UNIVERSIDADE FEDERAL DE PERNAMBUCO
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DOUTORADO EM BIOQUÍMICA E FISIOLOGIA

Acetilcolinesterase cerebral e eritrocitária como biomarcadores *in vitro* da exposição a pesticidas organofosforados e carbamatos.

CAIO RODRIGO DIAS DE ASSIS

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LISTA DE ABREVIATURAS

2-PAM – 2-pralidoxima

62c47 – diiodeto de 1,5-bis-(4-trimetilamônio-fenil) pentan-3-ona

A – forma assimétrica das enzimas colinesterases

AAA - aril-acilamidase amino-sensível

AChE – acetilcolinesterase

ADI – *Acceptable Daily Intake*

AE – *Activation Energy*

ANOVA – *Analysis of Variance*

ANVISA – Agência Nacional de Vigilância Sanitária

ASCh - acetiltiocolina

ATSDR – *Agency for Toxic Substances and Drug Registry*

BChE – butirilcolinesterase

BPMC - 2-sec-butilfenil N-metilcarbamato

BSCh – butiriltiocolina

bw – *body weight*

BW284c51 – dibrometo de 1,5-bis(4-alil-dimetilamônio-fenil) pentan-3-ona

CB – carbamato

CE - carboxilesterase

ChE – cholinesterase

ColQ – colágeno Q

CONAMA – Conselho Nacional de Meio Ambiente

DDM – cloridrato de ββ'-diclorodietil-N-metilamino

DEP – dimetil 2,2,2-tricloro-1-hidroxietilfosfonato

DFP – diisopropil fluorofosfato

DIMP – diisopropil metilfosfonato

DMSO – dimetil sulfóxido

DTNB – ácido 5,5'-ditiobis(2-nitrobenzóico)

EC – *Enzyme Commission*

EDTA – ácido etileno diamino tetracético

EFSA – *European Food Safety Agency*

EPN - O-etil O-4-nitrofenil fenilfosfonotioato

FAO – *Food and Agriculture Organization*

G – forma globular das enzimas colinesterases

GABA – ácido gama-aminobutírico

GTZ (atualmente GIZ) - Deutsche Gesellschaft für Internationale Zusammenarbeit

HDL – *High Density Lipoprotein*

IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

IDA – ingestão diária aceitável

IPCS – *Internation Programme on Chemical Safety*

Iso-OMPA – tetraisopropil pirofosforamida

IC_{20} - concentração que inibe a atividade enzimática em 20%

IC_{50} – *median inhibition concentration* concentração que inibe a atividade enzimática em 50%

Kcat – *turnover number*, número de renovação da enzima

Kcat/Km – Eficiência catalítica

kDa - kilodaltons

Ki – constante de inibição

Km – constante de Michaelis-Menten

Km ratio – razão Km

LC_{50} – *Median Lethal Concentration*

LMR – limite máximo de resíduo

MC7 – iodeto de 7-(dimetilcarbamiloxi)-N-metilquinolinio

MCL – *Maximum Concentration Level*

MEP – O,O-dimetil O-4-nitro-m-tolil fosforotioato (fenitrotion)

mM – milimolar

µM - micromolar

MPMC - 3,4-xilil metilcarbamato

MTMC - m-tolil metilcarbamato

NAC - 1-naftil metilcarbamato

NADH – nicotinamida adenina dinucleotídeo reduzida

NOAEL – *no-observed-adverse-effect-level*

Nu1250 – brometo de N-*p*-clorofenil-N-metilcarbamato de m-hidroxifeniltrimetilamônio

nd – not determined

NE ou ne – negligible effect

OP – organofosforado

P450 – complexo citocromo P450

PChE – propionilcolinesterase

PFAM – Protein Families database

PHC - 2-isopropoxifenil metilcarbamato

Phe - fenilalanina

PRiMA - proteína transmembranar de ancoragem rica em prolina

PSCh – propioniltiocolina

RBC – *Red Blood Cell*

REH – *Relative Efficiency of Hydrolisys*

RPR-II – ácido 2-butenóico 3-(dietoxifosfinotionil) metil éster

RPR-V - ácido 2-butenóico 3-(dietoxifosfinotionil) etil éster

SINITOX – Sistema Nacional de Informação Tóxico Farmacológica

SNA – sistema nervoso autônomo

SNC – sistema nervoso central

TBS – *Tris Buffer Saline*

TEPP – tetraetil pirofosfato

Tris – tris-hidróximetil aminometano

Trp – triptofano

U – unidade de atividade enzimática (1 µM de substrato processado por minuto)

USDA – *United States Departament of Agriculture*

USEPA - *United States Environmentl Protection Agency*

Vmax – velocidade maxima de catálise atingida por uma enzima

Vmax ratio – razão Vmax

WHO – *World Health Organization*

XMC - 3,5-xilil metilcarbamato

ÍNDICE

RESUMO

Organofosforados e carbamatos são as principais classes de inseticidas no mercado mundial. Sua rápida degradação e baixa estabilidade no meio ambiente fizeram com que substituíssem rapidamente outras classes importantes. Todavia, sua alta toxicidade em relação aos organismos não-alvo aliada às grandes quantidades utilizadas constituem uma ameaça à saúde humana e ambiental. O modo de ação de ambas as classes baseia-se na inibição de enzimas colinesterases. No presente trabalho, a acetilcolinesterase presente no cérebro de cinco espécies de peixes, *Colossoma macropomum* (tambaqui), *Arapaima gigas* (pirarucu), *Rachycentron canadum* (beijupirá), *Oreochromis niloticus* (tilápia do Nilo) e *Cichla ocellaris* (tucunaré) foi caracterizada fisico-quimica e cineticamente e foi exposta a pesticidas organofosforados, carbamatos e a íons. A acetilcolinesterase eritrocitária humana também foi exposta a esses pesticidas a fim de verificar a viabilidade de seu uso na detecção de pesticidas em agricultores residentes em localidades distantes dos laboratórios de análises. Os resultados demonstraram a possibilidade de estimação de parâmetros de eficiência catalítica (Kcat, Kcat/km, Energia de ativação e acréscimo na velocidade de reação) a partir do extrato bruto. Além disso, foi observada alta sensibilidade dos extratos enzimáticos de peixes aos pesticidas, principalmente ao diclorvós, carbofuran e tetraetil pirofosfato (TEPP). Também foi verificada a influência causada por alguns íons sobre a atividade dessas enzimas uma vez que eles são frequentemente encontrados em amostras ambientais. Nos ensaios com a enzima eritrocitária humana, constatou-se que a mesma, nas condições de ensaio propostas, foi sensível a concentrações dos pesticidas abaixo dos limites recomendados pela legislação nacional e internacional. Tais resultados contribuem para a determinação de condições ótimas experimentais e sugerem a utilização da acetilcolinesterase dessas fontes como biomarcador *in vitro* no monitoramento ambiental e da exposição ocupacional de alguns desses pesticidas.

Palavras-chaves: Organofosforados, carbamatos, biomarcador, acetilcolinesterase, eritrócito

ABSTRACT

Organophosphates and carbamates are the major classes of insecticides in the world market. Its rapid degradation and low stability in the environment caused the replacement of other important classes of pesticides by them. However, its high toxicity in relation to mammals and other non-target organisms, combined with the large quantities used constitutes a threat to human health and the environment. The mode of action of both classes is based on inhibition of cholinesterase enzymes. In this study, the acetylcholinesterase present in the brain of five species (tambaqui, arapaima, cobia, Nile tilapia and peacock bass) was physicochemical and kinetically characterized and exposed to organophosphorus and carbamate pesticides and to ions. Human erythrocyte acetylcholinesterase was also exposed to these pesticides in order to verify the feasibility of its use in the detection of pesticides in blood of farm workers living in remote locations. The results showed the possibility of estimating catalytic eficiency parameters (Kcat, Kcat/km, Activation energy and rate enhancement) using crude extracts. In addition, it was observed high sensitivity of the enzymes from fish to pesticides, mainly to dichlorvos, carbofuran, and tetraethyl pyrophosphate (TEPP). Also was observed the potential impact caused by some ions on the activity of these enzymes. Moreover, in the tests with human erythrocyte enzyme, it was found that, under the test conditions proposed, this enzyme was sensitive to concentrations of pesticides below the limits recommended by national and international regulations. Such results contribute to determine the optimal experimental conditions and suggest the use of these sources of acetylcholinesterase as *in vitro* biomarker of some of those pesticides in environmental screening and occupational health monitoring.

Key words: Organophosphorus, Carbamates, Biomarker, Acetylcholinesterase, erythrocyte.

1 – INTRODUÇÃO

Os recursos naturais têm sido tratados como fontes inesgotáveis de bens a serem aproveitados pela humanidade. Sua capacidade de suporte é sistematicamente ignorada mesmo em alguns países desenvolvidos. A escassez desses recursos e, sobretudo a contaminação ambiental proveniente de sua extração e transformação, têm sido as conseqüências diretas deste modelo tradicional de crescimento econômico que prossegue pouco alterado nos dias atuais.

A contaminação dos solos e das águas por agroquímicos e resíduos domésticos é um problema antigo de zonas rurais e urbanas e que não se restringe apenas às áreas causadoras da poluição. Desde a chamada "revolução verde", os pesticidas desempenham papel predominante no processo, sendo a causa de diversos distúrbios para a fauna e flora dos ecossistemas, bem como a causa da maioria dos casos de intoxicação humana, seja de natureza ocupacional ou não. Neste contexto, o controle e monitoramento desses compostos fazem-se necessários.

Os avanços na proteção ambiental obtidos são provenientes de políticas governamentais que atuam por meio de legislações e de incentivos às práticas ambientais e à pesquisa. Como resultado disso, o monitoramento do meio ambiente pode ser visto como uma abordagem capaz de dar cumprimento à legislação e como instrumento de controle na gestão ambiental de instituições públicas e privadas.

O monitoramento no manejo de qualquer área, seja ela protegida ou não, rural ou urbana, é de extrema necessidade na manutenção dos padrões de qualidade estabelecidos para os ecossistemas e para o desenvolvimento das diversas atividades humanas. Nesse contexto, as pesquisas acerca do monitoramento ambiental apresentam-se como tentativas de se alcançar métodos economicamente viáveis e de execução rotineira e eficiente.

As práticas de monitoramento ambiental são normalmente divididas em químicas e biológicas. Monitoramento químico é o conjunto de análises químicas que quantificam resíduos de contaminantes em um compartimento ou matriz ambiental (água, ar, solo, sedimentos e organismos animais ou vegetais) em uma escala temporal ou espacial. Por outro lado, quando o enfoque dado está em determinar a magnitude dos efeitos de tal contaminação sobre os organismos em nível individual (molecular ou celular), populacional ou de comunidades biológicas, temos o monitoramento biológico.

As substâncias conhecidas como biomarcadores conseguem unir as abordagens química e biológica, pois são compostos originados de seres vivos que, além de permitirem caracterizar quimicamente os poluentes e determinar suas concentrações, também podem estimar o impacto causado por esses poluentes aos organismos bioindicadores, que fornecem as substâncias em questão.

A acetilcolinesterase é um dos mais antigos biomarcadores e vem sendo testada na detecção da presença de pesticidas. Trata-se de uma enzima, do grupo das hidrolases, especializada na quebra de ésteres de colina (com maior afinidade pela acetilcolina) e que atua principalmente nos processos de transmissão de impulsos nervosos em vertebrados e invertebrados. Sua ação normal é interrompida quando exposta aos pesticidas das classes dos organofosforados e carbamatos. A estrutura da acetilcolinesterase apresenta variações inter e intraespecíficas, naturais e mutagênicas, o que justifica a necessidade de caracterizar sua atividade nas diversas espécies, bem como verificar sua sensibilidade aos compostos a serem monitorados.

As acetilcolinesterases de organismos aquáticos são bastante utilizadas como biomarcadores e as razões de sua utilização se ligam ao fato de poluentes de diversas fontes chegarem aos recursos hídricos e também à sua alta posição nas cadeias alimentares o que aumenta detecção de anticolinesterásicos bioacumuláveis. Os critérios de escolha das espécies se dão através de características como habitat, ecologia, hábitos alimentares, abundância e facilidade de captura. Além do monitoramento das diversas matrizes ambientais, essas enzimas, também presentes no sangue humano, podem ser utilizadas no diagnóstico de intoxicações ocupacionais por pesticidas, inclusive em localidades que não contam com infra-estrutura para essas análises.

Existe uma grande lacuna a ser preenchida quanto ao monitoramento da contaminação de solos e corpos d’água naturais, bem como de um programa sistemático de grande abrangência para prevenção e controle das intoxicações ocupacionais no campo, principalmente nos países em desenvolvimento.

2 – REVISÃO BIBLIOGRÁFICA

2.1. Enzimas colinesterases

Henry Hallett Dale, em 1914, sugeriu o possível envolvimento de uma enzima (presente em gatos, cães, coelhos e rãs) na interrupção dos efeitos muscarínicos atribuídos aos ésteres de colina. Em 1926, Loewi e Navratil demonstraram a hidrólise enzimática da acetilcolina em extratos aquosos de rã. Essas enzimas foram, pela primeira vez, chamadas de colinesterases por Stedman e colaboradores, em 1932. Alles e Hawes (1940) relataram discrepâncias na atividade dessas enzimas em relação à taxa de degradação de alguns substratos no plasma e nos eritrócitos, dando origem a estudos que concluíram que não poderia ser apenas um tipo de enzima a realizar essas tarefas (MASSOULIÉ e BONN, 1982; TAYLOR, 1991).

As colinesterases pertencem ao grupo das serino-esterases, mas são de uma família de enzimas que difere das serino-proteases, chamada COesterase PF00135 (na base de dados PFAM), apresentando mais identidade com outras esterases como as carboxilesterases, esterase microssomal de coelhos, esterase-6 da *Drosophyla* e proteínas com propriedades de adesão, entre outras propriedades, que provavelmente perderam a função catalítica ao longo da evolução como as neuroliguinas, neurotactinas, gliotactinas e tiroglobulina (CHATONNET e LOCKRIDGE, 1989; PEZZEMENTI e CHATONNET, 2010). As colinesterases são também glicoproteínas apresentando, em algumas formas, carboidratos em cerca de 10 a 15% de sua estrutura, os quais podem diferir quanto ao tipo e quantidade nos diferentes tecidos. A falta de tais cadeias, induzida por mutação, afetou fortemente a secreção da enzima, mas não afetou a atividade das moléculas que foram secretadas (CHATONNET e LOCKRIDGE, 1989; MASSOULIÉ et al., 1993). As colinesterases são classificadas como globulares (G) ou assimétricas (A) (Figs. 1). As formas globulares apresentam-se como monômeros, dímeros e tetrâmeros (G1, G2 e G4, respectivamente) que podem estar solúveis (formas hidrofílicas) no plasma, linfa e outros tecidos aos quais chegam através da circulação. Podem estar também ancoradas à membrana celular (formas anfifílicas) por meio de glicofosfolipídeos, lipídeos ou proteína transmembranar de ancoragem rica em prolina (PRiMA) (solubilizadas com detergentes) no coração, eritrócitos e linfócitos, no fígado e órgão elétrico de arraias do gênero *Torpedo* ou às membranas dos neurônios nas sinapses cerebrais (no caso da PRiMA)

(CHATONNET e LOCKRIDGE, 1989; TAYLOR, 1991; ZHANG e McCAMMON, 2005). As formas assimétricas são associadas a uma cauda de colágeno Q (ColQ) podendo conter 1, 2 ou 3 tetrâmeros (A4, A8 e A12, respectivamente) em sua extremidade, fixam-se à lâmina basal por meio de dois domínios de ligação à heparina que interagem com o heparan sulfato presente nas junções neuro-musculares (CHATONNET e LOCKRIDGE, 1989; DEPREZ, 2000). Tais formas não são passíveis de extração com detergentes, mas sim com tampões em alta concentração salina. São encontradas também no órgão elétrico de peixes Gymnotidae (CHATONNET e LOCKRIDGE, 1989).

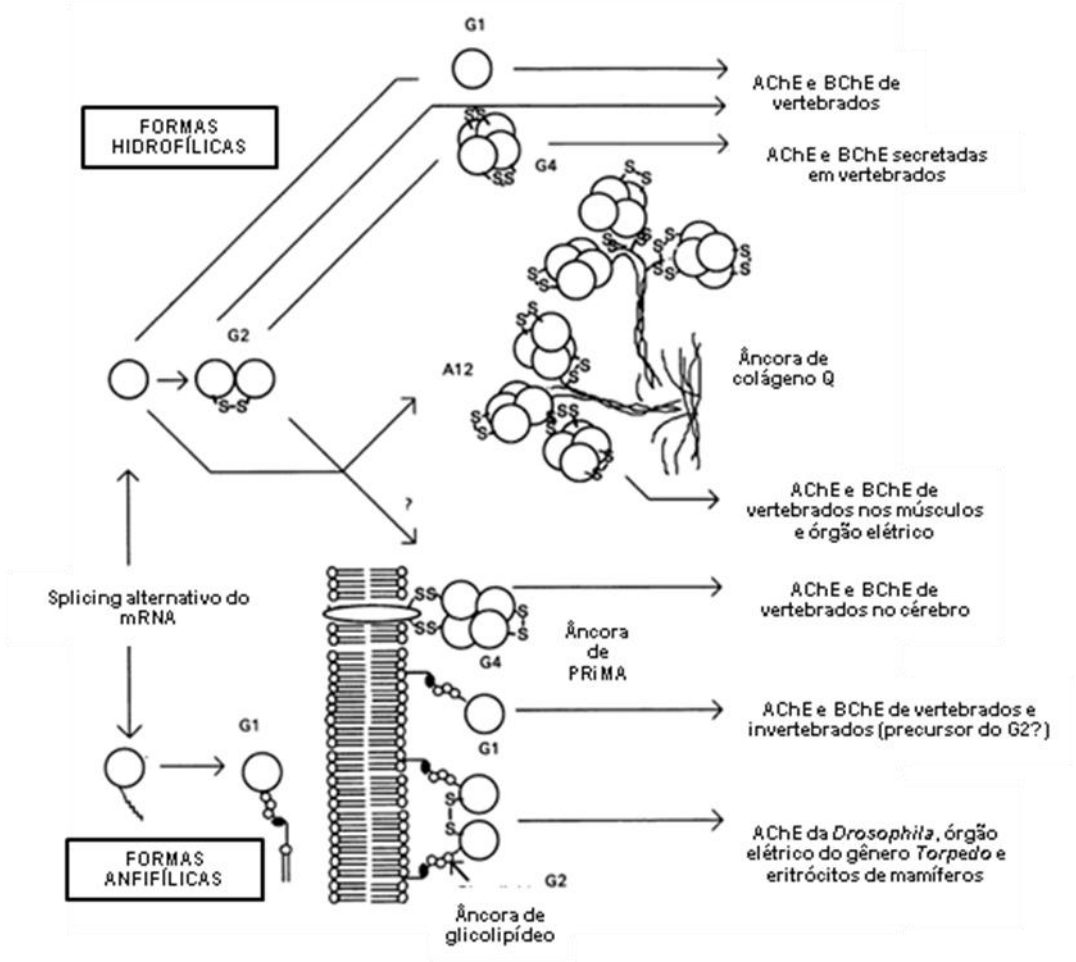


Figura 1 – Formas das colinesterases encontradas em vertebrados (adaptado de Chatonnet e Lockridge, 1989)

Existe ainda uma forma monomérica (G1) da BChE associada à albumina (similarmente ao que ocorre com a paraoxonase associada à HDL), fazendo com que alguns trabalhos atribuíssem atividade esterásica à albumina (FURLONG et al., 1988; LI et al., 2005; SALLES et al., 2006; MASSON e ROCHU, 2009).

Atualmente, são aceitos dois tipos de colinesterases, a acetilcolinesterase ou colinesterase verdadeira (AChE; EC 3.1.1.7) e a butirilcolinesterase, pseudocolinesterase ou colinesterase inespecífica (BChE; EC 3.1.1.8). A primeira, sintetizada no tecido nervoso e durante a eritropoiese, é encontrada nas fibras pré-ganglionares do SNA, fibras parassimpáticas pós-ganglionares e parte das fibras simpáticas pós-ganglionares e das sinapses interneurais do SNC, além do músculo esquelético e membrana dos eritrócitos e linfócitos, hidrolisa preferencialmente acetilcolina, enquanto a segunda é sintetizada pelo tecido hepático, continuamente exportada para a corrente sanguínea e, além de estar presente no fígado e plasma, é encontrada também no músculo liso, pâncreas, adipócitos, pele, massa branca do cérebro e coração (WESCOE et al., 1947; ROSENBERRY, 1975; MASSOULIÉ et al., 1993; CALDAS, 2000; COUSIN et al., 2005). Hidrolisa butirilcolina com mais eficiência e acetilcolina a uma taxa menor.

A principal e clássica função da AChE é a modulação dos impulsos nervosos responsáveis pela comunicação neuronal através da hidrólise do neurotransmissor acetilcolina, enquanto que as prováveis funções da BChE são a detoxificação (succinildicolina, pesticidas organofosforados e carbamatos, cocaína, aspirina, benactizina e drofenina) e a bioativação (bambuterol, heroína, irinotecan) de compostos exógenos (QUINN, 1987; CHATONNET e LOCKRIDGE, 1989; TÕUGU, 2001; ÇOKUGRAS, 2003). Eventualmente, a BChE pode substituir a AChE na hidrólise da acetilcolina, conforme foi observado em camundongos nulizigotos para AChE os quais apresentaram atividade BChE nas sinapses colinérgicas no cérebro e que não houve danos estruturais ao sistema nervoso (MESULAM, 2002).

Evidências também apontam para um possível papel dessas enzimas em atividades não colinérgicas (Tabela 1), como no crescimento e diferenciação neuronal, modulação da adesão celular e tumorigênese, abertura do canal de potássio na *substantia nigra* as quais são funcionalidades não dependentes da atividade catalítica normal, porém dependentes do sítio aniônico periférico da enzima e de um resíduo de 14 peptídeos próximos da extremidade C-terminal das enzimas (CHATONNET e LOCKRIDGE, 1989; TAYLOR, 1991; WEBB e GREENFIELD, 1992; STERNFELD et al., 1998; BIGBEE et al., 1999; BRIMIJOIN e KOENIGSBERGER, 1999; JOHNSON e MOORE, 2000; EMMET e GREENFIELD, 2004; BRIMIJOIN, 2005; SILMAN e SUSSMAN, 2005).

Outras funções catalíticas foram propostas para as colinesterases como a atividade hidrolítica amino-sensível de acilamidas aromáticas ou aril-acilamidase amino-sensível (AAA), a qual se correlaciona diretamente com a atividade esterásica normal e catalisa a quebra de ligações acilamida semelhantes às encontradas em fármacos como paracetamol e fenacetina. Essa atividade pode ser ativada por tiramina e inibida por serotonina e agentes anticolinesterásicos. Tal atividade pode representar uma ligação entre os sistemas de neurotransmissão colinérgico e serotoninérgico pricipalmente em estágios iniciais de desenvolvimento sugerindo uma função no desenvolvimento do cérebro e metabolismo de lipídeos (KUTTY, 1980; BALASUBRAMANIAN e BHANUMATHY, 1993; ÇOKUGRAS, 2003; BOOPATHY e LAYER, 2004).

Tabela 1 – Atividades biológicas propostas para as colinesterases (BALASUBRAMANIAN e BHANUMATHY, 1993)

Atividade biológica	**Colinesterases envolvidas**
Aril-acilamidase amino-sensível	AChE, BChE
Atividade metalocarboxipeptidase	AChE, BChE
Hidrólise de cocaína	BChE
Diferenciação de células neurais	AChE, BChE
Divisão celular e tumorigênese	AChE, BChE
Funções na *substantia nigra* (abertura do canal de potássio)	AChE
Interações célula-célula	AChE, BChE

Atividade proteolítica semelhante à metalocarboxipeptidase foi descrita em diversos trabalhos sem papel fisiológico definido sendo, todavia importante na patogênese da doença de Alzheimer através da clivagem do precursor da proteína β-amilóide (CHATONNET e MASSON, 1986; SMALL et al., 1991; BALASUBRAMANIAN e BHANUMATHY, 1993; BARBER et al., 1996; GUILLOZET et al. 1997; ÇOKUGRAS, 2003). Em outro trabalho, a atividade proteolítica foi considerada uma contaminação das amostras, uma vez que não sofreu imunoprecipitação com anticorpos anti AChE e BChE (CHECLER et al., 1994).

