (U/mg protein)
20
10
0
Urban & Industrial effluents
F
G
GST activity (U/mg protein)
350
250
150
50
H

Figure 2

Table I:

	LOCAL					SEASON			
	Agricultural area	***Industrial effluents***	***Reference 1***	***Reference 2***	***Urban and industrial effluents***	***Autumn***	***Winter***	***Spring***	***Summer***
AChE	---	*a*	*b*	---	*a*	*a*	*b*	*c*	*d*
LDH	*b*	*b*	*a*	*b*	*b*	*a*	*b*	*b*	*b*
EROD	*a*	*a*	*a*	*a*	*b*	*a*	*b*		*a*
GST	*a*	*b*	---	---	---	*a*	*b*	*c*	*c*

Table II:

	Industrial effluents	*Agricultural area*	*Reference 1*	*Reference 2*	*Urban and industrial effluents*
Industrial effluents	**26**	13	4	12	7
Agricultural area	7	**34**	2	12	3
Reference 1	6	4	**51**	15	12
Reference 2	6	5	2	**5**	4
Urban and industrial effluents	15	4	1	16	**34**
Total N	60	60	60	60	60
Discriminant power (%)	433	0.567	0.850	0.083	0.567
Mean discriminant power			**0.500**		