As colinesterases têm sido extensivamente estudadas pelo seu polimorfismo intra e interespecífico e por serem os alvos primários de diversos compostos utilizados em

agropecuária, medicina, campanhas de saúde pública e armas químicas (WHO, 1986a, 1986b; ATSDR, 2005; FORGET, LIVET e LEBOULENGER, 2002; RILEY, 2003; KELLAR, 2006).

2.2. Acetilcolinesterase

A acetilcolinesterase (Fig. 2) age na desativação do principal neurotransmissor do sistema nervoso, na maioria das espécies: a acetilcolina. A AChE hidrolisa rapidamente esse neurotransmissor, nas sinapses colinérgicas e junções neuromusculares, encerrando sua ação e garantindo a intermitência dos impulsos nervosos (Fig. 3) (QUINN, 1987; FUKUTO, 1990; TÕUGU, 2001; SILMAN e SUSSMAN, 2005). A AChE é freqüentemente descrita como uma enzima perfeita porque suas propriedades catalíticas se combinam para aproximar sua atividade do limite máximo de velocidade permitido pela própria difusão do substrato (QUINN, 1987; TÕUGU, 2001; MILLER e WOLFENDEN, 2002; SILMAN e SUSSMAN, 2005; RAMOS e TECHERT, 2005).

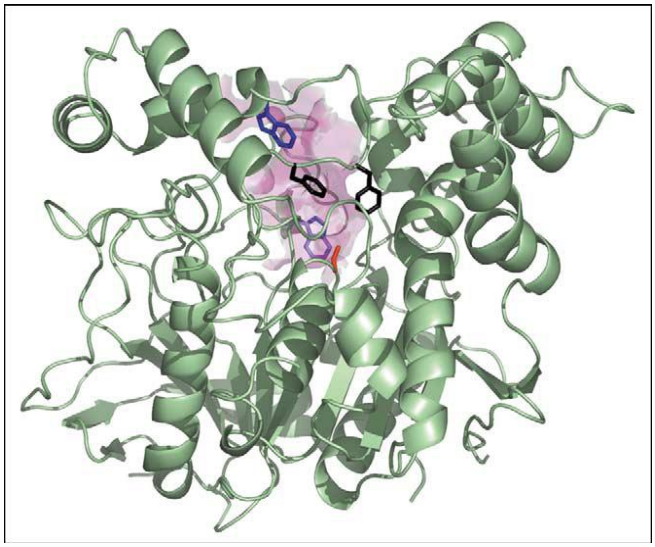
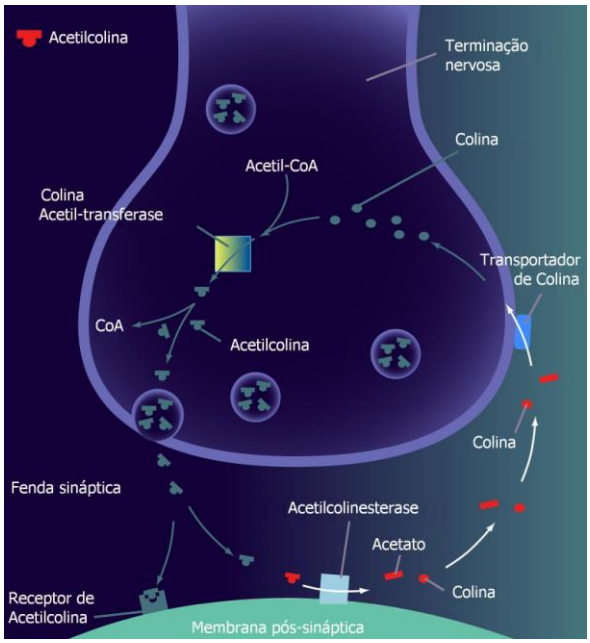


Figura 2 – Estrutura tridimensional da AChE da arraia elétrica *Torpedo californica*. A estrutura é apresentada como um diagram de fitas, com o N-terminal abaixo à esquerda e o C-terminal acima à direita. A entrada da cavidade do sítio ativo situa-se no topo e a superfície da cavidade é delineada em rosa. Triptofano 84, o resíduo-chave no sub-sítio aniônico do sítio ativo, está representado em roxo enquanto o triptofano 279, o resíduo-chave do sítio aniônico periférico está, em azul, na entrada da cavidade. O resíduo-chave do sub-sítio esterásico do sítio ativo, serina 200, é mostrado em vermelho enquanto os resíduos fenilalanina 288 e fenilalanina 290, que delineam a bolsa acil, são mostrados em preto (Silman e Sussman, 2005).



Adaptado de: CNSforum.com

Figura 3 – Desenho esquemático do ciclo da acetilcolina onde é possível observar o papel da acetilcolinesterase desativando o excesso desse neurotransmissor. Colina e acetato são liberados na fenda sináptica e a colina é reabsorvida pelo neurônio, onde a acetilcolina é ressintetizada pela ação da enzima Colina acetil-transferase. O acetato atravessa a barreira hemato-encefálica e é metabolizado em outros tecidos.

A acetilcolinesterase contem dois sub-sítios catalíticos, um sítio esterásico e um sítio aniônico. O sub-sítio esterásico da acetilcolinesterase situa-se no fundo de uma cavidade estreita (*active site gorge*) e é constituído de uma tríade catalítica formada pelos resíduos dos aminoácidos serina 200, histidina 440 e glutamato 327 (podendo variar ligeiramente suas posições, interespecificamente). Na catálise (representada nas Figs. 4 e 5), o sub-sítio aniônico (também chamado de sítio de ligação à colina), situado mais próximo à entrada do sítio ativo, atrai fortemente o nitrogênio quaternário, carregado positivamente, da acetilcolina. Uma vez dentro da cavidade catalítica, a acetilcolina sofre o ataque nucleofílico da serina (na figura 4, representada por O), desprotonada pelo resíduo histidina (representada por B), ao seu carbono carbonílico, criando um intermediário tetraédrico estabilizado por pontes de hidrogênio e pelo resíduo glutamato (representado por A), que num primeiro momento, forma serina acetilada e libera colina. Ao final do processo de clivagem da ligação éster, o grupo acetila é desligado pelo ataque nucleofílico da água, assistido pelo resíduo histidina, com formação de um segundo intermediário tetraédrico liberando ácido acético e regenerando o sítio catalítico (FUKUTO, 1990; TAYLOR et al., 1995; TÕUGU, 2001; VIEGAS Jr et al., 2004).

Na reação inicial mencionada acima, a ocorrência de pontes de hidrogênio entre o grupo carboxilato do glutamato e o N-1 do imidazol da histidina aumentam a habilidade do N-3 da histidina de atuar como uma base e extrair o H^+ do grupo hidroxila da serina.

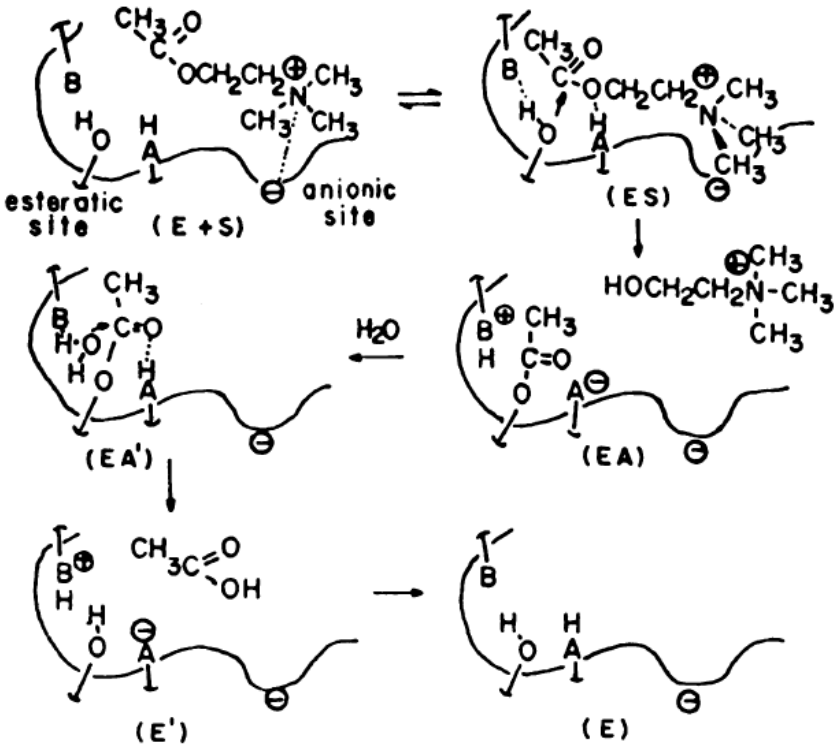


Figura 4 – Desenho esquemático da hidrólise da acetilcolina, catalizada pela AChE, na qual observa-se a ação dos sítios aniônico (atração e posicionamento do substrato) e esterásico (quebra da ligação éster) (fonte: Fukuto, 1990).

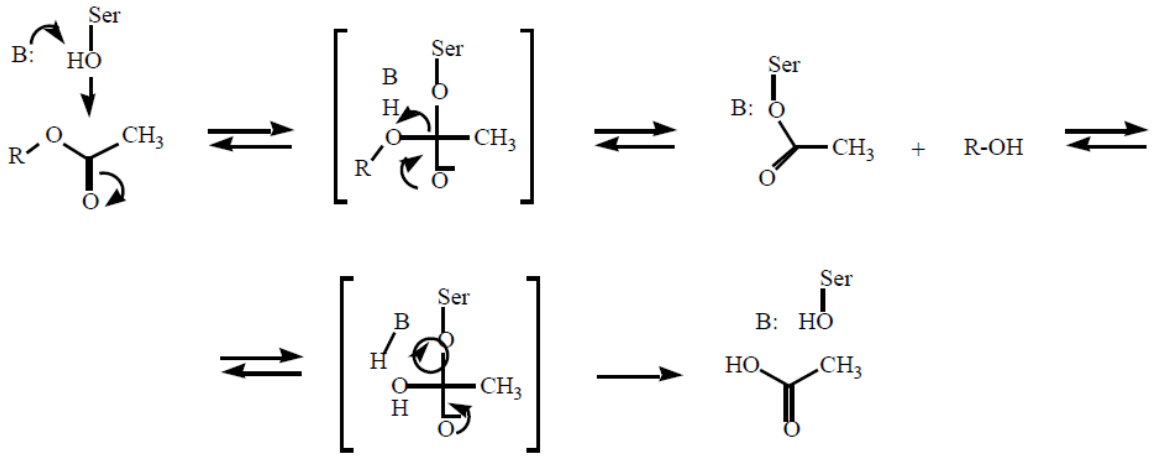


Figura 5 - Hidrólise de ésteres pela AChE. Acima, observa-se a acilação do sítio ativo da enzima e abaixo a desacilação. O esquema inicia-se com a formação do complexo enzima-substrato reversível e os intermediários tetraédricos similares ao estado de transição são mostrados entre colchetes (Fonte: Tõugu, 2001).

Esta cooperação torna o oxigênio da serina um nucleófilo forte, que ataca facilmente o carbono da carbonila da acetilcolina (TAYLOR e BROWN, 1994). Tudo isso ocorre num intervalo de tempo entre 62,5 a 138 microsegundos (AUGUSTINSSON, 1971; FUXREITER e WARSHEL, 1998).

Tentativas de explicar tal eficiência passaram por diversas teorias, desde a entrada alternativa para o sítio ativo (*putative back-door*), passando pela orientação eletrostática dos resíduos aromáticos (cerca de 14) que margeiam a cavidade do sítio e seu estado de dessolvatação com dipolos pré-orientados, até uma possível mobilidade da histidina da tríade catalítica (QUINN, 1987; WARSHEL, 1998; MILLIARD et al., 1999; TÕUGU, 2001). Tais características fariam com que o substrato se ligasse a uma área superficial da enzima e fosse guiado ao interior do sítio ativo, onde a energia das próprias interações contribuíssem para se atingir o estado de transição, sendo velocidade do processo limitada pelos efeitos da viscosidade do meio (QUINN, 1987). Além disso, Ramos e Techert (2005) observaram que uma menor quantidade de pontes de hidrogênio presentes no meio favoreciam o encontro entre enzima e substrato através da diminuição da concha de solvatação, aumentando a difusão do substrato por um aumento da mobilidade intramolecular da AChE.

A AChE apresenta inibição por excesso de substrato, através da ligação do mesmo a um sítio periférico formado por resíduos de aminoácidos que margeiam a entrada do sítio ativo central (MASSOULIÉ e BONN, 1982; EASTMAN et al., 1995).

A inibição desse mecanismo resulta no acúmulo do neurotransmissor nas sinapses do sistema nervoso central, nas junções neuromusculares, nas terminações nervosas parassimpáticas e simpáticas. Alta concentração de acetilcolina é então liberada aos seus receptores (TÕUGU, 2001). Essa inibição é uma reação específica, considerada o principal efeito da exposição aos pesticidas organofosforados (TAYLOR et al., 1995) e carbamatos (JARRARD et al., 2004).

Uma vez iniciada, a inibição tende à irreversibilidade, gerando quadros de intoxicação aguda ou crônica, dependendo do grau de exposição à substância. Um indivíduo agudamente intoxicado por qualquer inibidor de acetilcolinesterase pode morrer, pela superestimulação de seu sistema nervoso, convulsões e parada respiratória (TÕUGU, 2001). Segundo dados da Food and Agriculture Organization (FAO, 2007), uma inibição da atividade da AChE a partir de 20% caracteriza a ação de agentes anti-colinesterásicos, porém sinais clínicos geralmente aparecem após 50% de inibição e morte após 90%.

2.3. Organofosforados e carbamatos

Organofosforados (OP) e carbamatos (CB) são as classes de inseticidas mais utilizadas em todo mundo, juntos respondem por mais de 50% do que é comercializado (Tabela 2) e, em 2007, somente os organofosforados responderam por 35% de todos os inseticidas utilizados nos EUA (NAUEN E BRETENSCHNEIDER, 2002; ATSDR, 2005). São largamente utilizados nos países em desenvolvimento, de economia predominantemente agrícola, para o controle de pragas e em campanhas de combate a vetores de doenças (WHO, 1986a; 1989; USDA, 1997). Entretanto, alguns representantes da classe dos organofosforados constituem o princípio ativo de armas químicas como os gases neurotóxicos tabun, sarin, soman e VX (RILEY, 2003; KELLAR, 2006).

Esses pesticidas são inibidores típicos das enzimas colinesterases (ALDRIDGE, 1950; ALDRIDGE e DAVIDSON, 1952; WHO, 1986a; 1986b). Alguns são utilizados como medicamento no tratamento de doenças como *miastenia gravis*, glaucoma e mal de Alzheimer (FRANCIS et al., 1999; VIEGAS Jr. et al, 2004; CASIDA e QUISTAD, 2005; POPE, KARANTH e LIU, 2005; ALBUQUERQUE et al., 2006). Seu mecanismo de ação se dá através da ligação com o sítio ativo da acetilcolinesterase, com fosforilação para organosfosforados e carbamilação no caso dos carbamatos, produzindo a inibição da enzima (QUINN, 1987). A inibição por carbamatos é reversível e a regeneração da enzima pode levar de alguns minutos a horas. Já a inibição por organofosforados tende à irreversibilidade se não houver tratamento. Contudo, existe uma taxa de regeneração da enzima, que varia de composto para composto, enquanto a fração restante sofre o processo chamado de "envelhecimento" e não mais se regenera, podendo resultar em um efeito cumulativo ante exposições seguidas a esses compostos. A diferenciação entre as inibições promovidas por diferentes compostos se dá não apenas pela intensidade de inibição, mas também pela taxa de regeneração (WHO, 1986a; 1986b)

Esses pesticidas tiveram seu uso intensificado depois da proibição de utilização da maioria dos compostos organoclorados (ECOBICHON, 1996; USDA, 2002; MUKHERJEE e GOPAL, 2002), os quais são menos tóxicos, porém com maior bioacumulação no meio ambiente (NUNES e TAJARA, 1998; USDA, 2002).

Tabela 2 – Modo de ação dos 100 inseticidas/acaricidas mais vendidos no mundo e sua participação no mercado mundial (Nauen e Bretschneider, 2002).

Modo de Ação	1987 %	1999 %	Mudança %
Acetilcolinesterase*	71	52	- 20
Canais de Na^+ voltagem-dependente	17	18	+ 1,4
Receptores de acetilcolina	1,5	12	+ 10
Canais de Cl^- GABA-dependente	5,0	8,3	+ 3,3
Biossíntese de quitina	2,1	3,0	+ 0,9
NADH desidrogenase	0,0	1,2	+ 1,2
Desacopladores	0,0	0,7	+ 0,7
Receptores de octopamina	0,5	0,6	+ 0,1
Receptores de ecdisona	0,0	0,4	+ 0,4

* Organofosforados e carbamatos

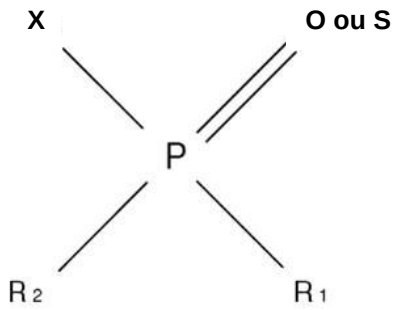


Figura 6 – Fórmula estrutural geral dos organofosforados.

Os OPs são ésteres, amidas ou derivados tióis dos ácidos fosfórico, fosfônico, fosforotióico ou fosfonotióico. Apresentam baixa solubilidade em água e são, em geral, facilmente hidrolizáveis em ambientes alcalinos. Na figura 6, R_1 e R_2 são usualmente radicais alquil ou aril e ambos podem estar ligados diretamente ao fósforo (nos fosfinatos) ou ligados via O- ou S- (nos fosfatos) ou ainda R_1 pode estar ligado diretamente e R_2 ligado por meio de um dos grupos acima (fosfonatos). Nos fosforamidatos, o carbono está ligado ao fósforo

através de um grupamento –NH. O grupo X pode ser qualquer grupamento alifático (ramificado ou não), aromático ou heterocíclico ligado ao fósforo de forma lábil (através de O- ou S-substituição) sendo o grupo de partida. Em relação ao átomo em ligação dupla com o fósforo, os OPs dividem-se em dois grupos: os fosfatos (forma oxon; P=O) e os fosforotioatos (forma tion; P=S) (VALE, 1998). Os primeiros são mais tóxicos devido à maior eletronegatividade do oxigênio em relação ao enxofre ao interagir com o sítio ativo da AChE. O segundo grupo é menos reativo, porém sua meia-vida no ambiente é mais longa proporcionando maior poder residual ao inseticida. Por essa razão, a maior parte dos OPs é comercializada na forma tion (WHO, 1986a; FUKUTO, 1990; VALE, 1998). Em geral, os OPs necessitam de biotransformação (dessulfuração por ação de isoformas do complexo citocromo P450, N-oxidação, S-oxidação e enzimas monoxigenases que contem flavina, além de fatores físicos como luz, pH e temperatura) para se tornarem toxicologicamente ativos (DAUTERMAN, 1971; WHO, 1986a). Tais biotransformações não ocorrem apenas no fígado (Fig. 7), mas também nos rins, pulmões e cérebro (MESNIL et al., 1984; CUNHA BASTOS et al., 1999; SARASQUETE e SEGNER, 2000; MONSERRAT, 2007).

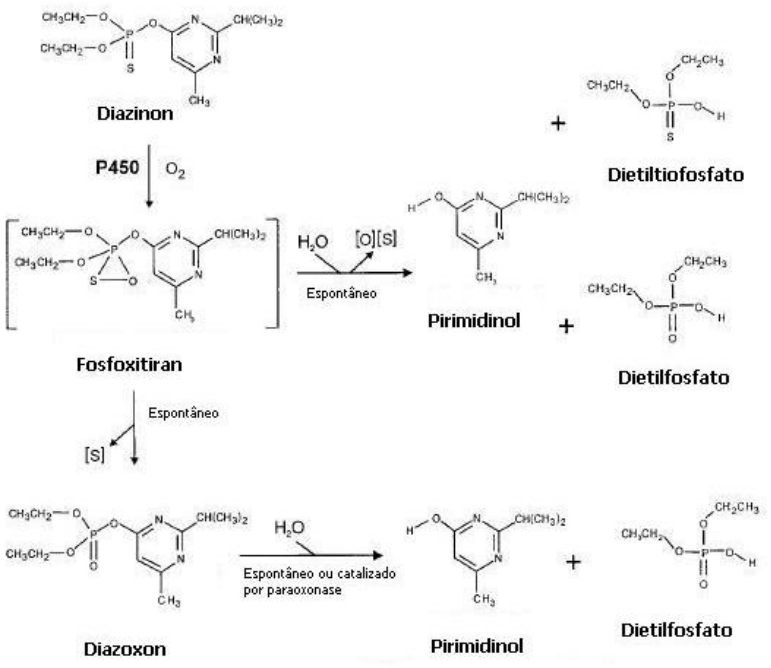


Figura 7 – Ativação do OP diazinon em fígado humano adaptado de Kappers et al. (2001)

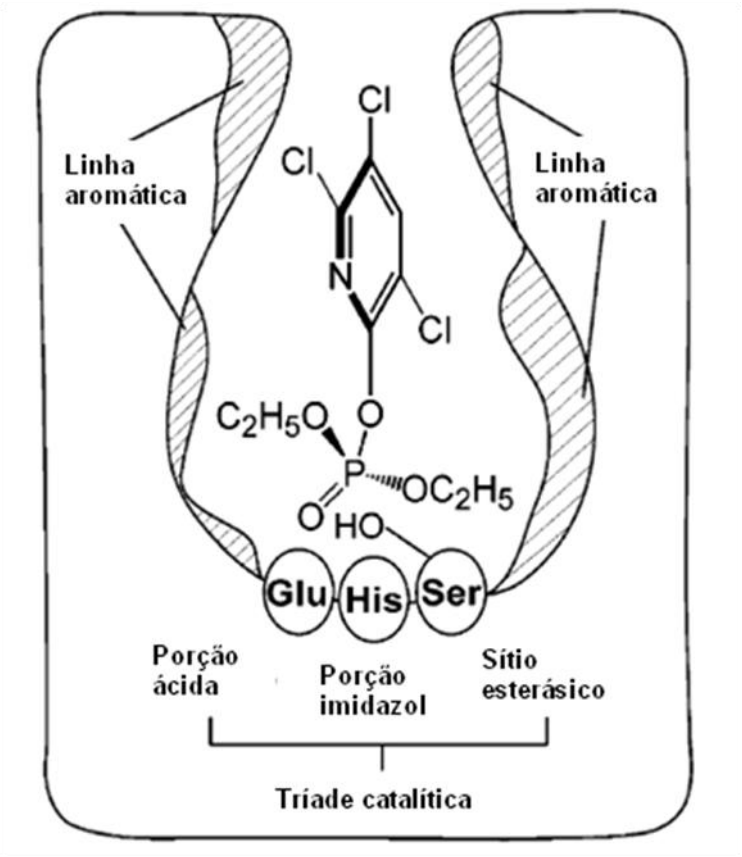


Figura 8 – Representação esquemática da interação entre o clorpirifós-oxon e o sítio ativo da AChE. A tríade catalítica situa-se próximo à base de uma cavidade estreita e profunda que alcança o centro da estrutura globular da proteína. Apesar da formação da dietilfosforil-enzima inativa ocorrer a partir da ligação com o resíduo serina, há o envolvimento (não em todos os casos) das porções imidazólica e ácida dos resíduos histidina e glutamato, respectivamente, assim como na catálise normal. O alinhamento aromático nas paredes da cavidade direciona o inibidor e serve como mecanismo guia para posicionar o átomo de fósforo. Adaptado de Casida e Quistad (2004).

A interação entre a acetilcolinesterase e seu inibidor organofosforado (Figs. 8, 9 e 10) envolve principalmente o sítio esterásico, formando um complexo bastante estável (WIENER e HOFFMAN, 2004; ATSDR, 2007). A estabilidade do complexo formado está relacionada fundamentalmente com a estrutura química do composto organofosforado. A ação anticolinesterásica dos compostos OP não está restrita à AChE do tecido nervoso central e periférico, ocorrendo de forma paralela a inibição da butirilcolinesterase (BChE) plasmática, da AChE eritrocitária (MUTCH, BLAIN e WILLIAMS, 1992).

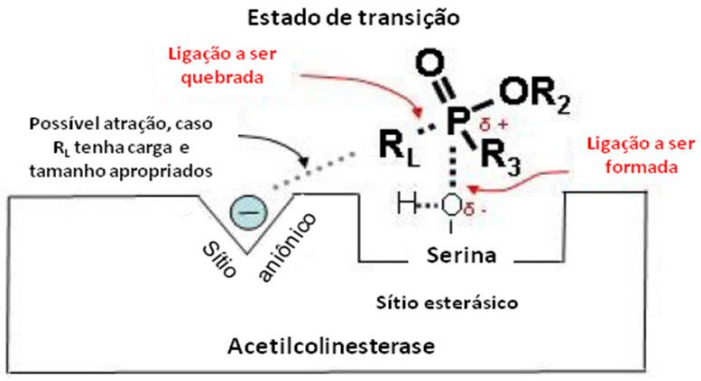


Figura 9 – Estado de transição na interação entre enzima e organofosforado. No detalhe, as ligações envolvidas. Adaptado de ATSDR (2007).

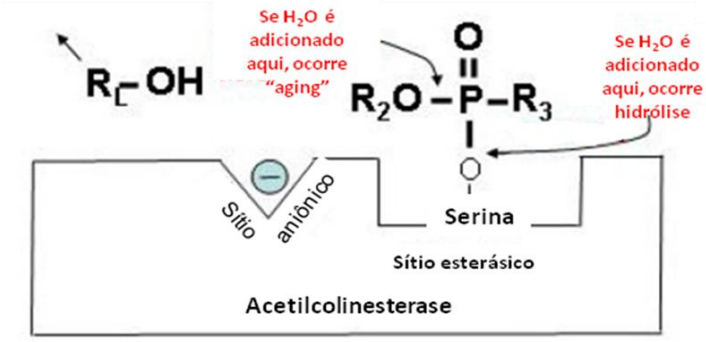


Figura 10 – Uma vez bloqueada pelo pesticida, a enzima pode: 1) hidrolisar o pesticida em processo lento; 2) sofrer o processo de "aging"; 3) ser reativada pela ação de uma oxima. Adaptado de ATSDR (2007).

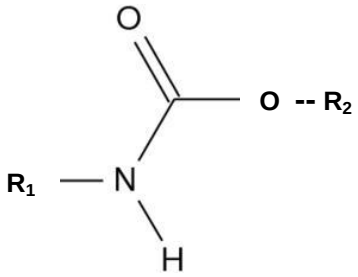


Figura 11 – Fórmula estrutural geral dos carbamatos.

Os carbamatos são ésteres ou derivados N-substituídos do ácido carbâmico. Na figura 11, R_2 pode conter radicais alquil ou aril. Os carbamatos inseticidas possuem um grupamento metil em R_1 enquanto os herbicidas possuem um radical aromático. Já os CBs fungicidas contêm um grupamento benzimidazol em R_1. Dentre esses, apenas os CBs inseticidas apresentam atividade anticolinesterásica (WHO, 1986b). Os CBs são inseticidas efetivos em virtude de inibirem a AChE no sistema nervoso, sem necessitar de biotransfomação. A ligação dos CBs com a AChE é instável e a regeneração da enzima carbamilada é relativamente rápida em relação à da enzima fosforilada pelos OPs uma vez que os carbamatos interagem mais com o sub-sítio aniônico, assim como os substratos, aumentando a possibilidade de hidrólise. A reativação espontânea das colinesterases carbamiladas, expressas como meia-vida a pH 7,0 e 25 °C variou entre 2 e 240 min para AChE e entre 2 e 17 min para BChE plasmática, mas esse período até a reativação pode ser bem maior para alguns compostos. Tal instabilidade da enzima carbamilada pode afetar a determinação do poder inibitório de alguns carbamatos (REINER, 1971; CALDAS, 2000). Outro motivo que torna os CBs menos perigosos que os OPs é o fato de que a diferença entre a dose requerida para produzir efeitos

mínimos e a dose letal é substancialmente maior nos CBs. Os CBs são instáveis e prontamente hidrolisáveis em ambiente alcalino, assim como os OPs, porém são mais solúveis em água (WHO, 1986b).

Os organofosforados e carbamatos são absorvidos pelo organismo por via oral, respiratória e cutânea levando a um conjunto de sintomas característicos (Tabela 3). A via oral é a maior causa de internações hospitalares de emergência e a cutânea, a causa mais comum nas intoxicações ocupacionais (CALDAS, 2000). O tratamento mais freqüente de intoxicações por agentes anticolinesterásicos, sobretudo os organofosforados, é feito através do uso do alcalóide atropina em combinação com oximas (Fig. 12). O primeiro bloqueia os receptores muscarínicos, impedindo que os mesmos sejam superestimulados pelo excesso de acetilcolina na fenda sináptica e a segunda, aplicada o mais cedo possível, reativa as enzimas fosforiladas por ter maior afinidade com as moléculas do pesticida, impedindo a irreversibilidade da inibição (KELLAR, 2006). Alguns trabalhos consideram contra-indicado o uso de oximas na reativação de colinesterases inibidas por carbamatos, porém em publicação do governo americano tal noção é considerada equivocada, uma vez que as oximas só não contribuíram para a regeneração das enzimas inibidas por um único carbamato: o carbaril (ATSDR, 2007).

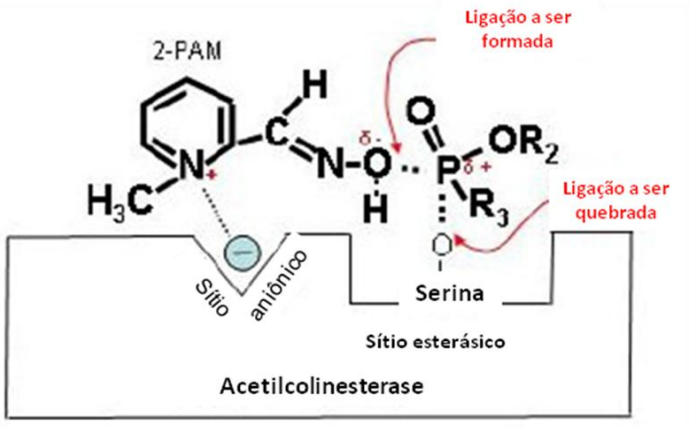


Figura 12 – Ação de uma oxima sobre a ligação fosfoéster entre a enzima e o organofosforado. Adaptado de ATSDR (2007).

Diversos pesticidas organofosforados e alguns carbamatos, incluindo alguns de seus metabólitos, são capazes de provocar malformações congênitas, afetar a fertilidade e produzir efeitos genéticos tóxicos, inclusive câncer (WHO, 1986a; 1986b). Casos de câncer foram evidenciados em 1992, em adultos jovens indígenas na Amazônia. Nestes jovens foram encontrados níveis elevados de organofosforados no sangue (MATOS et al., 1988; KOIFMAN et al., 1998). Os efeitos teratogênicos dos agrotóxicos podem resultar da

exposição intra-uterina do indivíduo em formação e mediante a ação mutagênica nos gametas dos progenitores nas primeiras etapas da gestação. Das malformações congênitas de fácil diagnóstico clínico, as que se destacaram pela influência de agrotóxicos em estudo realizado no Chile foram a síndrome de down, espinha bífida e hidrocefalia (ROJAS et al., 2000).

Tabela 3 - Sinais e sintomas das intoxicações por inseticidas organofosforados

Local	Sinais e sintomas
Sistema Nervoso Central	Distúrbios do sono, dificuldades de concentração, comprometimento da memória, ansiedade, agitação, convulsões, tremores, depressão respiratória, coma.
Sistema Nervoso Autônomo (efeitos muscarínicos)	No aparelho digestivo: perda de apetite, náuseas, vômitos, dores abdominais, diarréia, defecação involuntária. No aparelho respiratório: secreção bronquiolar, edema pulmonar. No sistema circulatório: bradicardia, bloqueio aurículo-ventricular. No sistema ocular: visão enfraquecida, pupilas puntiformes. No aparelho urinário: diurese freqüente e involuntária. Glândulas exócrinas: transpiração excessiva.
Sistema somático (efeitos nicotínicos)	Contração involuntária dos músculos, cãibras, enfraquecimento muscular generalizado.

Fonte: Larini (1999)

Com relação aos alvos principais dos organofosforados, três síndromes são descritas na literatura. Para os carbamatos em geral, apenas a primeira é descrita (WHO, 1986b). Todavia, a terceira síndrome já foi descrita para os carbamatos carbaril e carbofuran (CALDAS, 2000):

- *Síndrome colinérgica aguda*

Sintomatologia múltipla, efeito da superestimulação colinérgica

- fibras nervosas pós-ganglionares parassimpáticas (muscarínicos)
- fibras pré-ganglionares simpáticas e parassimpáticas (nicotínicos I)
- nervos motores somáticos (nicotínicos II)
- receptores de acetilcolina

- *Síndrome intermediária*

Efeito da hiperestimulação de longo período – 24 a 96 h após a síndrome aguda

- diminuição da força dos músculos proximais

- *Síndrome da neuropatia tardia*

Atinge a NTE, 'esterase-alvo', antiga 'esterase neurotóxica' causando degeneração dos neurônios do sistema nervoso central - 4 semanas após exposição.

2.4. Organofosforados e carbamatos no meio ambiente e alimentos

O uso excessivo de pesticidas na agricultura, desde a preparação do cultivo, até o armazenamento de produtos, é um fator determinante para a contaminação dos alimentos de origem vegetal. Os níveis de resíduos encontrados no meio ambiente e na alimentação refletem a freqüência de aplicação desses compostos, a qual varia com a cultura, estágio de desenvolvimento, nível de infestação da praga-alvo e fatores climáticos como pluviosidade e umidade relativa do ar.

Apenas 0,1% dos pesticidas aplicados atingem as pragas-alvo, de forma que o restante desse material contendo o princípio ativo se espalha pelas imediações, contaminando o ar e o solo (YOUNG, 1987; HART e PIMENTEL, 2002). OPs e CBs podem atingir os ecossistemas aquáticos e lençois freáticos (Fig. 13), carreados pelo escoamento superficial e lixiviação das águas da chuva, irrigação e drenagem, bem como através de pulverizações (USEPA, 1990 e 1999; DUBUS et al., 2000; MÜLLER et al., 2002, TOMITA e BEYRUTH, 2002). Uma vez presente no ambiente aquático, eles podem se associar ao material em suspensão, aos sedimentos no leito do corpo d'água ou ser absorvidos pelos organismos onde sofrerão bioacumulação ou detoxificação (NIMMO, 1985).

No prosseguimento da cadeia alimentar, os pesticidas chegam até os alimentos e demais produtos de origem agroindustrial utilizados pelos homens, tornando-se clara a necessidade de se monitorar tanto o meio ambiente quanto a qualidade dos alimentos. Particularmente pela alta toxicidade desses pesticidas em relação aos organismos aquáticos, os recursos hídricos devem ser continuamente monitorados (BEAUVAIS et al., 2002).

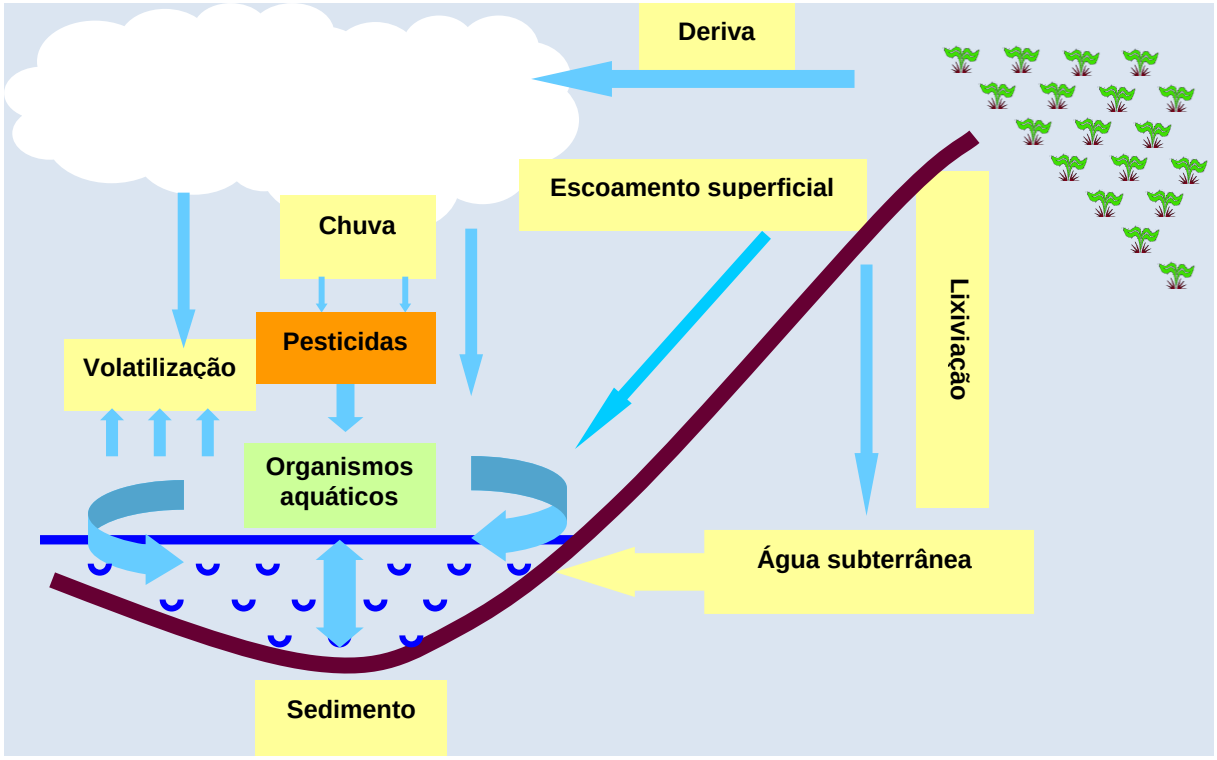


Figura 13 – Processos de entrada dos pesticidas em ambientes aquáticos (Adaptado de Tomita e Beyruth, 2002).

2.5. Monitoramento de pesticidas utilizando esterases animais e humanas

O monitoramento ambiental pode ser definido como o contínuo acompanhamento e mensuração dos impactos, bem como, reações ambientais às atividades e interferências humanas (IBAMA/GTZ, 2000). Uma aplicação prática do monitoramento ambiental seria a comparação temporal entre as condições ambientais de uma dada área, sujeita a variações devido à ação humana ou natural.

Normalmente, o monitoramento ambiental é dividido em químico e biológico. Monitoramento químico é o conjunto de análises químicas que quantificam resíduos de contaminantes em um compartimento ou matriz ambiental (água, ar, solo, sedimentos e organismos animais ou vegetais) em uma escala temporal ou espacial. Por outro lado, quando o enfoque dado está na determinação da magnitude dos efeitos de tal contaminação sobre os organismos em nível individual, populacional ou de comunidade biológica, temos o monitoramento biológico (HENRÍQUEZ-PÉREZ e SÁNCHEZ-HERNÁNDEZ, 2003).

Diversas ferramentas de monitoramento ambiental e alimentar vêm sendo avaliadas quanto à eficácia, praticidade e viabilidade econômica. Dentre elas, destacam-se as metodologias que utilizam moléculas provenientes de seres vivos como indicadores de substâncias nocivas, tendo em vista sua alta especificidade em relação a esses compostos (MARCO e BARCELÓ, 1996; ARIAS *et al*., 2007; MONSERRAT *et al*., 2003 e 2007).

Unir os enfoques metodológicos dos monitoramentos químico e biológico é uma tarefa de importância para a avaliação da contaminação ambiental e seus efeitos sobre o ecossistema. As substâncias conhecidas como bioindicadores conseguem unir as duas abordagens, pois são compostos de origem animal, vegetal, fúngica e microbiológica que, além de permitirem caracterizar quimicamente os poluentes e determinar suas concentrações, também podem estimar o impacto causado por esses poluentes aos organismos bioindicadores, que fornecem as substâncias em questão (WIJESURIYA e RECHNITZ, 1993; WATSON e MUTTI, 2004). Dentre essas substâncias, as enzimas representam papel importante, pelo alto grau de especificidade e rapidez na resposta às alterações pertinentes às substâncias-alvo. O uso de enzimas como bioindicadores baseia-se na interferência negativa ou inibitória, causada pelas substâncias-alvo, em sua atividade catalítica (MARCO e BARCELÓ, 1996).

As enzimas colinesterases têm sido testada, em diversos estudos, como bioindicador da presença de organofosforados e carbamatos na água ou da exposição de diversas espécies de animais a esses compostos. Sánchez-Hernández e Moreno-Sánchez (2002) utilizaram o lagarto *Gallotia galloti*, típico das Ilhas Canárias, como fonte da enzima para estudar a contaminação pelos pesticidas naquela localidade, tendo em vista que seu estudo em aves tornava-se bastante problemático devido ao tamanho das áreas percorridas pelas mesmas e pela dificuldade de captura de indivíduos contaminados e não contaminados.

Estudos utilizando peixes como a tilápia do Nilo, *Oreochromis niloticus* (RODRÍGUEZ-FUENTES e GOLD-BOUCHOT, 2000), o centrarquídeo norte-americano Bluegill, *Lepomis macrochirus* (BEAUVAIS et al., 2002), o salmão-prateado *Oncorhynchus kisutch* (JARRARD et al., 2004), a carpa comum *Cyprinus carpio* (CHANDRASEKARA e PATHIRATNE, 2005) e a correlação de alterações comportamentais com indicadores fisiológicos de várias espécies (SCOTT e SLOMAN, 2004) têm confirmado os peixes como uma fonte prática e economicamente viável de acetilcolinesterase, capazes de tornar rotineiros

os procedimentos de biomonitoramento de recursos hídricos (BOCQUENÉ, GALGANI e TRUQUET, 1990).

Silva (1997) estudou a exposição aos inseticidas de trabalhadores na atividade de desinsetização doméstica em Belo Horizonte, Minas Gerais e encontrou parâmetros físico-químicos de utilização da acetilcolinesterase extraída do sangue humano para maior confiabilidade dos resultados. A busca por essa caracterização físico-química é corroborada por Rodríguez-Fuentes e Gold-Bouchot (2004) e por Sturm et al. (1999a; 1999b), como forma de se obter uma resposta confiável das reações químicas.

Existe uma necessidade de se caracterizar a atividade dos diversos tipos de colinesterases, uma vez que a variabilidade de formas apresentadas por diferentes espécies e diferentes indivíduos é alta. Weill et al. (2003) encontraram um mecanismo de resistência à ação dos organofosforados, em populações de mosquitos *Anopheles gambiae* e *Culex pipiens*, que consistia na substituição de um único aminoácido na cadeia da acetilcolinesterase sintetizada por esses insetos, demonstrando que a enzima apresenta diferenças intraespecíficas de grande importância. Além disso, segundo Silman e Sussman (2005), o provável motivo para a acetilcolinesterase apresentar-se em uma série de formas moleculares em um mesmo indivíduo seria o de atender aos diversos tipos de sinapses colinérgicas presentes no tecido nervoso. AChE mutantes ou geneticamente modificadas já foram utilizadas como biodetectores da presença de inseticidas organofosforados (MARQUES et al., 2001; SOTIROPOULOU et al., 2005). A BChE em alguns casos, pode ser mais sensível do que a AChE, porém sua regeneração ocorre de forma mais rápida (REINER, 1971; CHAMBERS et al., 2002)

Os efeitos primários dos organofosforados e carbamatos não se restringem às colinesterases (Fig. 14): outras esterases do sistema nervoso central e periférico sofrem inibição, como a ‘esterase-alvo’ da neuropatia tardia por organofosforados (antiga ‘esterase neurotóxica’, *Neuropathy target esterase* – NTE; EC 3.1.1.5) (LOTTI, 1984; JOHNSON, 1990; JOHNSON e GLYNN, 1995; GLYNN, 1999; COSTA, 2006).

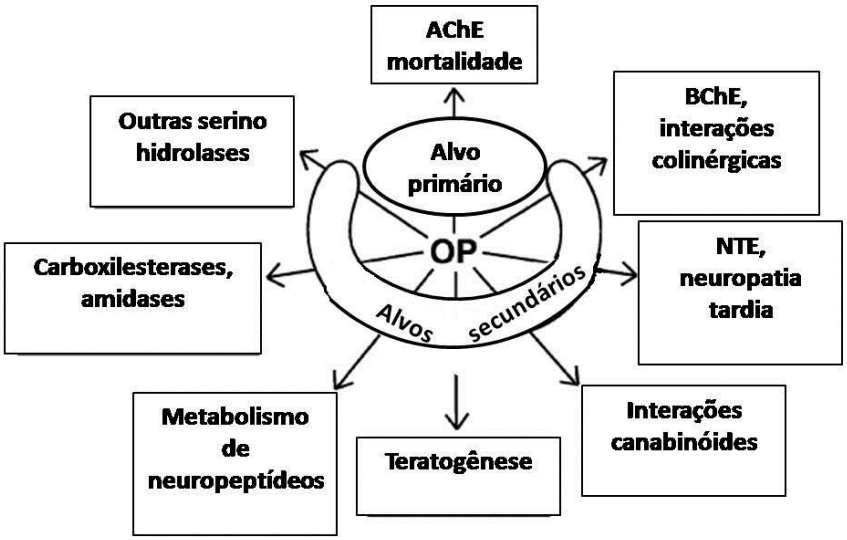


Figura 14 – Alvos primário e secundários dos organofosforados (adaptado de Casida e Quistad, 2004).

Cerca de 50 esterases podem ser inibidas, dentre elas algumas enzimas digestivas como a tripsina (EC 3.4.21.4), quimotripsina (EC 3.4.21.1) e carboxipeptidases A (EC 3.4.17.15) (KAM et al., 1979; FISCHER, 1988; CASIDA e QUISTAD, 2004 e 2005) e algumas carboxilesterases (CEs; EC 3.1.1.1), as quais catalisam a hidrólise de ésteres carbâmicos e carboxílicos presentes nos inseticidas carbamatos e piretróides, respectivamente (COHEN e EHRICH, 1976; SOGORB e VILANOVA, 2002).

As esterases do plasma sanguíneo foram divididas em A e B (ALDRIDGE, 1953a), ambas com a capacidade de hidrolisar carbamatos e piretróides, mas diferindo quanto à interação com organofosforados (ALDRIDGE, 1953a, 1953b; SOGORB e VILANOVA, 2002). Enquanto as do tipo A não sofrem inibição por organofosforados, as esterases B são inibidas por essa classe de compostos (ALDRIDGE, 1953a). As colinesterases são enquadradas no tipo B. Apesar de sua sensibilidade aos pesticidas, as carboxilesterases e demais esterases plasmáticas (com exceção das colinesterases) foram pouco caracterizadas havendo poucos dados sobre sua variação intra e interespecífica, não sendo, por isso, utilizadas com intensidade no monitoramento ambiental. Nesse contexto, a caracterização físico-química e cinética, bem como o efeito de pesticidas sobre as enzimas faz-se necessário para identificá-las como uma provável ferramenta de utilização no monitoramento ambiental e ocupacional.

Segundo o Governo Federal (IBAMA, 2002), ainda existe uma grande lacuna a ser preenchida em relação ao diagnóstico de áreas contaminadas por pesticidas, principalmente

em ecossistemas aquáticos. No Brasil, poucos trabalhos foram realizados na área, voltados para o biomonitoramento *in vitro* utilizando peixes e humanos. Além disso, o Sistema Nacional de Informação Tóxico Farmacológica (SINITOX) da Fundação Oswaldo Cruz, necessita sempre de dados atualizados sobre os casos de intoxicação de abrangência nacional.

3 - OBJETIVOS

3.1. Geral

Caracterizar físico-química e cineticamente a acetilcolinesterase cerebral de cinco espécies de peixes e investigar o efeito de pesticidas organofosforados e carbamatos sobre a atividade dessas enzimas, bem como verificar a sensibilidade da acetilcolinesterase eritrocitária humana exposta a tais pesticidas.

3.2. Específicos

- Definir o tipo de colinesterase presente no cérebro de cinco espécies de peixes (tambaqui, pirarucu, beijupirá, tilápia e tucunaré) e determinar suas propriedades físico-químicas e cinéticas;

- Propor um método prático e economicamente viável para determinação de parâmetros de eficiência catalítica (Kcat, Kcat/Km, Energia de ativação e acréscimo na velocidade de reação) das enzimas;

- Analisar o efeito de cinco pesticidas organofosforados (diclorvós, diazinon, clorpirifós, temefós e TEPP) e dois carbamatos (carbaril e carbofuran) sobre a atividade das enzimas das espécies estudadas, comparando os resultados de inibição com os valores de Limites Máximos de Resíduos (LMR) presentes na legislação nacional e internacional vigente;

- Analisar o efeito de íons (Al^{3+}, As^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, $EDTA^{2-}$, Fe^{2+}, Hg^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}) sobre a atividade das enzimas das espécies estudadas; e

- Analisar a sensibilidade da acetilcolinesterase eritrocitária humana a três pesticidas organofosforados (diclorvós, diazinon e clorpirifós) e dois carbamatos (carbaril e carbofuran), comparando os resultados de inibição com os valores de Ingestão Diária Aceitável (IDA) presentes na legislação nacional e internacional vigente.

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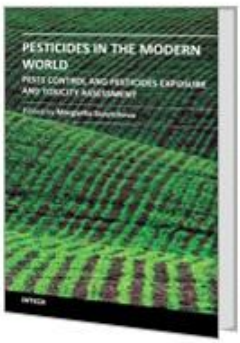
5 - CAPÍTULO I – FISH CHOLINESTERASES AS BIOMARKERS OF ORGANOPHOSPHORUS AND CARBAMATE PESTICIDES

CAPÍTULO PUBLICADO NO LIVRO PESTICIDES IN THE MODERN WORLD PESTS CONTROL AND PESTICIDES EXPOSURE AND TOXICITY ASSESSMENT

Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment



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13

Fish Cholinesterases as Biomarkers of Organophosphorus and Carbamate Pesticides

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5.1. Introduction

Due to reasons that last for decades, environmental monitoring of pesticides is an urgent need. Contamination by pesticides is an important public health problem, mainly in developing countries. It is estimated that only 0.1% of the applied pesticides in fact reach the target pests, while the rest spreads throughout the environment (Hart and Pimentel, 2002). In addition, among the 500,000 deaths a year related to pesticides in the developing world, approximately 200,000 occur due to the use of organophosphorus (OP) and carbamates (CB) pesticides (Eddleston et al., 2008). These are among the most important classes of insecticides/acaricides in usage and billing (Nauen and Bretschneider, 2002). The primary and most known target for the action of organophosphorus and carbamate compounds is a family of enzymes (Cholinesterases; ChEs) formed by: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). The first is synthesized in hematopoiesis, occurs in the brain, endplate of skeletal muscle, erythrocyte membrane, and its main function is to regulate neuronal communication by hydrolyzing the ubiquitous neurotransmitter acetylcholine in synaptic cleft (Quinn, 1987; Silman and Sussman, 2005). The second is synthesized in liver and is present in plasma, smooth muscle, pancreas, adipocytes, skin, brain and heart (Çokugras, 2003). Although its physiological function is not well defined, BChE is pointed as one of the main detoxifying enzymes able to hydrolyze or scavenge a broad range of xenobiotic compounds like cocaine, heroine, anaesthetics, and pesticides (Soreq and Zakut, 1990; Taylor, 1991; Çokugras, 2003; Nicolet et al., 2003). Some studies hypothesize that one of the functions of BChE is to protect AChE against anticholinesterasic agents (Whitaker, 1980; Whitaker, 1986). Pezzementi and Chatonnet (2010) reported that ChEs emerged from a family of proteins with adhesion properties. Both play other roles in the neuronal tissue, particularly in neuronal differentiation and development, cell growth, adhesion and signalling. In addition, AChE participates even in hematopoietic differentiation (Chatonnet and Lockridge, 1989; Taylor, 1991; Johnson and Moore, 2000; Silman and Sussman, 2005).

Moreover, AChE and BChE are different concerning several other aspects: while AChE has an *in vivo* half-life of 120 days, BChE lasts 7-12 days. AChE is inhibited by substrate excess and BChE is activated by substrate excess (Lopez-Carillo and Lopez-Cervantes, 1993; Çokugras, 2003). AChE is selectively inhibited by propidium, DDM, caffeine, Nu1250, 62c47

and BW284c51 while BChE is selectively inhibited by percaine, isopestox, ethopropazine, Iso-OMPA, bambuterol and haloxon (Adams and Thompson, 1948; Austin and Berry, 1953; Aldridge, 1953; Bayliss and Todrick, 1956; Chatonnet and Lockridge, 1989; Harel et al., 1992; Kovarik et al., 2003). BChE has a larger space in its active site, which can hydrolize or be inhibited by a range of compounds. AChE has a more specific active site (Çokugras, 2003). Some of these features are governed by crucial differences in the structure of the enzymes such as: 1) the difference in size of active site can be explained by six aromatic residues lining the active site of AChE that are missing in BChE; 2) two of these (Phe-288 and Phe-290) are replaced by leucine and valine, respectively, in BChE. This feature prevents the entrance of butyrylcholine in the AChE active site; 3) peripheral site specific-ligands such as propidium does not inhibit BChE because the residue Trp-279, which is part of the peripheral anionic site located at the entrance of the active site gorge in AChE, is absent in BChE (Harel et al., 1992). According to Rosenberry (1975), AChE is more sensitive to the size of the acyl group than to the alcohol moiety (whether charged or neutral) of the substrate, while for BChE the opposite is observed. Both are inhibited by 50 μM of physostigmine (eserine), which is a condition that affords to discriminate cholinesterases (ChEs) from other esterases (Augustinsson, 1963).

The class of AChEs is more homogeneous in terms of their primary structure than the class of BChEs (Rosenberry, 1975). Despite of these differences, the amino acid sequence identity between AChE and BChE from vertebrates ranges from 53 to 60%, even in evolutionarily distant species (Chatonnet and Lockridge, 1989; Taylor, 1991). In addition, a study promoted the replacement of only two amino acids by site-directed mutagenesis in AChE for it to develop BChE activity (Harel et al., 1992). Both enzymes present the active site within a deep and narrow gorge, approximately in the middle of its globular structure, which apparently could disturb the substrate traffic. However, in fact this structure follows a rational organization which entraps substrate and transports it to the active site through the arrangement of amino acids lining the gorge. And all this occurs very efficiently (Quinn, 1987; Tõugo, 2001).

To characterize ChE, some studies used the kinetic parameters Km and Vmax, more specifically the Km and Vmax ratios for acetyl and butyrylcholine hydrolysis and their analogues by the enzymes. According to the expected values for these ratios, AChE has a low Vmax ratio and a Km ratio ≥ 1, because it presents excess substrate inhibition. BChE does not show this feature, its Vmax ratio is ≥ 1, and Km ratio < 1. (Pezzementi et al., 1991; Rodríguez-Fuentes and Gold-Bouchot, 2004).

Table 1 summarizes Km and Vmax of fish AChEs from brain, muscle and electric organ reported in the literature. The Km values varied from 0.085 (Rainbow trout brain) up to 3.339 mM (Brazilian flathead brain), whereas Vmax ranged from 0.116 (arapaima brain) up to 0.524 U/mg protein (female hornyhead turbot muscle).

Table 2 presents the values for optimum pH and maximum temperature of fish enzymes. pH values ranged from 7.5 to 8.5 for all reported species, while temperatures varied from 26ºC (bluegill brain) to 45ºC (tambaqui and pirarucu brains).

The Km values of fish BChEs presented in table 3 ranged from 0.033 (Nile tilapia liver) to 1.61 mM (tambaqui brain) and Vmax were from 0.04 (tambaqui brain) up to 0.231 U/mg protein (piaussu serum). Several studies have described that AChE accounts for most of the brain cholinesterasic activity (Rodríguez-Fuentes, 2004; Varò et al., 2004; Varò et al., 2007; Jung et al., 2007). However, our studies on brain ChEs from some fish reveal that certain

Scientific and common name	Km (mM)	Vmax (U/mg protein)	Source	Reference
Ictalurus punctatus – Channel catfish	0.375 ± 0.002	0.212 ± 0.002	Brain	Carr and Chambers, 1996
Oreochromis niloticus – Nile tilapia	0.101 ± 0.03	0.229 ± 0.014	Brain	Rodríguez-Fuentes and Gold-Bouchot, 2004
Pseudorasbora parva – topmouth gudgeon, Stone moroko	0.113 ± 0.11	0.490 ± 0.024	Brain	Shaonan et al., 2004
Carassius auratus – goldfish	0.112 ± 0.09	0.504 ± 0.027	Brain	Shaonan et al., 2004
Oncorhynchus mykiss – rainbow trout	0.085 ± 0.06	0.266 ± 0.023	Brain	Shaonan et al., 2004
Genidens genidens – guri sea catfish	0.236	nd	Brain	Oliveira et al., 2007
Paralonchurus brasiliensis – banded croaker	0.228	nd	Brain	Oliveira et al., 2007
Haemulon steindachneri – chere-chere grunt	1.035	nd	Brain	Oliveira et al., 2007
Pagrus pagrus – red porgy, common seabream	1.087	nd	Brain	Oliveira et al., 2007
Menticirrhus americanus – Southern kingcroaker	1.579	nd	Brain	Oliveira et al., 2007
Cynoscion striatus – striped weakfish	1.595	nd	Brain	Oliveira et al., 2007
Dules auriga (*Serranus auriga*)	1.624	nd	Brain	Oliveira et al., 2007
Merluccius hubbsi – Argentinean hake	3.259	nd	Brain	Oliveira et al., 2007
Percophis brasiliensis - Brazilian flathead	3.339	nd	Brain	Oliveira et al., 2007
Limanda yokohomae – Marbled sole	0.365 ± 0.16	nd	Brain	Jung et al., 2007
Limanda yokohamae – Marbled sole	0.18 ± 0.11	nd	Muscle	Jung et al., 2007
Pleuronectes vetulus - English sole	1.689 ± 0.26	0.482 ± 0.034	Muscle	Rodríguez-Fuentes et al., 2008
Pleuronichthys verticalis – hornyhead turbot	0.303 ± 0.07 (female); 0.226 ± 0.06 (male)	0.524 ± 0.032 (female); 0.120 ± 0.008 (male).	Muscle	Rodríguez-Fuentes et al., 2008
Colossoma macropomum – tambaqui	0.43 ± 0.02	0.129 ± 0.005	Brain	Assis et al., 2010
Arapaima gigas - pirarucu	0.42 ± 0.09	0.116 ± 0.002	Brain	not published results
Rachycentron canadum - cobia	0.43 ± 0.14	0.243 ± 0.02	Brain	not published results
Oreochromis niloticus – Nile tilapia	0.39 ± 0.2	0.218 ± 0.007	Brain	not published results

U = µmol of substrate hydrolyzed per minute; and nd = not determined

Table 1. Kinetic parameters of AChE from several freshwater and marine species

Scientific and common name	Optimum Temp	Optimum pH	Source	Reference
Solea solea – common sole	nd	7.5	Brain	Bocquené et al.,1990
Pleuronectes platessa – plaice	32 – 34°C	8.5	Brain	Bocquené et al.,1990
Scomber scomber – mackerel	nd	7.5 – 8.5	Brain	Bocquené et al.,1990
Lepomis macrochirus – bluegill	26 – 27°C	nd	Brain	Beauvais et al., 2002
Clarias gariepinus – African sharptooth catfish	nd	8.0	plasma	Mdegela et al., 2010
Colossoma macropomum – tambaqui	40 - 45°C	7.0 – 8.0	Brain	Assis et al., 2010
Oreochromis niloticus – Nile tilapia	35°C	8.0	Brain	not published results
Arapaima gigas - pirarucu	45°C	8.0	Brain	not published results
Rachycentron canadum - cobia	35°C	8.0	Brain	not published results

nd = not determined

Table 2. Values of optimal pH and temperature for AChE from several species of fish

Scientific and common name	Km (mM)	Vmax (U/mg protein)	Source	Reference
Oreochromis niloticus – Nile tilapia	0.033± 0.004	0.063 ± 0.001	Liver	Rodríguez-Fuentes and Gold-Bouchot, 2004
Oreochromis niloticus – Nile tilapia	0.123± 0.051	0.224 ± 0.016	Muscle	Rodríguez-Fuentes and Gold-Bouchot, 2004
Leporinus macrocephalus – piaussu	0.047	0.231 ± 0.008	Serum	Salles et al., 2006
Limanda yokohamae – Marbled sole	0.068 ± 0.35	nd	Muscle	Jung et al., 2007
Colossoma macropomum – tambaqui	1.61 ± 0.01	0.04 ± 0.001	Brain	not published results

U = µmol of substrate hydrolyzed per minute; nd + not determined.

Table 3. Kinetic parameters of BChE from several freshwater and marine species

species can present brain BChE or AChE with wider active sites. This is in accordance with Pezzementi and Chatonnet (2010), who reported atypical ChE activity in some fish species. Data about optimal pH and temperature of fish BChE are not presented here due to scarcity.

5.2. Organophosphorus and carbamates action on fish cholinesterases

OPs and CBs act by phosphorylating or carbamoylating the serine residue at the active site of the ChEs. Their structures present either similarities to the substrates or their hydrolytic intermediates and interact very slowly with the enzyme by forming stable conjugates (Quinn, 1987; Tõugu, 2001). This mechanism hinders the normal functioning of the enzyme, which cannot prevent the accumulation of the neurotransmitter in the synaptic cleft. The overstimulation caused by acetylcholine continuously firing its receptors generates a range of signs and symptoms. Because of their low environmental persistence and high toxicity, particularly to aquatic organisms, water must be continuously monitored (Beauvais et al., 2002).

Environmental monitoring may be chemical and/or biological. Chemical monitoring is the set of chemical analysis that quantify waste contaminants in a compartment or environmental matrix in a temporal or spatial scale. On the other hand, when the focus is to determine the magnitude of the effects of this contamination on organisms at individual or population level, biological monitoring is adopted (Henriquez Pérez and Sánchez-Hernández, 2003). The combined use of chemical and biological approaches in environmental monitoring is an important task for the assessment of contamination and its effects on an ecosystem. This is the basis of the concept of bioindicators.

In this scenario, when determining chemical characteristics of pollutants and their concentrations, organisms and their biomolecules represent a useful choice as bioindicators, since they afford to employ both the chemical and the biological approaches in environmental biomonitoring. Moreover, they also allow estimating the impact of these pollutants to such species that provide the target molecules (Wijesuriya and Rechnitz, 1993; Watson and Mutti, 2003). Among these compounds, enzymes play an important role due to their degree of specificity and fast response to relevant changes in the surrounding medium. The use of enzymes as bioindicators is based on the inhibition or negative interference in catalytic activity triggered by analytes (Marco and Barceló, 1996). Cholinesterase inhibition has been used as biomarker of organophosphorus and carbamate exposure. AChE is one of the oldest environmental biomarkers (Payne et al., 1996).

In general, the higher the concentration of pesticides and longer exposure time, the greater are the negative impacts, since these are the conditions when higher levels of biological organization, such as communities and ecosystems, are affected by pesticides. The effects of contaminants on low levels of biological organization (e. g., molecular and biochemical responses) occur more quickly, and the specificity of responses is generally higher. The effects on such levels can be directly related to exposure to pollutants. The presence of chemical residues and metabolites is a direct indicator of the availability of contaminants to organisms (Arias et al., 2003). In the monitoring of pesticides and other contaminants in water resources, several techniques that use organisms as bioindicators have been developed, either by estimation of population density and behavioral changes or by assessment of physiological characteristics of these organisms that make them sensitive to certain pollutants. These organisms are chosen based on features like habitat, ecology, food habits, species abundance and ease of capture (Henríquez Pérez and Sánchez-Hernández, 2003). There are two main approaches: 1) The *in vivo* approach, which exposes live specimens to the analyzed substance and collect tissues for analysis after the exposure period and 2) the *in vitro* approach, which exposes tissues or purified biomolecules directly to the analytes.

Each technique has its own advantages. In the first approach, the slow interaction between enzyme and pesticides is behind the ability ChEs has to signal inhibition several days or weeks after exposure, even when the concentrations in the water are negligible. On the other hand, the *in vitro* approach makes it possible to gain more precision in the correlation between pesticide concentrations and the resulting inhibition. In addition, the *in vitro* conditions avoid the contact between pesticides and the detoxificant complex of other tissues, allowing the use of target cholinesterases enzymes as biocomponents in electrochemical and optical devices and increasing the accuracy of data acquisition in biosensors.

In the aquatic environment pesticides and other xenobiotics can attach to suspended matter, sediments in bed of water body or be absorbed by the aquatic organisms where they undergo detoxification or bioaccumulation (Nimmo, 1985). Thus, AChE from aquatic organisms has been used due to its ability to assess the environmental impact when these compounds are not present in the water (Morgan et al., 1990; Sturm et al., 1999; Ferrari et al., 2004; Wijeyaratne and Pathiratne, 2006). Among these organisms are fish (Rodríguez-Fuentes and Gold-Bouchot, 2000; Fulton and Key, 2001; Oliveira et al, 2007; Rodríguez-Fuentes, Armstrong and Schlenk, 2008). Fish are part of ecosystems that are constantly affected by pollution from various sources, including crop fields and their pesticides and fertilizers. They occupy intermediate or higher positions in their food chains, thus undergoing accumulation of xenobiotics in their tissues and becoming a feasible alternative for environmental biomonitoring. Though it is unlikely that significant amounts of organophosphorus compounds could persist after the digestion and therefore be stored successively by higher members of the food chain, the position in the chain can influence strongly the pesticide bioaccumulation (Flint and Van der Bosch, 1981). And though the persistence of OPs in the environment is relatively short, residual life of some OP pesticides such as leptophos and fenamiphos is longer. Moreover, in general OPs may have their half-lives extended multiple times in acidic pH (WHO/IPCS/INCHEM, 1986a).

There is a lack of specificity in cholinesterase inhibition by pesticides. Several compounds are capable of inhibit them in a manner almost indistinguishable at first sight. However, such substances show different patterns of enzyme inhibition represented by time for covalent binding and type or duration of recovery. Some anticholinesterasic pesticides can interact with both active and allosteric sites of the enzyme expressing mixed inhibition mechanisms.

ChE inhibition by OP compounds follows different behaviors depending on pesticide chemical structure. OP compounds include esters, amides or thiol derivatives of phosphoric, phosphonic, phosphorotioic or phosphonotioic acids (WHO/IPCS/INCHEM, 1986a). As for the phosphoester moiety, two main groups of organophosphorus pesticides are present, the phosphate group (oxon form; P=O) and the phosphorothioate group (thion form; P=S). The first exerts direct inhibition, due to the greater electronegativity of oxygen in relation to sulphur when interacting in the active domain of the enzyme. The second group is less toxic and requires biotransformation to their oxo-analogues to become biologically active. This biotransformation occurs by oxidative desulfuration mediated by cytochrome P450 (CYP450) isoforms and flavin-containing mono-oxigenase enzymes, by N-oxidation and S-oxidation (WHO/IPCS/INCHEM, 1986a; Vale, 1998). The second group is synthesized in this form in order to resist the environmental factors and to increase the residual power of the compound, since OPs, in general, present a short half-life in the environment after biotransformation.

OPs effects can also be divided in terms of the kind of phosphorylation that takes place in the active site. Most of these pesticides contain two methyl or two ethyl (less often isopropyl) ester groups bonded to the phosphorus atom (Table 4). Depending on their structure, they can dimethyl- or diethyl-phosphorylate the serine hydroxyl group in the active center. After the release of the leaving group, dimethyl-phospho-ChE can be spontaneously reactivated slowly (starting from 0.7 hours) while diethyl-phosphoenzymes can recover their activity spontaneously in 31 hours. However, in diethyl OP compounds this recovery occurs in a minor fraction of the enzyme and this fraction can be reinhibited so that it is necessary to use oximes or other reactivation agents. On the other hand, diisopropyl-phospho-ChE has no measurable recovery (WHO/IPCS/INCHEM, 1986a; Vale, 1998; Eddlestone, 2002; Paudyal, 2008). It means that diethyl and diisopropyl-organophosphorus are able to inhibit the enzyme in long term.

Dimethyl OP	Diethyl OP	Diisopropyl OP
Dichlorvos	Diazinon	Diisopropyl fluorophosphates (DFP)
Temephos	Chlorpyrifos	Diisopropyl methylphosphonate (DIMP)
Methyl parathion	Tetraethyl pyrophosphate (TEPP)	
Malathion	Parathion	
Fenthion	Coumaphos	
Dimethoate	Sulfotepp	
Methamidophos	Ethion	

Table 4. Examples of organophosphorus pesticides according to ester groups bonded to phosphorus atom

Another feature of the interaction of OP compounds with the tissues is that most of them are lipophilic. According to Vale (1998), they are rapidly absorbed and accumulated in fat, liver, kidneys and salivary glands. Phosphorothioate compounds are more lipophilic than phosphates (Table 5).

More lipophilic	Less lipophilic
Chlorpyrifos, Diazinon, Temephos, Malathion, Parathion, Methyl-Parathion, Fenthion, Coumaphos, Dimethoate, Ethion, Sulfotepp	Tetraethyl pyrophosphate (TEPP), Trichlorfon, Dichlorvos, Methamidophos, Fenamiphos, Phosphamidon, Monocrotophos

Table 5. Examples of organophosphorus pesticides according to the lipophilicity

The loss of an alkyl group from the phosphoester bond in the enzyme-OP complex leads to the so-called aging process, which is time dependent. This process is mainly influenced by type of OP compound, pH and temperature. Since dimethyl OPs present less time for recovery, its aging half life is also short (3.7 hours). On the other hand, for diethyl OPs long time for recovery implies a longer aging half life, which may be up to 33 hours (Worek et al., 1997; Worek et al., 1999).

Oximes are nucleophilic agents which present more affinity for the OP molecules than the active center of cholinesterases. They catalyze the reactivation of enzyme and decrease the availability of enzymes subjected to the process of aging (Eddlestone, 2002). After aging, the

enzyme is not responsive to oximes treatment. Wilson (1951) reported reactivation of tetraethyl pyrophosphate-inhibited AChE by choline and hydroxylamine.

Some organophosphorus coumarinic compounds such as haloxon and coroxon present a type of inhibition which acts by phosphorylating the active site of AChE, concomitantly interacting with the peripheral site responsible for the inhibition by substrate excess. Despite being a more efficient inhibitor for BChE, haloxon and its analogues display unusual inhibition kinetics for AChE (Aldridge and Reiner, 1969).

CB pesticides are *N*-substituted esters of carbamic acid capable of readily inhibiting cholinesterases without metabolic activation, so they can induce acute toxicity effects faster than most of OP compounds. Although most CBs are not very stable in aquatic environments, some are soluble in water and can bioaccumulate in trophic levels, being particularly toxic to fish because they are metabolized slowly in such animals (Vassilieff and Ecobichon, 1982). Compared to OP compounds, CBs require larger doses to produce mortality or poisoning symptoms, because they do not bind to cholinesterases as stable as OP and do not promote aging. The half life of carbamoylated cholinesterases ranges from 0.03 to 4 h, depending on the compound (WHO/IPCS/INCHEM, 1986b).

There are two main reasons to use fish cholinesterase as biomarker. The first concerns the availability of this source: in 2009, the world fisheries and aquaculture production was 145.1 million tones, and most of the fish waste reused comes from tissues other than those that provide ChEs (FAO, 2010). Moreover, studies found very high AChE concentrations in the electric organs of the ray *Torpedo marmorata* and the eel *Electrophorus electricus* (Nachmansohn and Lederer, 1939; Leuzinger and Baker, 1967). Up to now the electric organs of *Torpedo* rays and *Electrophorus* eels (actually, they are Gymnotiformes, closer to knifefish than true eels) are still considered the most abundant source of this enzyme. These tissues are composed of structural units called electrocytes, electroplaques or electroplax, which consist in thin, flat plates of modified muscle that assemble as two large, wafer-like, roughly circular or rectangular surfaces. Each single *E. electricus* electroplaque generates a small charge because they present a potential difference of 100 mV. However, when they are piled in rows as a Voltaic pile (the arrangement in its body) they can generate a potential of approximately 600 V since there are from 5,000 to 6,000 electroplaques in its electric organ, which constitutes around 4/5 of its length. The sensitivity of fish ChEs under OP and CB exposure can be seen in tables 6, 7 and 8, which shows some differences between species *in vitro* and *in vivo*.

When measuring cholinesterases activity and inhibition, numerous differences between methodologies and laboratories become apparent, and many concerns rouse about what could be a normal level of activity for each species (Fairbrother and Bennet, 1988). In order to address these differences, some studies expressed results in terms of percentage of residual activity (Cunha Bastos et al., 1999; Villatte et al., 2002; Assis et al., 2007; Assis et al., 2010) or percentage of inhibition. According to the Food and Agriculture Organization (2007), 20% inhibition of brain AChE activity is considered the endpoint to identify the no-observed-adverse-effect-level (NOAEL) in organisms, while signs and symptoms appear when AChE is inhibited by 50% or more. Death occurs above 90% inhibition.

The most used assay for ChE activity is the Ellman method (1961). It consists in a dye-binding reaction occurring when the chromogenic reagent DTNB joins the choline or thiocholine moieties released after cholinesterases substrates breakdown. Over the years, the assay has been improved by the contribution of several works and some will be listed here.

Species	IC_{50} (µmol/L)	Ki (µmol/L)	Source	Reference
ORGANOPHOSPHATE				
Azinphos ethyl				
Cyprinus carpio	34.6	-	Muscle	Sato et al., 2007
Azinphos methyl				
Cyprinus carpio	53.7	-	Muscle	Sato et al., 2007
Chlorpyrifos				
Cyprinus carpio	810	-	Brain	Dembélé et al., 2000
Colossoma macropomum	7.6	2.61×10^{-2}	Brain	Assis et al., 2010
Arapaima gigas	7.87	2.69×10^{-2}	Brain	not published results
Rachycentron canadum	30.24	5.94×10^{-2}	Brain	not published results
Oreochromis niloticus	26.78	0.161	Brain	not published results
*Electrophorus electricus***	0.03	2.18×10^{-4}	Electric organ	not published results
Chlorpyrifos-oxon				
Gambusia affinis	0.05	-	Brain	Boone and Chambers, 1997
Gambusia affinis	0.006	-	Muscle	Boone and Chambers, 1997
Chlorpyrifos ethyl				
Cyprinus carpio	9.12	-	Muscle	Sato et al., 2007
Chlorpyrifos methyl				
Cyprinus carpio	35.48	-	Muscle	Sato et al., 2007
Chlorfenvinfos				
Cyprinus carpio	19	-	Brain	Dembélé et al., 2000
Clarias gariepinus	0.03	-	Brain	Mdegela et al., 2010
DEP				
Cyprinus carpio	12.02	-	Muscle	Sato et al., 2007
Diazinon				
Pimephales promelas	5000	-	Muscle	Olson and Christensen, 1980
Oncorhynchus mykiss	2.5	-	Brain	Keizer et al., 1995
Danio rerio	20.0	-	Brain	Keizer et al., 1995
Poecilia reticulate	7.5	-	Brain	Keizer et al., 1995
Cyprinus carpio	0.2	-	Brain	Keizer et al., 1995
Cyprinus carpio	19	-	Brain	Dembélé et al., 2000
Cyprinus carpio	2.95	-	Muscle	Sato et al., 2007
Clarias gariepinus	0.15	-	Brain	Mdegela et al., 2010
Colossoma macropomum	-	-	Brain	Assis et al., 2010
Arapaima gigas	1500	5.13	Brain	not published results
Rachycentron canadum	-	-	Brain	not published results
Oreochromis niloticus	-	-	Brain	not published results
*Electrophorus electricus***	0.3	2.18×10^{-3}	Electric organ	not published results
Diazoxon				
Cyprinus carpio	0.019	-	Muscle	Sato et al., 2007

Species	IC_{50} (µmol/L)	Ki (µmol/L)	Source	Reference
Dichlorvos				
Alburnus alburnus	0.63	-	Brain	Chuiko, 2000
Leuciscus idus	0.31	-	Brain	Chuiko, 2000
Esox lucius	0.31	-	Brain	Chuiko, 2000
Dicentrarchus labrax	33.4	-	Brain	Varò et al., 2003
Dicentrarchus labrax	44.8	-	Muscle	Varò et al., 2003
Cyprinus carpio	1.78	-	Muscle	Sato et al., 2007
Colossoma macroponum	0.04	1.37×10^{-4}	Brain	Assis et al., 2010
Arapaima gigas	2.32	7.92×10^{-3}	Brain	not published results
Rachycentron canadum	6.9	1.36×10^{-2}	Brain	not published results
Oreochromis niloticus	5.4	3.26×10^{-2}	Brain	not published results
*Electrophorus electricus***	0.16	1.16×10^{-3}	Electric organ	not published results
Dimethoate				
Clarias gariepinus	190	-	Brain	Mdegela et al., 2010
EPN oxon				
Cyprinus carpio	0.055	-	Muscle	Sato et al., 2007
Ethoprofos				
Cyprinus carpio	37.15	-	Muscle	Sato et al., 2007
Fenitrothion				
Clarias gariepinus	0.2	-	Brain	Mdegela et al., 2010
Iprobenfos				
Limanda yokohamae	1.11	-	Muscle	Jung et al., 2007
Isoxathion oxon				
Cyprinus carpio	0.00068	-	Muscle	Sato et al., 2007
Leptophos				
Cyprinus carpio	26.02	-	Muscle	Sato et al., 2007
Malaoxon				
Pimephales promelas	18	-	Muscle	Olson and Christensen, 1980
Oreochromis niloticus	0.02	-	Brain	Pathiratne and George, 1998
Pseudorasbora parva	0.81	-	Brain	Shaonan et al., 2004
Carassius auratus	0.76	-	Brain	Shaonan et al., 2004
Oncorhynchus mykiss	0.34	-	Brain	Shaonan et al., 2004
Cyprinus carpio	0.049	-	Muscle	Sato et al., 2007
Malathion				
Pimephales promelas	5700	-	Muscle	Olson and Christensen, 1980
Oreochromis niloticus	1000	-	Brain	Pathiratne and George, 1998
Cyprinus carpio	169.8	-	Muscle	Sato et al., 2007
MEP oxon				
Cyprinus carpio	2.14	-	Muscle	Sato et al., 2007

Species	IC_{50} (µmol/L)	Ki (µmol/L)	Source	Reference
Monocrotophos				
Sciaenops ocellatus	0.72	-	Brain	Ru et al., 2003
Paraoxon				
Gambusia affinis	0.27	-	Brain	Boone and Chambers, 1997
Gambusia affinis	0.06	-	Muscle	Boone and Chambers, 1997
Paraoxon ethyl				
Cyprinus carpio	0.14	-	Muscle	Sato et al., 2007
Paraoxon methyl				
Gambusia affinis	8.4	-	Brain	Boone and Chambers, 1997
Gambusia affinis	0.54	-	Muscle	Boone and Chambers, 1997
Cyprinus carpio	0.60	-	Muscle	Sato et al., 2007
Genidens genidens	0.45	-	Brain	Oliveira et al., 2007
Paralonchurus brasiliensis	0.47	-	Brain	Oliveira et al., 2007
Haemulon steindachneri	0.27	-	Brain	Oliveira et al., 2007
Pagrus pagrus	0.12	-	Brain	Oliveira et al., 2007
Menticirrhus americanus	0.29	-	Brain	Oliveira et al., 2007
Cynoscion striatu	0.21	-	Brain	Oliveira et al., 2007
Dules auriga	0.16	-	Brain	Oliveira et al., 2007
Merluccius hubbsi	0.11	-	Brain	Oliveira et al., 2007
Percophis brasiliensis	0.10	-	Brain	Oliveira et al., 2007
Parathion ethyl				
Cyprinus carpio	380	-	Muscle	Sato et al., 2007
Parathion methyl				
Cyprinus carpio	602.5	-	Muscle	Sato et al., 2007
Phoxim				
Cyprinus carpio	3.80	-	Muscle	Sato et al., 2007
Pirimiphos methyl				
Clarias gariepinus	0.003	-	Brain	Mdegela et al., 2010
Profenofos				
Clarias gariepinus	0.002	-	Brain	Mdegela et al., 2010
Temephos				
Colossoma macropomum	ne	-	Brain	Assis et al., 2010
Arapaima gigas	ne	-	Brain	not published results
Rachycentron canadum	ne	-	Brain	not published results
Oreochromis niloticus	ne	-	Brain	not published results
*Electrophorus electricus***	7.6	5.51×10^{-2}	Electric organ	not published results
TEPP				
Colossoma macropomum	3.7	1.27×10^{-2}	Brain	Assis et al., 2010
Arapaima gigas	0.009	3.07×10^{-5}	Brain	not published results

Species	IC_{50} (µmol/L)	Ki (µmol/L)	Source	Reference
Rachycentron canadum	8.1	1.59×10^{-2}	Brain	not published results
Oreochromis niloticus	20.75	0.125	Brain	not published results
*Electrophorus electricus***	0.06	4.35×10^{-4}	Electric organ	not published results
Triazophos oxon				
Pseudorasbora parva	0.13	-	Brain	Shaonan et al., 2004
Carassius auratus	0.16	-	Brain	Shaonan et al., 2004
Oncorhynchus mykiss	0.042	-	Brain	Shaonan et al., 2004
CARBAMATES				
BPMC				
Cyprinus carpio	0.76	-	Muscle	Sato et al., 2007
Carbaryl				
Pimephales promelas	10.0	-	Muscle	Olson and Christensen, 1980
Colossoma macropomum	33.8	0.116	Brain	Assis et al., 2010
Arapaima gigas	12.25	4.18×10^{-2}	Brain	not published results
Rachycentron canadum	8.31	1.63×10^{-2}	Brain	not published results
Oreochromis niloticus	9.2	5.55×10^{-2}	Brain	not published results
Electrophorus electricus	0.6	-	Electric organ	Tham et al., 2009
Clarias batrachus	0.59	-	Muscle	Tham et al., 2009
Clarias gariepinus	0.003	-	Brain	Mdegela et al., 2010
Carbofuran				
Cyprinus carpio	0.45	-	Brain	Dembélé et al., 2000
Colossoma macropomum	0.92	3.15×10^{-3}	Brain	Assis et al., 2010
Arapaima gigas	0.75	2.56×10^{-3}	Brain	not published results
Rachycentron canadum	0.082	1.61×10^{-4}	Brain	not published results
Oreochromis niloticus	0.19	1.15×10^{-3}	Brain	not published results
*Electrophorus electricus***	0.005	3.63×10^{-5}	Electric organ	not published results
Electrophorus electricus	0.02	-	Electric organ	Tham et al., 2009
Clarias batrachus	0.03	-	Muscle	Tham et al., 2009
MPMC				
Cyprinus carpio	0.98	-	Muscle	Sato et al., 2007
MTMC				
Cyprinus carpio	3.89	-	Muscle	Sato et al., 2007
NAC				
Cyprinus carpio	0.93	-	Muscle	Sato et al., 2007
PHC				
Cyprinus carpio	0.95	-	Muscle	Sato et al., 2007
XMC				
Cyprinus carpio	2.24	-	Muscle	Sato et al., 2007

ne – negligible effect.

Table 6. Pesticide IC_{50} and Ki* values for *in vitro* AChE from freshwater and marine fish.

Species	IC_{50} (µmol/L)	Source	Reference
Dichlorvos			
Alburnus alburnus	0.0063	Serum	Chuiko, 2000
Leuciscus idus	0.0016	Serum	Chuiko, 2000
Abramis ballerus	0.0008	Serum	Chuiko, 2000
Abramis brama	0.001	Serum	Chuiko, 2000
Rutilus rutilus	0.0016	Serum	Chuiko, 2000
Blicca bjoerkna	0.0008	Serum	Chuiko, 2000
Iprobenfos			
Limanda yokohamae	0.306	Muscle	Jung et al., 2007
Malathion			
Ictalurus furcatus	31	Liver	Aker et al., 2008
Ictalurus furcatus	50.2	Muscle	Aker et al., 2008
Parathion			
Gasterosteus aculeatus	0.00343a	Liver	Wogram et al., 2001
Gasterosteus aculeatus	0.00343b	Muscle	Wogram et al., 2001
Gasterosteus aculeatus	0.00343c	Gills	Wogram et al., 2001

a – 60% inhibition; b – 30% inhibition; c – 30% inhibition.

Table 7. Pesticide IC_{50} and Ki* values for *in vitro* BChE from freshwater and marine fish.

Species	Inhibition report	Source	Reference
ORGANOPHOSPHATES			
Azinphos methyl			
Sparus aurata	IC_{50} 72h - 0.0096 µM	Larvae	Arufe et al., 2007
Chlorpyrifos			
Oreochromis mossambicus	LC_{50} 96h – 0.07 µM Caused 88% inhibition in brain and gill	Brain and gill	Rao et al., 2003
Gambusia yucatana	0.43 µM 96h inhibited 80 and 50% (muscle and head, respectively)	Muscle and head	Rendón-von Osten et al., 2005
Oreochromis niloticus	IC_{50} 48 h - 0.011 µM	Brain	Chandrasekara and Pathiratne, 2007
Chlorpyrifos methyl			
Poecilia reticulate	LC50 96 h – 4.89 µM	-	Selvi et al., 2005
Diazinon			
Micropterus salmoides	295 µM 24h - 48.2%	Brain	Pan and Dutta, 1998
Cyprinus carpio	LC_{50} 96h for larvae –	Embryos and	Aydin and

Species	Inhibition report	Source	Reference
	5.03 µM and for embryos – 3.25 µM	larvae	Köprücü, 2005
Oreochromis niloticus	67% inhibition at 0.33 µM on the first day	Muscle	Durmaz et al., 2006
Oreochromis niloticus	3.3 µM - 62,5% inhibition after 24h	Brain	Üner etal., 2006
Cyprinus carpio	55.51% inhibition at 0.00012 µM after 5 days	Muscle, gill and kidney	Oruç and Usta, 2007
Dichlorvos			
Dicentrarchus labrax	LC_{50} 96h – 15.83 µM	Fingerling	Varò et al., 2003
Sparus aurata	0.23 µM 24h - 40.95% inhibition	Fingerling brain + dorsal muscle	Varò et al., 2007
Malathion			
Oreochromis niloticus	LC_{50} 96h – 6.66 µM	Brain	Pathiratne and George, 1998
Monocrotophos			
Oreochromis mossambicus	LC_{50} 96h – 51.5 µM This concentration caused 79 (brain), 89 (gill) and 43.8% (muscle) inhibition, in 24h exposure	Brain, gill and muscle	Rao, 2004
Oreochromis mossambicus	1/10 LC_{50} 96h caused 21 (liver), 40 (brain) and 28.6% (gill) inhibition in 24h exposure	Brain, liver and gill	Rao., 2006a
Parathion			
Danio rerio	0.0007 µM after 142 days inhibited 27.4%	Whole fish	Roex et al., 2003
RPR-II			
Oreochromis mossambicus	LC_{50} 96h – 0.75 µM This concentration caused 58 (brain), 90.2 (gill) and 68.5% (muscle) inhibition, in 24h exposure	Brain, gill and muscle	Rao., 2004
Oreochromis mossambicus	1/10 LC_{50} 96h caused approx. 33 (brain), 57 (gill) and 43% (muscle) inhibition, in 72h exposure	Brain, gill and muscle	Rao., 2006c
RPR-V			
Oreochromis mossambicus	LC_{50} 96h – 0.78 µM	Brain, gill and	Rao., 2004

Species	Inhibition report	Source	Reference
	This concentration caused 70.6 (brain), 86.3 (gill) and 54.8% (muscle) inhibition, in 24h exposure	muscle	
Oreochromis mossambicus	1/10 LC_{50} 96h caused approx. 30 (brain), 50 (gill) and 36% (muscle) inhibition, in 72h exposure	Brain, gill and muscle	Rao., 2006c
Temephos			
Oreochromis niloticus	ne	Head	Antwi, 1987
Sarotherodon galilaea	ne	Head	Antwi, 1987
Alestes nurse (*Brycinus nurse*)	ne	Head	Antwi, 1987
Schilbe mystus	ne	Head	Antwi, 1987
Trichlofon			
Cyprinus carpio	0.97 µM 24h - 52% inhibition	Brain	Chandrasekara and Pathiratne, 2005
Oreochromis niloticus	0.97 µM 8h - 73,6% inhibition	Axial muscle	Guimarães et al., 2007
CARBAMATES			
Aldicarb			
Danio rerio	LC_{50} 96h – 52.9 µM	-	Gallo et al., 1995
Poecilia reticulata	LC_{50} 96h – 3.5 µM	-	Gallo et al., 1995
Carbaryl			
Oncorhynchus mykiss	1.24 µM 96h inhibited 60.8%	Brain	Zinckl et al., 1987
Danio rerio	LC_{50} 96h – 46 µM	-	Gallo et al., 1995
Poecilia reticulata	LC_{50} 96h – 12.5 µM	-	Gallo et al., 1995
Oncorhynchus mykiss	3.72 µM 96h inhibited 50%	Larvae	Beauvais et al., 2001
Oncorhynchus mykiss	EC_{50} 96h – 0.095 µM	Brain and muscle	Ferrari et al., 2007
Carbofuran			
Oreochromis niloticus	LC_{50} 24h – 1.13 µM 96h – 2.17 µM	-	Stephenson et al., 1984
Carassius auratus	0.22 µM 48h inhibited 28% (brain) and 2.26 µM 48h inhibited 92% (muscle)	Brain and muscle	Bretaud et al., 1999
Gambusia yucatana	1.13 µM 24h	Muscle and	Rendón-von Osten

Species	Inhibition report	Source	Reference
	inhibited 50 and 30% (muscle and head, respectively)	head	et al., 2005
Tinca tinca	60% inhibition after 20 days of exposure of *Tinca tinca* to carbofuran at 0.1 µg/mL	Brain	Hernández-Moreno et al., 2010

ne – negligible effect.

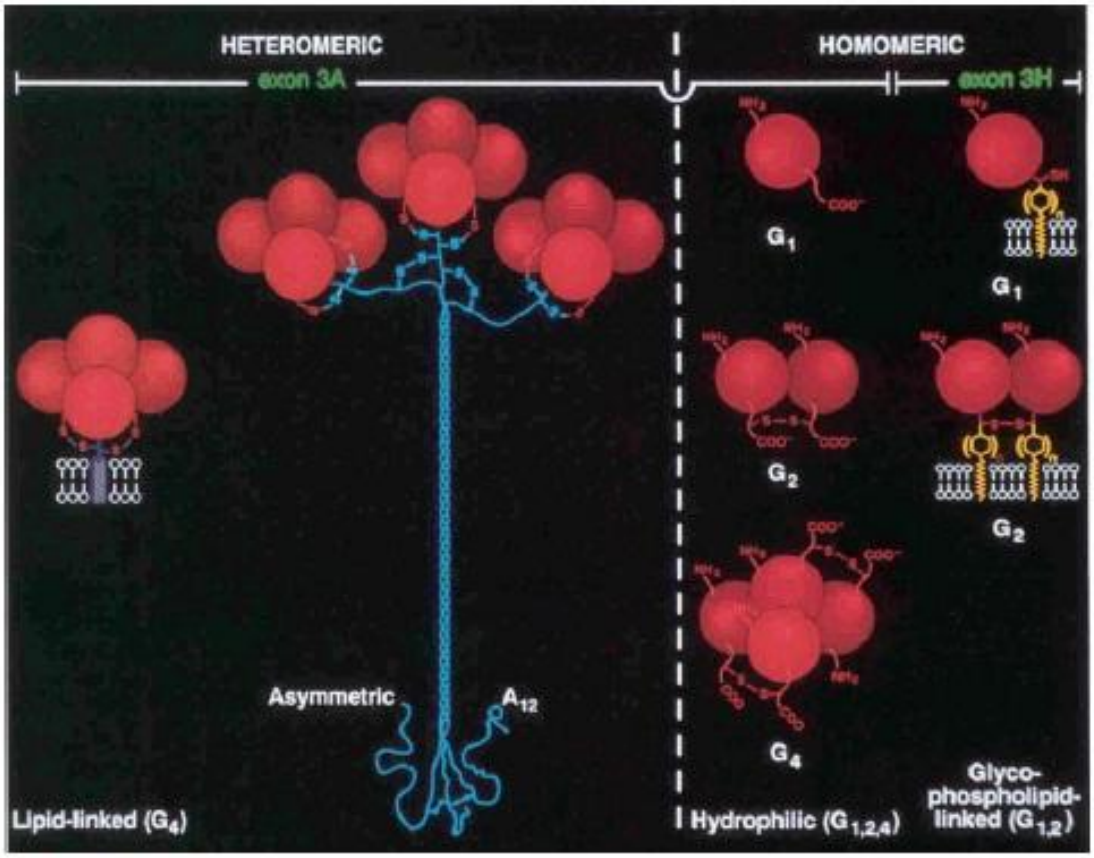
Table 8. Pesticide inhibition for *in vivo* AChE from freshwater and marine fish.

In 1960, Blaber and Creasey used ethopropazine in crude extract to prevent BChE activity when measuring AChE recovery *in vivo* (control with ethopropazine inhibited AChE by 13.7%, while BChE was inhibited by 91.5%).

Ions can alter cholinesterase activity inhibiting or activating so that some authors even propose the enzymes as biomarkers of heavy metals and other pollutants (Abou-Donia and Menzel, 1967; Mukherjee and Bhattacharya, 1974; Olson and Christensen, 1980; Tomlinson et al., 1981; Hughes and Bennett, 1985; Gill et al., 1990; 1991; Payne et al., 1996; Devi et al., 1996; Najimi, 1997; Reddy et al., 2003). This fact is not always taken into account during the use of cholinesterases as biomarkers of pesticides and can lead to false positives or negatives and misinterpretation of results. Tomlinson et al. (1981) described that activation by ions is only observed in conditions of low ionic strength, while inhibition can be noted in both low and high ionic strength.

Thus, heavy metals and ions can be present in samples of environmental matrices, as well as in food samples. Also, they are important interfering components in pesticide analysis using cholinesterases, since some of them are inhibitors or positive effectors. Nevertheless, the use of non-inhibitor chelating agents and ions with protecting enzyme activity effect could overcome these interferences.

Bocquené, Galgani and Truquet (1990) found that Tris buffer was the best extractor for fish AChE. Najimi and coworkers (1997) reported that using Tris the increasing doses of heavy metals resulted in different AChE activities though such result was not observed with phosphate buffer. It could be concluded that phosphate is the best buffer for pesticide assays and that Tris is the best alternative for heavy metals assays. However, Tomlinson et al. (1981) reported that EDTA has a protective action against divalent metallic cations which can cause some interference. Chatonnet and Lockridge (1989) reviewed cholinesterases and reported the different extracting strategies caused by ChEs molecular polymorphism: the globular forms G1, G2 and G4 are extractable in low ionic strength buffers (G2 glycophospholipid-linked is the form found in erythrocytes and in *Torpedo* electric organ, while G4 lipid-linked is present in vertebrates brain). The globular forms tightly bound to membranes require detergent for solubilization. Asymmetric forms (found mainly in vertebrate muscle and in some electric organs) are solubilized with buffers with high salt concentration. These forms contain tetrameric subunits (A4, A8 and A12) attached by disulphide bonds to a collagen-like tail (Figure 1).



Source: Taylor (1991)

Fig. 1. Molecular polymorphism of cholinesterases

Working with brain AChE, Ho and Ellman (1969) were able to solubilize the enzyme using triton X-100 and treatment with proteases. Nevertheless, in cholinesterase assays with pesticides, triton X-100 interacts with OP (oxon-form) and CB compounds or influences its rate of AChE inhibition (Marcel et al., 2000; Rosenfeld, Kousba and Sultatos, 2001).

For pesticides with larger acyl chains or higher lipophilic characteristic (for which only a small fraction reaches the target tissues), BChE can be more sensitive than AChE. The use of BChE offers some advantages, such as the facilitated plasma (its main source) separation from the other blood components and the possibility to collect samples without killing specimens. Furthermore, several studies have tried, with some success, to establish sharp correlations between inhibition in blood cholinesterases and in peripheral and central nerve tissues cholinesterases (Pope et al., 1991; Pope and Chakraborti, 1992; Chauldhuri et al., 1993; Padilla et al., 1994). Padilla (1995) working with paraoxon and chlorpyrifos, described that the strongest correlations occurred when measuring cholinesterase activity in steady-state inhibition, which is the peak inhibition time. This time depends on the inhibitor under analysis (4 hours post-dosing for paraoxon and 1-3 weeks post-dosing for chlorpyrifos).

Another concern about using fish cholinesterase as biomarker of organophosphorus and carbamate pesticides is that cyanobacterial blooms are very common in rivers, lakes and reservoirs when eutrophication raises nutrient contents in water. Some species of

cyanobacteria (*Anabaena flos-aquae* and *Anabaena lemmermannii*) produce anticholinesterasic metabolites such as anatoxin-a(s), which can be considered natural OP compounds and whose toxicity can be approximately 1000-fold higher than that of the insecticide paraoxon (Mahmood and Carmichael, 1986; Villatte et al., 2002). Moreover, cholinesterases inhibited by anatoxin-a(s) cannot be reactivated by oximes, because they are true irreversible inhibitors of these enzymes. The structure of anatoxin-a(s) resembles the shape of the organophosphorus dealkylated within the active site of the enzyme forming almost instantly an aged complex. A study obtained aged cholinesterase after 20-min incubation with this toxin (Villatte et al., 2002). However, by washing the brains before (with a solvent that does not transport it into the cells and does not affect enzymatic activity), such toxins do not interfere on *in vivo* assays using cholinesterase from this tissue, since it was observed that anatoxin-a(s) does not cross the blood-brain barrier (Cook et al., 1988; Rodríguez et al., 2006).

When comparing the use of crude extract to the use of purified enzyme, advantages and disadvantages can be observed in both, depending on the purpose. First of all, purified enzymes allow determining activity and inhibition more acutely without endogenous interfering agents. Moreover, they can be immobilized on a range of materials in particles or electrodes in order to produce electrochemical devices. Nevertheless purified enzymes require a medium to mimetize *in vivo* conditions and stabilize its activity. Besides, they are more susceptible to exogenous ions and non target compounds. The crude extract has the disadvantage of exposing the enzyme not only to the analyte. However, as mentioned before, much of OP pesticides are produced in the thion form (P=S), requiring bioactivation to reach their full toxic potential. Before biotransformation, the thion group exhibits little power of inhibition (WHO/IPCS/INCHEM, 1986a) which could hinder the correlation between pesticide concentration and ChE inhibition. Considering this, many studies use brain homogenates, since they also provide enzymatic complexes such as CYP P450 capable to transform the pesticide to its oxo-form (Mesnil, Testa and Jenner, 1984; Iscan et al., 1990; Ghersi-Egea et al., 1993).

According to Zahavi et al. (1971) and Carr and Chambers (1996), the reasons behind the species' differences in inhibitory potency has been reported to be the result of steric exclusion of the inhibitor from the active site of the enzyme. However, the difference in sensitivity between species occurs not only due to the structural diversity of inhibitors and between species cholinesterases, but also due to the balance between the activities of the detoxication complex and enzymes that promote the biotransformation of OPs. This balance can be part of enantiostatic responses to external agents which act as a device protecting against intoxication (Cunha Bastos et al., 1999; Monserrat et al., 2007).

Several attempts have been reported worldwide, in search for the best enzyme and fish source to establish methods to detect diverse organophosphorus and carbamate pesticides. In this sense, it is possible to improve monitoring protocols, obtaining data about the activation/detoxification complex of each species in use.

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6 - CAPÍTULO II – A SIMPLE ESTIMATION METHOD FOR CHARACTERS OF CATALYTIC EFFICIENCY OF BRAIN CHOLINESTERASES IN FOUR TROPICAL FISH

ARTIGO ENVIADO À REVISTA FRESHWATER BIOLOGY

A simple estimation method for characters of catalytic efficiency of brain cholinesterases in four tropical fish

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Running header: Catalytic efficiency of brain cholinesterases from four fish.

Key words: Butyrylcholinesterase, Acetylcholinesterase, Selective inhibition, Fish

A simple estimation method for characters of catalytic efficiency of brain cholinesterases in four tropical fish

Summary

1. Brain cholinesterase (ChE) content from four fish, pirarucu (*Arapaima gigas*), tambaqui (*Colossoma macropomum*), cobia (*Rachycentron canadum*) and Nile tilapia (*Oreochromis niloticus*) were characterized using the substrates acetylthiocholine, propionylthiocholine, and S-butyrylthiocholine iodide.

2. Selective inhibitors were used: BW284c51 as acetylcholinesterase (AChE) inhibitor, Iso-OMPA as butyrylcholinesterase (BChE) inhibitor and neostigmine bromide and eserine as total ChE inhibitor. By the exposure to these compounds was possible to estimate their IC_{50} and Ki for these enzymes.

3. Activation energy (AE) and rate enhancement produced by the enzymes as well as parameters of catalytic efficiency like first (Kcat, the turnover number) and second (Kcat/Km) order rate constants were estimated in crude extract through a simple methodology proposed here.

4. Despite the BChE-like activity, values of Vmax ratio and Km ratio pointed to the existence of only AChE in the brain of at least three of the species under study. Moreover, selective inhibition suggests that *C. macropomum* brain ChE present atypical activity due to different behaviour in presence of ChE selective inhibitors (the same IC_{50} and Ki in presence of BW284c51 and Iso-OMPA).

5. Data from AE showed that AChE from the species under analysis increased the rate of reaction from 10^4 to 10^7-fold while the BChE-like activity increased the rate from 10^7 to 10^{12}-fold in relation to nonenzymatic reaction. The Kcat and Kcat/Km values found were in accordance with results in literature for experimental and computational data.

6. Results from zymograms showed more than one AChE isoforms in each species and their molecular weight were, respectively, 246 and 287 kDa (*C. macropomum*); 202 and 299 kDa (*A. gigas*); 207, 218 and 244 kDa (*R. canadum*); 207, 218, 229 and 252 (*O. niloticus*).

Introduction

Currently, there are two accepted types of cholinesterases: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). The first, mainly present in nerve tissue, muscle and erythrocytes, hydrolyzes acetylcholine, while the second, present mainly in liver and plasma, hydrolyzes acetylcholine and butyrylcholine, with preference for the latter. These enzymes belong to the family of serine esterases that hydrolyze specifically choline esters and are classified as globular or asymmetric associated with lipids, glycophospholipids and collagen-like tails. The globular forms present as monomers, dimers and tetramers can be attached to the basal lamina or anchored to the cell membrane or soluble in the nervous system, muscles, plasma, erythrocytes, liver and other organs where they comes through the circulation in vertebrates and invertebrates (Massoulié and Bon, 1982; Taylor, 1991).

AChE main and classical function is modulating the nerve impulses responsible for neuronal communication by the deactivation of neurotransmitter acetylcholine in cholinergic synapses and neuromuscular junctions (Quinn, 1987; Tõugo, 2001; Silman and Sussman, 2005). BChE physiological function is not well defined although studies point to the possibility of acting in detoxification and activation of xenobiotics like succinylcholine, aspirin, cocaine, heroin, bambuterol, irinotecan and as scavenger of pesticides and other anticholinesterasic agents (Soreq and Zakut, 1990; Taylor, 1991; Çokugras, 2003; Nicolet et al., 2003) and in some cases can be more sensitive to pesticides than AChE (Chambers et al., 2002). Evidences also suggest a possible role of both enzymes in the developing nervous system, particularly in neuronal differentiation and development, cell growth, adhesion and signalling. In addition, BChE is allowed to hydrolyze acetylcholine in

early developing nervous system (Chatonnet and Lockridge, 1989; Taylor, 1991; Sternfeld et al., 1998; Brimijoin and Koenigsberg, 1999; Bigbee et al., 1999; Brimijoin, 2005; Silman and Sussman, 2005).

Moreover, AChE and BChE have many other differences: while AChE has an in vivo half-life of 120 days, BChE lasts 7-12 days. AChE is inhibited by substrate excess and BChE is activated by substrate excess (Lopez-Carillo and Lopez-Cervantes, 1993; Çokugras, 2003). AChE is selectively inhibited by DDM, caffeine, Nu1250, 62c47 and BW284c51 and BChE is selectively inhibited by DFP, percaine, isopestox, ethopropazine and Iso-OMPA (Adams and Thompson, 1948; Austin and Berry, 1953; Aldridge, 1953; Bayliss and Todrick, 1956; Chatonnet and Lockridge, 1989). BChE has a larger space in its active site which can hydrolize or be inhibited by a range of compounds. AChE has a more specific active site (Çokugras, 2003). Cholinesterases have been extensively studied for its intra and interspecific polymorphism and for being the primary targets of compounds used in agriculture, medicine, public health campaigns and chemical weapons (Antwi, 1987; Forget et al., 2002; Kellar, 2006; Chauhan et al., 2008; Ross et al., 2008).

Cholinesterase inhibition is a widely known biomarker of exposure to organophosphorus and carbamate pesticides in aquatic environments due to its sensibility to such compounds (Payne et al., 1996; Rodríguez-Fuentes and Gold-Bouchot, 2000; Fulton and Key, 2001; Rodríguez-Fuentes, Armstrong and Schlenk, 2008). These enzymes have been assayed in several aquatic organisms due to its ability to assess the environmental impact even when these compounds are not present in the water, particularly for organophosphorus binding (Sturm et al., 1999). This work aimed to characterize the cholinesterases in brain of five tropical fish as well as to provide estimation methods for catalytic efficiency parameters and rate enhancements produced by such enzymes without purification.

Methods

Materials

AChE from electric eel *Electrophorus electricus* type VI-S, acetylthiocholine iodide, S-butyrylthiocholine iodide, propionylthiocholine iodide, tetraisopropyl pyrophosphoramide (Iso-OMPA), 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51), neostigmine bromide, eserine, bovine serum albumin, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), tris (hydroxymethyl) aminomethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Was used a microplate spectrophotometer xMark™ and a pre stained broad range standards (catalog 161-0318) for electrophoresis from Bio-Rad (Hercules, CA, USA) and Amersham full-range Rainbow Molecular Weight Markers (Code: RPN800E) GE Healthcare. Laboratory mixer IKA RW-20 digital (Staufen, Germany). The juvenile specimens of *C. macropomum* (30.0 ± 4.2 cm; 512.5 ± 123.7 g), *A. gigas* (76.8 ± 8.7 cm; 4,118 ± 207.9 g) and *O. niloticus* (12.0 ± 3.0 cm; 7.9 ± 1.2 g) were supplied by the Fisheries and Aquaculture Department of Universidade Federal Rural de Pernambuco (Recife, PE, Brazil). *R. canadum* (51.67 ± 1.5 cm; 1,575 ± 329.6 g) was supplied by Aqualider Ltda. (Recife, PE, Brazil).

Enzyme Extraction

The juvenile fishes were sacrificed in ice. The brains were immediately excised, pooled and homogenized in 0.5 M Tris-HCl buffer, pH 8.0, maintaining a ratio of 20 mg of tissue per ml of buffer using a Potter-Elvehjem tissue disrupter. The homogenates were centrifuged for 10 min at 1,000 x *g* (4ºC) and the supernatants (crude extracts) were frozen at -20ºC for further assays.

Enzyme activity and protein determination

According to Assis et al. (2010). Briefly, 200 μL of 0.25 mM DTNB in 0.5 M tris-HCl buffer, pH 7.4 was added to the crude extract (20 μL), and the reaction started by adding 20 μL of 62 mM acetylthiocholine iodide (ASCh), S-butyrylthiocholine iodide (BSCh) or propionylthiocholine iodide (PSCh). Enzyme activity was determined by reading the increase in absorbance at 405 nm during 180 s. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 μM of substrate per minute. A blank was prepared with 0.5 M Tris-HCl buffer, pH 8.0 instead of crude extract sample. For kinetic parameters (Michaelis-Menten constant - Km and maximum velocity - Vmax) determination, fourteen substrate final concentrations were used ranging from 0 to 20.88 mM. Protein content was estimated according to

Sedmak and Grossberg (1978), using bovine serum albumin as the standard.

Selective inhibition

Samples of the crude extract from the species analyzed were exposed to selective inhibitors: BW284c51 for AChE, Iso-OMPA for BChE and Neostigmine bromide and eserine for total cholinesterases. The samples (10 µL) were exposed to five concentrations of each inhibitor (10 µL) during 1 h. The five concentrations ranged from 0.001 to 10 mM and each subsequent concentration was 10-fold higher than the previous one. After incubation the reaction was carried out and followed spectrophotometrically as described above. The concentration capable of inhibiting half of the enzyme activity - IC_{50} - was estimated for each inhibitor using MicroCal™ Origin® Version 8.0. The inhibition constant (Ki) of each compound for the enzymes was calculated using the Cheng and Prusoff equation (1973).

Activation energy (*AE*) *and rate enhancement produced by AChE from studied species*

The reactions were carried out under different temperatures in order to estimate the decrease of activation energy (AE, using Arrhenius plot from 15 to 35 or 45 ºC depending on the species) caused by the enzymes studied in the hydrolysis of the substrates. From these data was possible to calculate the rate enhancement promoted by the enzymes in relation to the non-enzymatic reaction using the following equation replacing AE in enzymatic conditions (and in non-enzymatic conditions using the Arrhenius plot data of blanks):

$$k = (\mathbf{k}T/h) \times e^{(-AE/R)}$$

where, *k* is the rate constant; **k** is the Boltzmann constant; T corresponds to the absolute temperature; *h* represents the Planck constant; AE is the activation energy and R is the gas constant.

Estimation of catalytic efficiency of the enzymes

The enzyme content (Et) of the extracts was estimated using a commercial *E. electricus* AChE standard curve and the same method of *Enzyme activity* topic. The activity in this standard curve was linear (r^2 = 0.994) in the range from 0.01 to 6 µg/mL (20 µL) of commercial enzyme and using 62 mM acetylthiocholine iodide (20 µL) and 0.25 mM DTNB (200 µL). The activity of the extracts was then converted to µg/mL obtaining the enzyme content. With this variable in addition to Km and Vmax was possible to estimate the first order rate constant or turnover number (Kcat) and second order rate constant (Kcat/Km) of the enzymes.

Non-denaturing gel electrophoresis and staining of AChE activity

The fractions 0-90% of ammonium sulfate from brain ChE extracts of each species were applied in a polyacrylamide gradient gel (6–10%) with 4% stacking gel, at 11 mA for approximately 2 h at 4 °C. The activity of ChEs was stained on the gel according to Karnovsky and Roots (1964). After electrophoresis, the gel was washed three times with 50 mM tris-HCl buffer, pH 7.5 and was incubated in substrate buffer solution over night at room temperature. Substrate buffer was prepared according to Mohamed et al. (2007): 50 mg of acetylthiocholine iodide dissolved in 65 mL of 100 mM tris-HCl buffer, pH 7.5, adding the following components in this sequence: 5 mL of 100 mM sodium citrate, 10 mL of 30 mM copper sulfate, 10 mL distilled water and 10 mL of 5 mM potassium ferricyanide. After overnight incubation and bands visualization, substrate buffer was replaced by 10% acetic acid. Pre stained broad range standards were used to estimate the molecular weight of the enzymes.

Results

Apparent content of BChE can be observed in the brain of *C. macropomum* in **figure 1A** that shows the higher residual activity of its extract which retained about 10% activity even at 10 mM of BW284c51, while the other extracts presented negligible activity at 1 mM. **Figure 1B** displays the results for exposition to Iso-OMPA where *C. macropomum* was the only species that presents a sharp drop in its activity at 1 mM.

By using two total ChE inhibitor, neostigmine inhibited the enzymes in a way stronger than eserine. In addition, it can be seen again the discrepant behaviour of *C. macropomum* ChE (**Figs. 1C** and **1D**) retaining almost 60% of its activity whereas the other species were fully inhibited at 0.001 mM neostigmine.

According to **Table 1**, the brain enzymes presented preference for acetylthiocholine since the Km

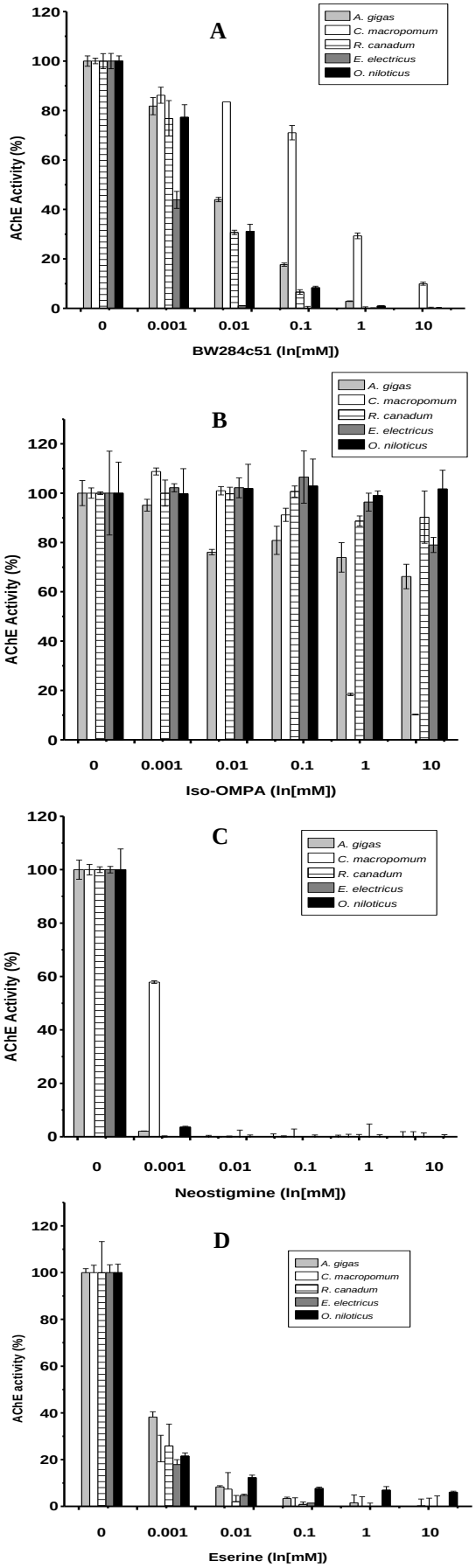

Figure 1 – Effect of A) 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51); B) Tetraisopropyl pyrophosphoramide (Iso-OMPA); C) neostigmine bromide; D) eserine on brain ChE activity from five species.

is lower for this substrate than using propionyl and butyrylthiocholine and the Vmax for acetylthiocholine is more than 2-fold for the other two. The lower Km value between the species under study was 0.37 mM (*O. niloticus* AChE activity) while the higher one was 5.14 mM (*A. gigas* BChE-like activity). The greater affinity for the substrates butyryl and propionylthiocholine was presented by the species *C. macropomum* (Km of 1.61 and 0.63 mM, respectively).

The IC_{50} and Ki related to each inhibitor are presented in **Table 2**. The values showed that, excepting *C. macropomum*, the enzymes from all species were highly susceptible to BW284c51 while Iso-OMPA did not reach 50% inhibition in the concentration range of the assays (0.001 to 10 mM). Also, it can be seen values that confirmed the stronger inhibitory activity of neostigmine comparing to eserine.

The estimation of activation energy (AE) is displayed in **Table 3** which compares the energy required to overcome the transition state between enzymatic and non enzymatic reaction. By these data was possible to estimate the rate enhancement of the reactions promoted by the enzymes. It can be seen that the higher enhancement occurred by the BChE-like activity from *A. gigas* (10^{12}) whereas the lower occurred by the AChE activity from *O. niloticus* (10^4). **Table 4** displays the Kcat and Kcat/Km of AChE activity from the species of the present work. The Kcat values ranged from 2,850 s^{-1} (*A. gigas*) to 6,500 s^{-1} (*C. macropomum*). The Kcat/Km for the same species ranged from 6.65 x 10^6 to 1.51 x 10^7 $M^{-1}s^{-1}$, respectively.

The profile of cholinesterases from the four species was displayed in non-denaturing gel where two bands in each lane can be seen for *A. gigas* and *C. macropomum* while for *E. electric*, *R. canadum* and *O. niloticus* appeared one, three and four bands, respectively (**Fig. 2**). Molecular weights of the bands were calculated considering it as isoforms of AChE (according to the results of substrate specificity - Vmax and Km ratios showed in Table 1**)** and they were, respectively, 246.24 and 287.72 kDa (*C. macropomum*); 202.65 and 299.12 kDa (*A. gigas*); 207.56, 218.05 and 244.57 kDa (*R.*

canadum); 207.56, 218.05, 229.14 and 252.95 (*O. niloticus*); 266.12 kDa (*E. electricus*).

activity described in fish (Pezzementi and Chatonnet, 2010). This species presented the same susceptibility to

Table 1 - Substrate specificity in brain ChE from five species

Substrate		*Oreochromis niloticus*	*Colossoma macropomum*	*Arapaima gigas*	*Rachycentron canadum*	*Electrophorus electricus*[a]
ASCh (A)						
Vmax	mU/mg protein	147 ± 2	129 ± 5	116 ± 2	283.5 ± 7.4	67820 ± 198*** 52290 ± 66****
Km	mM	0.37 ± 0.05	0.43 ± 0.02	0.42 ± 0.09	0.57 ± 0.12	0.45 ± 0.11*** 0.08 ± 0.1****
PSCh (P)						
Vmax	mU/mg protein	59.55 ± 3.2	63.19 ± 19.4	64.48 ± 2.12	53.07 ± 3.31	NA
Km	mM	2.90 ± 0.54	0.63 ± 0.03	1.02 ± 0.18	2.24 ± 0.56	NA
Vmax P/A		0.41	0.49	0.55	0.19	-
Km ratio P/A		7.83	1.46	2.43	3.93	-
BSCh (B)						
Vmax	mU/mg protein	NA	40.96 ± 1.23	54.4 ± 6.5	31.9 ± 10.4	NA
Km	mM	NA	1.61 ± 0.01	5.14 ± 0.18	4.23 ± 4.2	NA
Vmax B/A		-	0.32	0.47	0.11	-
Km ratio B/A		-	3.74	12.24	7.42	-

[a]Obtained from Sigma; NA – No activity; ***hyperbolae model; ****two-site binding model

Discussion

Many studies have described negligible or no BChE activity in brain of fish (Varò et al., 2003; Varò et al., 2007; Jung et al., 2007), markedly in percids, esocids, cichlids and few cyprinids like *Cyprinus carpio*, *Carassius carassius*, *Pelecus cultratus* and *Danio rerio* (Chuiko et al., 2003; Rodríguez-Fuentes and Gold-Bouchot, 2004; Pezzementi and Chatonnet, 2010). However, in the present work, some species like *C. macropomum* and *A. gigas* seems to present a BChE-like activity in brain as can be seen in some of the results.

The behaviour of *C. macropomum* brain ChE may be in accordance with atypical cholinesterase the selective inhibitors BW284c51 and Iso-OMPA (IC_{50} of 318.4 µM and Ki of 2.19 µM). Such feature could represent a structurally different AChE to accommodate compounds with greater carbon chains like Iso-OMPA in its active center. Rodríguez-Fuentes and Gold-Bouchot (2004) also found equal sensitivity to the selective inhibitors BW284c51 and Iso-OMPA in ChE from liver and muscle of *Oreochromis niloticus*.

To characterize ChE, some studies used the kinetic parameters Vmax ratio (also known as relative efficiency of hydrolysis – REH) and Km ratio for acetyl and butyrylcholine (and their analogues) hydrolysis by the enzymes. According to the expected values for these

ratios, AChE has a low Vmax ratio and a Km ratio ≥ 1, because it presents excess substrate inhibition. BChE does not show this feature, its Vmax ratio is ≥ 1, and Km ratio < 1 (Pezzementi et al., 1991; Rodríguez-Fuentes and Gold-Bouchot, 2004). Thus, the values found here point for considering the bands as AChE isoforms since, excepting *O. niloticus* and *E. electricus*, the values of Vmax ratio in all other cases (species and substrates) were lower (< 0) while Km ratios in the same cases were > 0. Only *C. macropomum* enzyme affinity could be considered close to a propionylcholinesterase (PChE). However, the existence of PChE was not completely proved and its EC number is the same of BChE (3.1.1.8). In animals, the closest ChE to PChE seems to be the *Drosophyla* ChE whose active center was described as intermediary between the center of AChE and BChE (Gnagey et al., 1987) and the ChEs from the molluscs *Limnaea stagnalis* (Grigorieva, 1973) and *Murex brandaris* (Talesa et al., 1990).

Table 2 – Selective inhibitors* IC50 and Ki** values for *in vitro* freshwater and marine fish in the present work.

Species	IC50 (µM)	Ki (µM)
BW284c51		
Colossoma macropomum	318.4	2.19
Arapaima gigas	7.52	0.05
Rachycentron canadum	3.96	0.045
Oreochromis niloticus	4.12	0.02
Electrophorus electricus▲	0.85	0.006
Iso-OMPA		
Colossoma macropomum	318.4	2.19
Arapaima gigas	-	-
Rachycentron canadum	-	-
Oreochromis niloticus	-	-
Electrophorus electricus ▲	-	-
Neostigmine		
Colossoma macropomum	0.59	0.004
Arapaima gigas	0.15	0.001
Rachycentron canadum	0.17	0.002
Oreochromis niloticus	0.16	0.0009
Electrophorus electricus▲	0.17	0.001
Eserine		
Colossoma macropomum	0.31	0.0021
Arapaima gigas	0.37	0.0025
Rachycentron canadum	0.49	0.0055
Oreochromis niloticus	0.76	0.0045
Electrophorus electricus▲	0.52	0.0037

* - Purity degree varied from 97.4% to 99.9%; ** - According to Cheng & Prusoff (1973); – less than 50% inhibition at 10 mM; ▲Obtained from Sigma.

ChEs are described as perfect enzymes since they hydrolyse the substrate close to the diffusion-controlled limit in the medium (Quinn, 1987; Tõugu, 2001; Miller and Wolfenden, 2002). They can decrease the AE enough to produce high rate enhancements in the catalyzed reactions. The AE estimated for each enzyme were decreased in more than a half which implies a gain of several orders of magnitude in the rates of reactions. The values in the present work are compatible with AE of

9.0 kcal/mol in the reaction catalized by AChE from the nematode *H. bacteriophora* (Mohamed et al., 2007). The enzymes were capable for decrease AE in up to 10.2 (*A. gigas* AChE activity) and to 17.4 kcal/mol (*A. gigas* BChE-like activity) from non-catalyzed reactions. The increased the non-enzymatic reaction rate. However, the BChE-like activity achieved such enhancement in *R. canadum* and *A. gigas* enzymatic hydrolysis due to the higher stability of S-butyrylthiocholine in assay conditions.

Table 3 – Activation energy (AE) in brain ChE from four species and respective rate enhancements in buffer tris-HCl pH 7.4 at 25ºC

Species				
Reaction	**AChE-ASCh Kcal/mol**	**Rate enhancement**	**BChE-like-BSCh Kcal/mol**	**Rate enhancement**
C. macropomum	8.0646	10^6	11.8492	10^7
A. gigas	7.0858	10^7	5.2512	10^{12}
R. canadum	7.4937	10^7	7.1701	10^{11}
O. niloticus	11.5772	10^4	-	-
Non enzymatic reaction	17.3637		22.6511	

Table 4 – Turnover number (Kcat) and catalytic efficiency (Kcat/Km) of AChE from four species.

Species	**Kcat [s-1]**	**Kcat/Km [M-1s-1]**
Oreochromis niloticus	2.24×10^3	6.05×10^6
Colossoma macropomum	6.50×10^3	1.51×10^7
Arapaima gigas	2.85×10^3	6.78×10^6
Rachycentron canadum	5.85×10^3	1.03×10^7

rate enhancement values found here for acetylthiocholine hydrolysis were similar to the values (>10^8) found by Tõugu (2001) combining results of the works of Wright (1968) and Nolte et al. (1980) for *E. electricus*. The values found in the present work also agreed with those found for rate enhancement in computational simulations for AChE activity (from 10^7 to 10^{11}) in other study by Fuxreiter and Warshel (1998) in spite of different assay conditions in measuring non-catalyzed reaction. Here, this measure was performed in the same assay conditions of the enzymatic reaction, in buffer pH 7.4, which

The estimation method for total enzyme content (Et) applies to tissues where AChE is abundant. Through this method was possible to estimate Kcat and Kcat/Km. The Kcat values are close to the ones described by Augustinsson (1971) (1.2×10^4) with *E. electricus* and were lower than those found in other works such as 1.6×10^4 by Fuxreiter and Warshel (1998) using computational simulations and 1.4×10^4 by Fersht (1999) whereas the Kcat/Km values estimated here are very similar (excepting *O. niloticus*) to 10^7 described by Miller and Wolfenden (2002) and lower than 10^9 found by Nolte et

al. (1980) using *E. electricus*. Such difference is justified by the fact that ChEs are enzymes whose rate of catalysis

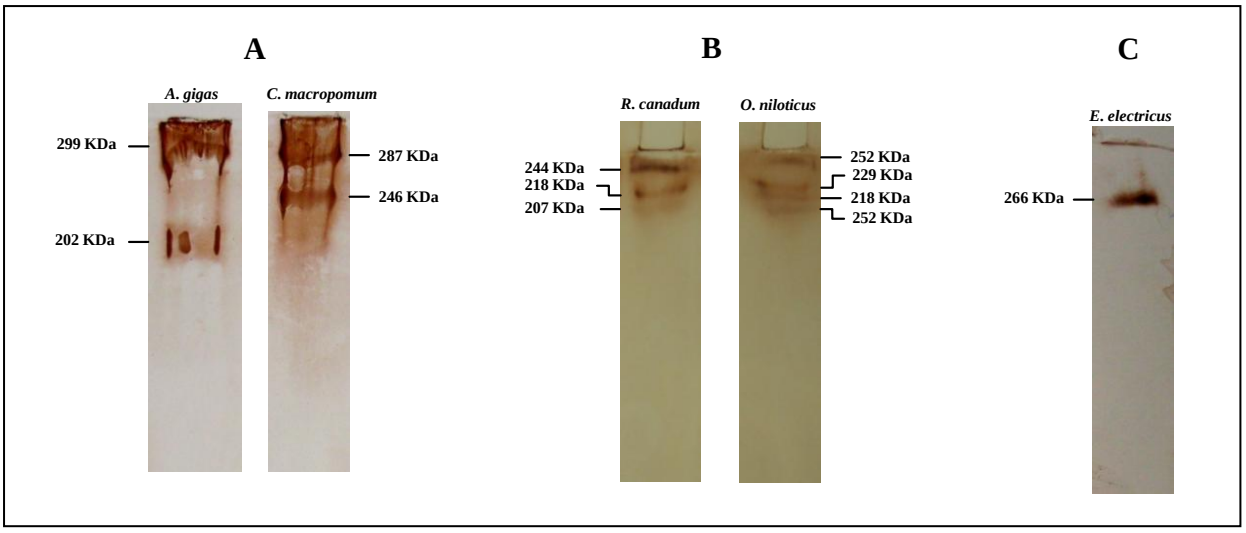

Figure 2 - Zymograms of ChE activity. Polyacrylamide gel 6-10% gradient: **A** – brain ChE from pirarucu (*A. gigas*) and tambaqui (*C. macropomum*) in dialyzed 0-90% fraction of $(NH_4)_2SO_4$; **B** – brain ChE from cobia (*R. canadum*) and Nile tilapia (*O. niloticus*) in dialyzed 0-90% fraction of $(NH_4)_2SO_4$; **C** – commercial AChE from electric eel (*E. electricus*).

are controlled by the substrate diffusion limit as mentioned before and this limit is restricted by the viscosity of the medium (brain extract buffered in the present work). However, this condition is more realistic in the sense of being closer to the *in vivo* functioning of these enzymes.

The reason to use commercial AChE from electric organ of *E. electricus* (A12 form, three tetramers linked to a collagen-like tail) in the Et estimation of brain AChE from the other species is that it undergoes a protease treatment to separate the tail and the tetramers (Chatonnet and Lockridge, 1989), thus becoming compatible with brain tetramers. The molecular weight of *E. electricus* commercial AChE found here (266 kDa) is similar to that described by Augustinsson (1971) (260 kDa) for the same species. Due to the low specificity for butyrylthiocholine as showed in the selective substrates analysis and the incipient inhibition by iso-OMPA (excepting *C. macropomum*), the bands in non-denaturing gel were classified as AChE isoforms. The only band that could be classified as PChE or BChE in view of its unusual behavior when exposed to selective inhibitors and substrates would be the upper band in *C. macropomum* lane. However, considering that BChE tetramers (the forms found in vertebrates brain) can reach approximately 340-360 kDa (Augustinsson, 1971; Lockridge, 1987a; 1987b), *C. macropomum* upper band cannot be classified as BChE also regarding its greater preference for the substrate acetylthiocholine and its sensibility to BW284c51.

The present study shows methods less expensive and less time-consuming to determine parameters of catalytic efficiency of the enzymes under analysis in the crude extract with results not far from those present in literature using purified enzymes and computational simulations. In addition, was found that at least three of the four studied species presented only AChE in their brain. The results with *C. macropomum* require more details in further studies to provide correct interpretations when using this species as source of ChEs in environmental monitoring for pesticides detection since it presented very high sensitivity to widely used pesticides like dichlorvos and carbofuran (Assis et al., 2010).

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7 - CAPÍTULO III – CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM THE BRAIN OF THE AMAZONIAN TAMBAQUI (*Colossoma macropomum*) AND IN VITRO EFFECT OF ORGANOPHOSPHORUS AND CARBAMATE PESTICIDES

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Abstract—In the present study, acetylcholinesterase (AChE) from the brain of the Amazonian fish tambaqui (*Colossoma macropomum*) was partially characterized and its activity was assayed in the presence of five organophosphates (dichlorvos, diazinon, chlorpyrifos, and tetraethyl pyrophosphate [TEPP]) and two carbamates (carbaryl and carbofuran) insecticides. Optimal pH and temperature were 7.0 to 8.0 and 45°C, respectively. The enzyme retained approximately 70% of activity after incubation at 50°C for 30 min. The insecticide concentration capable of inhibiting half of the enzyme activity (IC50) for dichlorvos, chlorpyrifos, and TEPP were calculated as 0.04 μmol/L, 7.6 μmol/L, and 3.7 μmol/L, respectively. Diazinon and temephos did not inhibit the enzyme. The IC50 values for carbaryl and carbofuran were estimated as 33.8 μmol/L and 0.92 μmol/L, respectively. These results suggest that AChE from the juvenile *C. macropomum* brain could be used as an alternative biocomponent of organophosphorus and carbamate biosensors in routine pesticide screening in the environment. Environ. Toxicol. Chem. © 2010 SETAC

Keywords—Organophosphorus pesticide Carbamate pesticide Acetylcholinesterase Biomarkers *Colossoma macropomum*

INTRODUCTION

Organophosphorus and carbamate are major classes of pesticides in use throughout the world. Together, they share about 50% of the world market of insecticides/acaricides. Their relatively fast hydrolysis and low persistence in the environment have supported their increasing use. However, their toxicity to mammals and other nontarget organisms, together with the large amounts used, constitute a threat to human health and the environment. Both classes are cholinesterase inhibitors, and several methodologies have been developed using these enzymes from various species to monitor their environmental presence. These neurotoxic agents have been distributed throughout the world without control in recent decades and, due to misuse and a lack of specificity, have become a serious problem to both humans and the environment [1]. Therefore, methods for organophosphorus and carbamate detection using either organisms or their enzymes as bioindicators and biomarkers, respectively, have been evaluated [2,3]. The cholinesterase group stands out among such molecules [4–6].

Acetylcholinesterase (AChE; enzyme classification 3.1.1.7) is widely known as a specific biomarker of organophosphorus and carbamate pesticides due to the inhibition of its activity [7]. This enzyme is responsible for modulating neural communication in the synaptic cleft by hydrolyzing the ubiquitous neurotransmitter acetylcholine. A lack of AChE activity causes central and peripheral nervous system disorders and death [8].

Studies have confirmed cholinesterases as suitable for monitoring the occurrence of these pesticide classes in environmental compartments [6,9–11]. For example, biosensors have been proposed based on AChE from electric eel and both genetically engineered (B394) and wild-type strains of *Drosophila melanogaster* [12]. However, the high interspecific and intraspecific polymorphism of these enzymes cause varied responses to insecticide compounds, thereby hindering the evaluation and comparison of results from different studies [13]. Consequently, it is necessary to characterize AChE activity in each species and type of tissue.

In previous work, AChE from the brain of the juvenile Amazonian fish tambaqui (*Colossoma macropomum*) was shown to be sensitive to dichlorvos [14]. This enzyme source could be proposed as a feasible alternative for setting up biosensors once it is located in a discarded tissue (brain) of this fish, which is the third most farmed species in Brazil (30,598 tons in 2007, according to the Brazilian Ministry of Environment; http://www.ibama.gov.br/recursos-pesqueiros/documentos/estatistica-pesqueira/).

The aims of the present study were to partially characterize some kinetic and physicochemical parameters of this enzyme, and to evaluate the effect of seven relevant organophosphorus and carbamate pesticides on its activity, to propose it as the biocomponent of an in vitro biosensor.

MATERIALS AND METHODS

Acetylthiocholine iodide, bovine serum albumin, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), Tris (hydroxymethyl) aminomethane, and dimethyl sulfoxide were purchased from Sigma. Analytical grade dichlorvos (98.8%), diazinon (99.0%), chlorpyrifos (99.5%), temephos (97.5%), tetraethyl pyrophosphate (97.4%), carbofuran (99.9%), and carbaryl (99.8%) were obtained from Riedel-de-Haën, Pestanal®. Disodium hydrogen

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phosphate and HCl were obtained from Merck. Trisodium citrate was acquired from Vetec. Glycine was acquired from Amersham Biosciences. The spectrophotometer used was Bio-Rad Smartspec™ 3000. The juvenile specimens of *C. macropomum* were supplied by Mar Doce Piscicultura e Projetos. Tambaqui specimens, 16.5 ± 3.7 cm in length and 93.8 ± 7.9 g in weight, were captured from a 750-m^3 pond.

Enzyme extraction

Twenty juvenile fish were acclimatized in 100-L aquaria (dissolved oxygen 8.04 ± 0.05 mg/L, temperature 26.04 ± 0.07°C, pH 6.93 ± 0.22, salinity 0.17 g/L) for one week and then sacrificed by immersion in an ice bath (0°C). The brains were immediately removed, joined in pairs, and homogenized in 0.5 mol/L Tris-HCl buffer, pH 8.0, maintaining a ratio of 20 mg of tissue per ml of buffer using a Potter–Elvehjem tissue disrupter. The homogenates were centrifuged for 10 min at 1,000 *g* (4°C) and the supernatants (crude extracts) were frozen at −20°C.

Enzyme activity and protein determination

The crude extract (30 µl) was added to 500 µl of 0.25 mmol/L DTNB dissolved in 0.5 mol/L Tris-HCl buffer, pH 7.4, and the reaction started by the addition of 0.125 mol/L acetylthiocholine iodide (30 µl) [14]. Enzyme activity (quadruplicate) was spectrophotometrically determined by following the absorbance at 405 nm for 180 s, in which the reaction exhibited a first-order kinetics pattern [14]. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 µmol of substrate per minute. A blank assay was similarly prepared except that 0.5 mol/L Tris-HCl buffer, pH 8.0, replaced the crude extract sample. Protein content was estimated according to a modified dye-binding method [15], using bovine serum albumin as the standard.

Optimal pH and temperature

Assays were performed using DTNB solutions in a pH range from 2.5 to 9.5 by using citrate-HCl (2.5 to 4.5), citrate-phosphate (4.0 to 7.5), Tris-HCl (7.2 to 9.0) buffers. Substrate nonenzymatic hydrolysis (in basic pH) was corrected by subtracting their values from the activities. Optimum temperature was established by assaying the enzyme activity at temperatures ranging from 5 to 70°C for 180 s.

Thermal stability

Thermal stability of juvenile *C. macropomum* AChE was evaluated by exposing crude extract samples for 30 min at temperatures ranging from 25 to 80°C and assaying the activity retained after 5 min of equilibration at 25°C (room temperature).

Inhibition assay

Acetylcholinesterase inhibition was assayed using five organophosphates (dichlorvos, diazinon, chlorpyrifos, temephos, and tetraethyl pyrophosphate [TEPP]) and two carbamates (carbaryl and carbofuran). The insecticides were first dissolved in dimethyl sulfoxide and then diluted in distilled water to five final concentrations ranging from 0.001 to 10 ppm, with each subsequent concentration 10-fold higher than the previous concentration. These concentrations correspond respectively: 0.0045 to 45.2 µmol/L (dichlorvos); 0.0032 to 32.8 µmol/L (diazinon); 0.0028 to 28.5 µmol/L (chlorpyrifos); 0.0021 to 21.4 µmol/L (temephos); 0.0034 to 34.5 µmol/L (TEPP); 0.0061 to 61.3 µmol/L (carbaryl); and 0.0045 to 45.2 µmol/L (carbofuran). The insecticide solutions (10 µl) were incubated with crude extract (20 µl) for 1 h [14] and the residual activity (%) was determined as previously described, using the absence of pesticide as 100% activity. All enzymatic and inhibition assays were carried out at room temperature (25°C). Five crude extracts from 10 fish brains were analyzed in triplicate for each insecticide concentration and data were expressed as mean ± standard deviation. These data were statistically analyzed by nonlinear regression fitted to polynomial or exponential decay ($p > 0.05$) modeling using the software MicroCal® Origin Version 8.0. The concentration capable of inhibiting half of the enzyme activity (IC50) was estimated for each pesticide.

RESULTS AND DISCUSSION

Optimum pH for juvenile *C. macropomum* AChE was found to be in the range 7.0 to 8.0 (Fig. 1A) similar to those described in the literature for other fishes (Table 1): *Solea solea* (7.0), *Scomber scomber* (8.0), and *Pleuronectes platessa* (8.5) [9]; *Cymatogaster aggregate*[16] and *Hypostomus punctatus* [17] (between 7.0 and 7.2). Optimum temperature was estimated as 45°C (Fig. 1B). Bocquené et al. [9] found temperatures in the range 32 to 34°C for *Pleuronectes platessa*; Beauvais et al. [4] at 25°C for *Lepomis macrochirus*, and Hazel [18] at 35°C for *Carassius auratus*. In the present study, AChE from juvenile

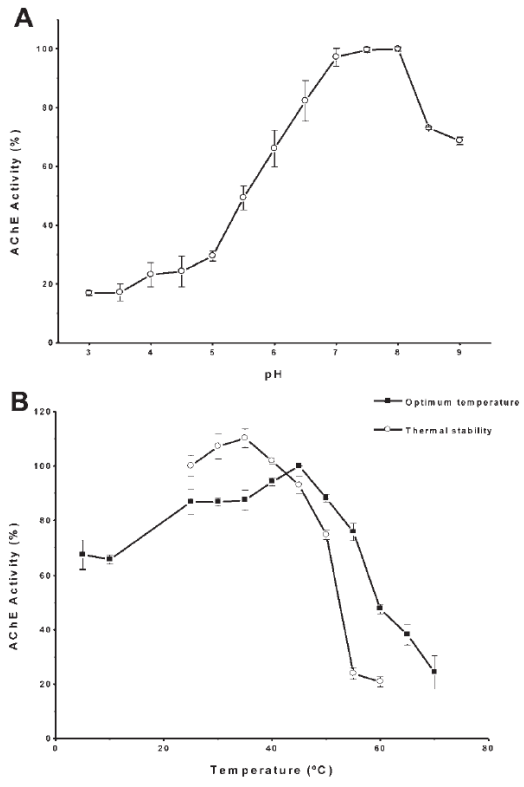

Fig. 1. Effect of pH (**A**) and temperature (**B**) on the AChE from brain of juvenile *C. macropomum*. The pH range was attained by using citrate-HCL, citrate-phosphate, and tris-HCl buffers, whereas the temperature effect was investigated either on the enzyme activity (optimum temperature, ■) or on the enzyme preparation (thermal stability, ○) for 30 min; after 5 min (25°C equilibrium), its activity was estimated. AChE = acetylcholinesterase.

Table 1. Kinetics and physicochemical parameters of AChE from some freshwater and marine species[a]

Species [reference]	K_m (mmol/L)	V_{max} (U/mg of protein)	Optimum pH	Optimum temperature (°C)	Source	Life stage
Oreochromis niloticus[5]	0.10 ± 0.03	0.229 ± 0.014	ND	ND	Brain	Juvenile 48.2 ± 3.9 g
Pleuronectes vetulus[6]	1.69 ± 0.26	0.482 ± 0.034	ND	ND	Muscle	Juvenile 13.5–29.5 cm
Pleuronychtis verticalis [6]	0.30 ± 0.07[b] 0.23 ± 0.06[c]	0.524 ± 0.032[b] 0.120 ± 0.08[c]	ND	ND	Muscle	Juvenile
Solea solea [9]	ND	ND	7.5	ND	Brain	ND
Pleuronectes platessa [9]	ND	ND	8.5	33	Brain	ND
Scomber scomber [9]	ND	ND	8.0	ND	Brain	ND
Colossoma macropomum [present work]	0.43 ± 0.02	0.13 ± 0.05	7.5	45	Brain	Juvenile 16.6 ± 3.7 cm

[a] AChE = acetylcholinesterase; K_m = Michaelis–Menten constant; V_{max} = maximum velocity of enzyme activity; ND = not determined.
[b] Female specimens.
[c] Male specimens.

C. macropomum after being incubated for 30 min at 50°C retained about 70% of its activity at 35°C (Fig. 1B). Zinkl et al. [19] reported absence of cholinesterase activity in the brain of *Oncorhyncus mykiss* (formerly known as *Salmo gairdneri*) subjected to temperatures higher than 45°C.

The Michaelis–Menten kinetics is displayed in Figure 2, from which the maximal velocity (V_{max}) and apparent bimolecular constant (K_m) were 0.128 ± 0.005 U/mg protein and 0.434 ± 0.025 mmol/L, respectively, using acetylthiocholine iodide as substrate. The Lineweaver–Burk plot is also presented. Varó et al. [20] reported acetylthiocholine iodide inhibition at concentrations greater than 5.12 mmol/L in brain tissue from *Sparus aurata*, in contrast to muscle tissue, for which inhibition occurred at 20.48 mmol/L. Rodríguez-Fuentes and Gold-Bouchot [5] found acetylthiocholine inhibition at 4.89 mmol/L in AChE from the brain of *Oreochromis niloticus*. However, in the present study, no substrate inhibition was observed even at the 15 mmol/L acetylthiocholine iodide. According to Table 1, the apparent Michaelis–Menten constant of the juvenile *C. macropomum* AChE was lower than that estimated for *Pleuronectes vetulus* muscle and higher than *Pleuronychtis verticalis* muscle and *Oreochromis niloticus* brain, whereas the maximum velocity was smaller than those reported for these mentioned tissues.

Among the anticholinesterasic agents, organophosphates and its analogues play a different role in the metabolic paths before reaching sites of neuronal transmission. Some of them are produced in a less toxic form (thion form, P = S) which is more stable in the environment. When absorbed by an organism, this form of pesticide undergoes bioactivation to a more toxic form (oxon form, P = O) by monooxigenases from the cytochrome P450 complex present in some organs/tissues including liver, kidneys, lungs, and brain. Therefore, this phenomenon and the diverse effect of the resulting products on the AChE can determine differences in the behavior of the enzyme.

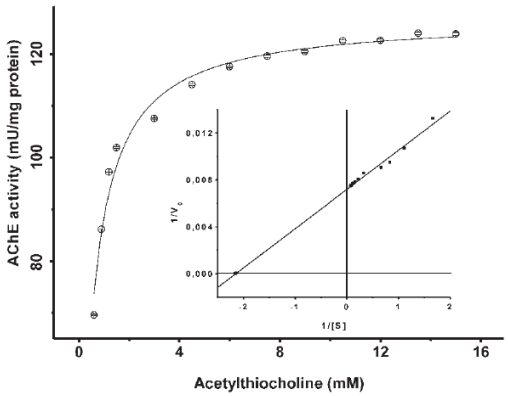

Fig. 2. Michaelis–Menten plot of the AChE from brain of juvenile *C. macropomum* acting on acetylthiocholine. Data are expressed as the mean ± standard deviation of three replicates from four homogenates. The inset shows the Lineweaver–Burk plot. AChE = acetylcholinesterase.

The Food and Agriculture Organization [21] recommends that 20% inhibition is the relevant end-point to determine acceptable daily intakes of an anticholinesterasic compound. In the present study, some of the compounds analyzed were highly toxic to tambaqui AChE, and the inhibition they caused could rapidly reach the above-mentioned levels.

Results from inhibition assays are displayed in Figure 3 and Table 2 and summarize the IC50 values estimated from these data for the five organophosphates (dichlorvos, diazinon, chlorpyrifos, temephos, and TEPP) and two carbamate insecticides (carbaryl and carbofuran). Dichlorvos as previously demonstrated [14] was shown to strongly inhibit the juvenile *C. macropomum* AChE. Among the investigated pesticides in the present study, this insecticide presented the lowest IC50 value (0.04 μmol/L; 0.01 ppm) and the lowest value compared with those reported in the literature for other fish species. Chuiko [22] estimated the IC50 value of 0.31 μmol/L for *Leuciscus idus* and *Esox lucius*, and 0.63 μmol/L for *Alburnus alburnus*. Dichlorvos is a direct inhibitor of AChE. It is an oxon organophosphate compound [23] and does not require bioactivation for enzyme inhibition in contrast with thion compounds, for which only a fraction of the total amount is activated in the tissues [24,25]. Chlorpyrifos also displayed lower IC50 value (7.6 μmol/L) than that reported for *Cyprinus carpio* [26]. Diazinon and temephos did not show inhibition effect on the juvenile *C. macropomum* AChE under the experimental conditions used in the present study. According to a number of studies, acute toxicity from phosphorothionate pesticides such as diazinon and chlorpyrifos is strongly influenced by differences in the activity of cytochrome P450-mixed oxidase systems, which bioactivate these compounds [27,28]. Nevertheless, these influences only determine toxic effects through the balance between activation and detoxification pathways: P450 dearylation, carboxylesterase and butyrylcholinesterase phosphorylation, and oxonase-mediated hydrolysis [29]. Thus, the contrast between high sensitivity to oxons and apparent lower oxidation activity possibly could be a *C. macropomum* enantiostatic mechanism when facing xenobiotic threats [30].

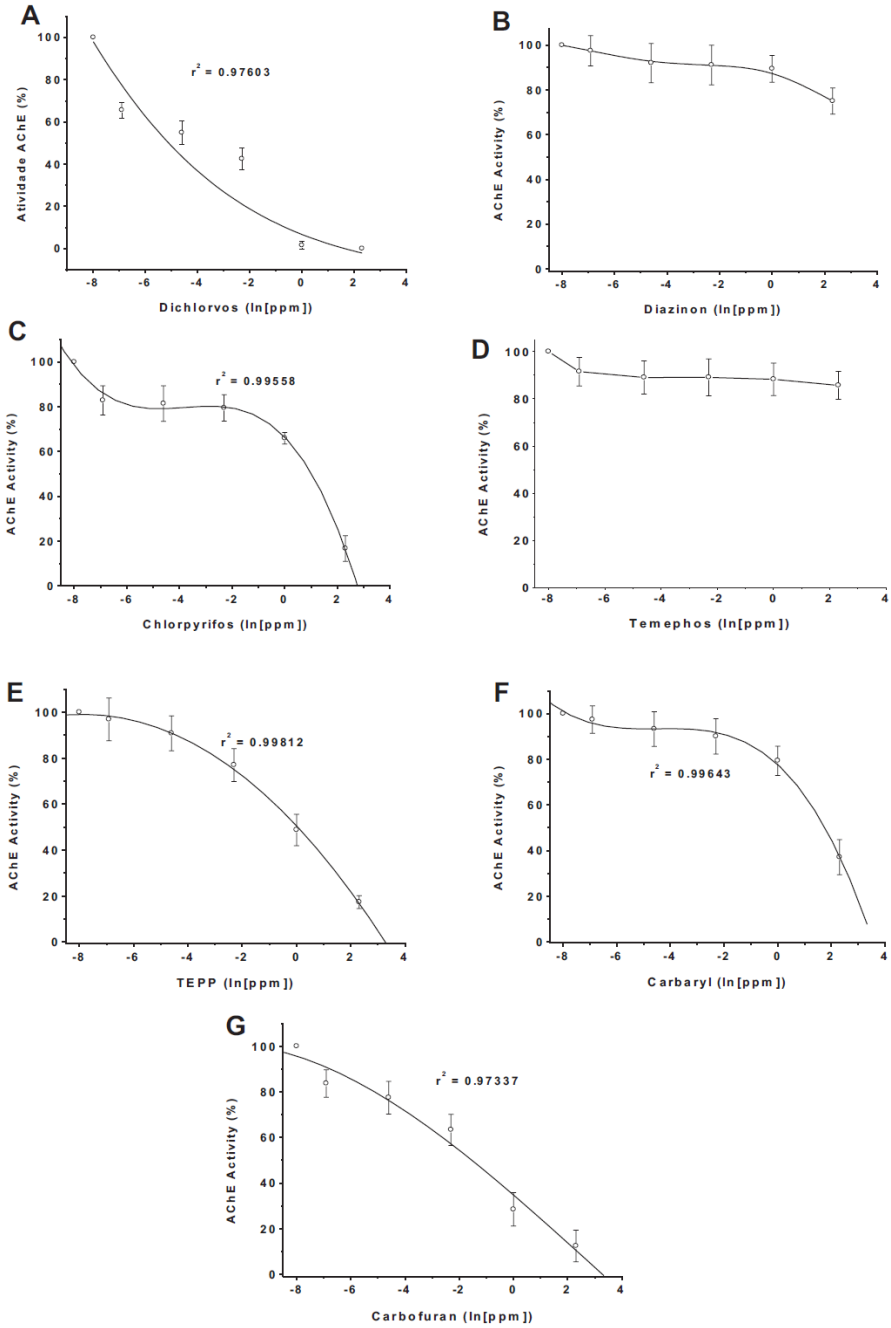

Fig. 3. Effect of organophosphates and carbamates on the activity of AChE from brain of juvenile *C. macropomum*. Dichlorvos (**A**), diazinon (**B**), chlorpyrifos (**C**), temephos (**D**), TEPP (**E**), carbaryl (**F**), and carbofuran (**G**) concentrations ranged from 0.001 to 10 ppm. All the assays were performed at 25°C, and the experimental points are the mean ± standard deviation of triplicate of four crude extracts. AChE = acetylcholinesterase; TEPP = tetraethyl pyrophosphate.

Another condition that may cause discrepancies, particularly in case of chlorpyrifos, is that this compound accumulates in tissues, which likely affects other results. Antwi [31] also found no statistical differences in four fish species (*Oreochromis niloticus*, *Sarotherodon galilaea*, *Alestes nurse*, and *Schilbe mystus*) between controls and individuals living in areas treated weekly with temephos over a six-year period. Temephos is also a thion compound, but the reasons for such results are not only caused by the circumstances mentioned for diazinon and chlorpyrifos. This pesticide is known to exhibit moderate to low

Table 2. Pesticide IC50 values for in vitro freshwater fish[a,b]

Species [reference]	IC50 (μmol/L)
Dichlorvos	
Alburnus alburnus [23]	0.63
Leuciscus idus [23]	0.31
Esox lucius [23]	0.31
Colossoma macropomum [14][c]	0.36
Colossoma macropomum [present study]	0.04
Chlorpyrifos	
Cyprinus carpio [27]	810
Colossoma macropomum [present study]	7.6
Diazinon	
Oncorhynchus mykiss [26]	2.5
Danio rerio [26]	20.0
Poecilia reticulata [26]	7.5
Cyprinus carpio [26]	0.2
Colossoma macropomum [present study][d]	No effect
Temephos	
Oreochromis niloticus, Sarotherodon galilaea, Alestes nurse, and *Schilbe mystus* [32]	No effect
Colossoma macropomum [present study]	No effect
TEPP	
Colossoma macropomum [present study]	3.7
Carbaryl	
Colossoma macropomum [present study]	33.8
Carbofuran	
Cyprinus carpio [27]	0.45
Colossoma macropomum [present study]	0.92

[a] IC50 = insecticide concentration capable of inhibiting 50% of enzyme activity; TEPP = tetraethyl pyrophosphate.
[b] Pesticide purity degree varied from 97.4 to 99.9%.
[c] Commercial formulation.
[d] Up to 1.0 ppm.

toxicity to mammals and other nontarget organisms, and is commonly used in potable water treatment against mosquito larva vectors of diseases in public health campaigns [31]. Tetraethyl pyrophosphate displayed an IC50 value of 3.7 μmol/L. This is an organophosphorus known to be highly toxic to mammals; it is the biotransformation product of another pesticide, which is classified as extremely hazardous by the World Health Organization [32]. Table 3 displays its in vivo LC50 for other fish species provided by the U.S. Environmental Protection Agency Ecotoxicology Database (http://cfpub.epa.gov/ECOTOX/), which reflects the high toxicity of this compound (6.8 h at 25°C) [33]. Tetraethyl pyrophosphate is currently classified as an obsolete pesticide [32], but in fact is responsible for part of the toxic action in some organophosphate products, such as diazinon, chlorpyrifos, parathion, and demeton, where it appears as an impurity or breakdown product due to the manufacturing process or unsuitable storage conditions [33]. The two analyzed carbamate insecticides, carbaryl and carbofuran, presented IC50 values of 33.8 μmol/L and 0.99 μmol/L, respectively. The latter IC50 value is similar to that reported by Dembélé et al. for in vitro, *Cyprinus carpio* [26], namely, 0.45 μmol/L (0.1 ppm).

Table 3. TEPP LC50 in several fish species[a,b]

Species	TEPP (%)	LC50 (mg/L)
Carassius auratus	40.0	21.00
Gambusia affinis	40.0	2.84
Ictalurus punctatus	40.0	1.60
Lepomis macrochirus	40.0	0.79
Pimephales promelas	40.0	1.00
Poecilia reticulata	40.0	1.80
Oncorhynchus tshawytscha	40.0	0.31

[a] TEPP = tetraethyl pyrophosphate; LC50 = concentration resulted in death for half of the animals.
[b] Source: U.S. Environmental Protection Agency ECOTOX Database.

The monitoring of pesticides such as organophosphates and carbamates can be evaluated by using organisms in aquatic environments (in vivo assays). In these cases, tanks, animal manipulation, feeding demands, and specially trained personnel are required. Otherwise, animals can be collected from their environment and these toxic components analyzed in their tissues. The use of enzymes, namely, cholinesterases, allows in vitro procedures that are less costly, less time-consuming, less laborious, and more sensitive. The analysis of reactions can take place without interfering compounds present in tissues or animal sensors that could interact with anticholinesterasic agents, thereby causing false positives or negatives. Moreover, biosensors based on these enzymes can be built and used in environmental monitoring. The findings described here confirm previous findings [14] related to the sensitivity of AChE from the brain of the juvenile Amazonian tambaqui towards dichlorvos, and its possible use as the biocomponent of in vitro sensor for this pesticide, and also for chlorpyrifos, carbaryl, and carbofuran.

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8 - CAPÍTULO IV – CHARACTERIZATION OF BRAIN ACETYLCHOLINESTERASE FROM FOUR TROPICAL FISH AND EFFECT OF PESTICIDES ON ITS ACTIVITY

ARTIGO ENVIADO À REVISTA JOURNAL OF ENVIRONMENTAL MONITORING

Running header: Pesticides on acetylcholinesterase from four fish.

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Characterization of brain acetylcholinesterase from four tropical fish and effect of pesticides on its activity

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Abstract

Acetylcholinesterase (AChE; EC 3.1.1.7) from four tropical fish: pirarucu (*Arapaima gigas*), cobia (*Rachycentron canadum*), electric eel (*Electrophorus electricus*) and Nile tilapia (*Oreochromis niloticus*) were characterized and their activities were assayed in the presence of seven pesticides (five organophosphates: dichlorvos, diazinon, chlorpyrifos, temephos, tetraethyl pyrophosphate - TEPP and two carbamates: carbaryl and carbofuran). Optimal pH and temperature were found to be 8.0 and 35-45ºC, respectively. The pirarucu, cobia and Nile tilapia AChE retained approximately 81%, 21% and 66 %, respectively, of the activity after incubation at 50ºC for 30 min. The IC50 of TEPP estimated for the pirarucu AChE was 0.009 µmol/L. The electric eel was sensitive to carbofuran (IC50 of 0.005 µmol/L). These results provide more information regarding the use of AChE from juvenile *A. gigas*, *R. canadum*, *E. electricus* and *O. niloticus* as biocomponent of organophosphorus and carbamate biosensors in pesticide routine screening in the environment.

Key words: Organophosphorus, carbamates, Acetylcholinesterase, Biomarkers, Fish

8.1. Introduction

Cholinesterase (ChE) inhibition is a widely known biomarker of exposure to organophosphorus (OP) and carbamate (CB) pesticides in aquatic environments due to its sensibility to such compounds [1, 2]. In its typical role, acetylcholinesterase (AChE; EC 3.1.1.7) works onto the cholinergic synapses interrupting the chemical signal by the hydrolysis of the neurotransmitter acetylcholine. These pesticides bind to the catalytic site of the enzyme through phosphorylation or carbamoylation, causing inhibition, which tends to irreversibility, in the case of organophosphate exposure. The disruption of the nerve function occurs by the accumulation of the acetylcholine in the synaptic cleft. Loss of AChE activity may lead to a range of effects resulting from excessive nervous stimulation and culminating in respiratory failure and death [3].

Organophosphorus and carbamate pesticides still remain as the most important classes of insecticides in terms of usage and billing. In 2002, they shared about 50% of the world market [4]. These compounds have been distributed throughout the world without control in recent decades and due to misuse have become a serious problem to both humans and the environment [5].

Although these pesticide classes are more biodegradable and less persistent than organochlorines they are highly toxic to non-target organisms such as birds and aquatic organisms [6, 7]. They can persist for days in the environment but their action on tissues can last weeks making it more advantageous to measure ChEs than chemical residues for environmental monitoring programs.

ChE inhibition has been assayed in several species including aquatic organisms due to its ability to assess the environmental impact of pesticides even when these compounds are not present in the water [8].

The investigation and characterization of brain AChE are relevant to identify it as a suitable tool for use in environmental and food screening [9-11]. Several studies described that AChE is responsible for almost all cholinesterasic activity in brain and in some cases this enzyme is more sensitive to pesticides than butyrylcholinesterase (BChE; EC 3.1.1.8) [10, 12-14]. Monitoring at the biochemical and physiological level

can detect the presence of contaminants faster and specifically detecting substances before adverse effects are evident at higher levels of biological organization, offering an early warning [15].

However, for a suitable use of sentinel organisms and its biomolecules, preliminary studies are necessary to characterize its biochemical behavior under normal conditions for further analysis of changes induced by toxic substances [1, 8, 10, 11, 16, 17].

AChEs from different sources present high inter and intra-specific polymorphism that can cause varied responses to the same insecticide compounds thereby, hindering the evaluation and comparison of results from different studies [18].

The aim of the present study was to characterize the kinetic and physicochemical parameters of the brain AChEs *in vitro* from the following tropical fish: pirarucu (*Arapaima gigas*), cobia (*Rachycentron canadum*), Nile tilapia (*Oreochromis niloticus*) and to evaluate the effect of organophosphorus and carbamate pesticides on their activities comparing the results with a commercial and purified AChE from electric eel (*Electrophorus electricus*).

8.2. Materials and Methods

8.2.1. Materials

AChE from electric eel type VI-S, Acetylthiocholine iodide, S-butyrylthiocholine iodide, bovine serum albumin, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), tris (hydroxymethyl) aminomethane and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade dichlorvos (98.8%), diazinon (99.0%), chlorpyrifos (99.5%), temephos (97.5%), TEPP (97.4%) (Tetraethyl pyrophosphate), carbofuran (99.9%) and carbaryl (99.8%) were obtained from Riedel-de-Haën, Pestanal® (Seelze, Germany). A Bio-Rad x Mark (Hercules Ca, USA) microplate spectrophotometer and a IKA RW - 20 digital (Staufen, Germany) tissue disrupter were used. The sub-adult juvenile specimens of pirarucu (76.8 ± 8.7 cm; 4,118 ± 207.9 g) and Nile tilapia (12.0 ± 3.0 cm; 7.9 ± 1.2 g) were supplied by Fisheries and Aquaculture Department of the Federal Rural University of Pernambuco. Sub-adult Cobia (51.67 ± 1.5 cm; 1,575 ± 329.6 g) were supplied by Aqualider Ltda. (Recife, Brazil).

8.2.2. Methods

8.2.2.1. Enzyme extraction

Sub-adult Juvenile fish enzymes were extracted according to Assis at al. [19]. The juvenile fishes were sacrificed in an ice bath. The brains were immediately excised, pooled and homogenized in 0.5 mol/L Tris-HCl buffer, pH 8.0, maintaining a ratio of 20 mg of tissue per ml of buffer using a Potter-Elvehjem tissue disrupter. The homogenates were centrifuged for 10 min at 1000 x *g* (4ºC) and the supernatants (crude extracts) were frozen at -20ºC for further assays. Triton X-100 was not used in this method since its kinetic interaction with organophosphorus oxon compounds can affect the results [20].

8.2.2.2. Enzyme activity and protein determination

AChE and BChE activities were carried out by a modification of the colorimetric method [21]. Briefly: 200 μL of 0.25 mmol/L DTNB in 0.5 mol/L tris-HCl buffer, pH 7.4 was added to the crude extract (20 μl), and the reaction started by the addition of either 62 mmol/L acetylthiocholine iodide or S-butyrylthiocholine iodide (20 μl) for AChE and BChE, respectively. Enzyme activity was determined by reading the increase in absorbance at 405 nm for 180 s. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 μmol of substrate per minute. Protein content was estimated according to Sedmak and Grossberg [22], using bovine serum albumin as the standard.

8.2.2.3. Kinetic parameters

The kinetic parameters Km and Vmax were estimated using increasing acetylthiocholine concentrations from 0.8 to 20.8 mmol/L final concentration and fitting data to non-linear regression using the software MicroCal™ Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

8.2.2.4. Optimal pH, temperature and thermal stability

Assays were performed with DTNB solutions in a pH range from 2.5 to 9.0 by using citrate-HCl (2.5 - 4.5), citrate-phosphate (4.0 – 7.5), tris-HCl (7.2 – 9.0) buffers. Substrate non-enzymatic hydrolysis (in basic pH)

was corrected by subtracting their values from the activities. Optimum temperature was established by assaying the enzyme activity at temperatures ranging from 0 to 80ºC for 180 s.

Thermal stability of fish AChE was evaluated by exposing crude extract samples for 30 min at temperatures ranging from 25 to 80ºC and assaying the remanent activity after 15 minutes at 25ºC (room temperature) equilibration.

8.2.2.5. Inhibition assay by pesticides

AChE inhibition assays for the species analized were carried out using the organophosphates dichlorvos, diazinon, chlorpyrifos, temephos and TEPP and the carbamate insecticides carbaryl and carbofuran as inhibitors. The insecticides were diluted to seven final concentrations ranging from 0.001 to 1000 ppm increasing the concentration ten times at each subsequent concentration. These concentrations corresponded to: 0.0045 to 4520 µmol/L (dichlorvos); 0.0032 to 3280 µmol/L (diazinon); 0.0028 to 2850 µmol/L (chlorpyrifos); 0.0021 to 2140 µmol/L (temephos); 0.0034 to 3450 µmol/L (TEPP); 0.0061 to 6130 µmol/L (carbaryl); and 0.0045 to 4520 µmol/L (carbofuran). The insecticide solutions (10 µl) were incubated with crude extract (10 µl) for 1 h [21] and the residual activity was determined considering the activity in absence of pesticide as 100%. All assays were carried out at room temperature (25ºC) and quadruplicates. Residual activity percentage was plotted versus *ln* of insecticide concentration in ppm. Statistical analysis were carried out by linear and non-linear regression fitted to sigmoidal (Boltzmann) or exponential decay using MicroCal® Origin® Version 8.0. The IC50 was estimated for each pesticide by using the same software and Ki was calculated using the Cheng and Prusoff equation [23].

8.3. Results and discussion

Optimal pH for the four species under analysis were found to be in the range 7.5-8.0 (**Fig. 1**). Activities decreased after pH 8.5. This is in accordance with the ionization constant of the ring nitrogen from the imidazole group present in the catalytic triad which is the pH-dependent step in the hydrolytic process [24, 25]. These results are next to the values found for some studies showed in **Table 1**: *Solea solea* (7.0), *Scomber scomber* (8.0) and *Pleuronectes platessa* (8.5) [9]. In other works, values for *Cymatogaster aggregate* [26], *Hypostomus punctatus* [27] were found between 7.0 and 7.2 while 7.5 and 8.0 for *C. macropomum* [21].

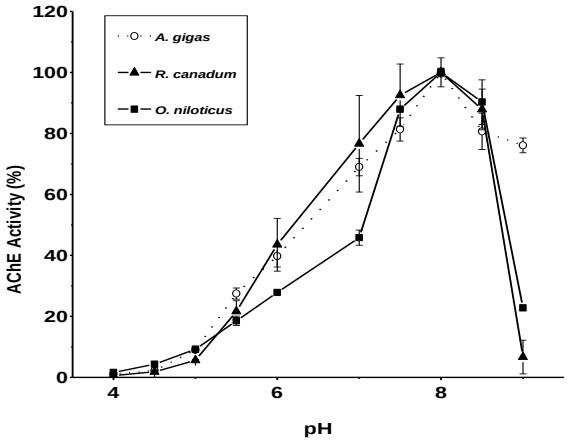

Figure 1 – Effect of pH on the AChE activity from brain of three species. The pH range was attained by using citrate-HCl, citrate-phosphate and tris-HCl buffers.

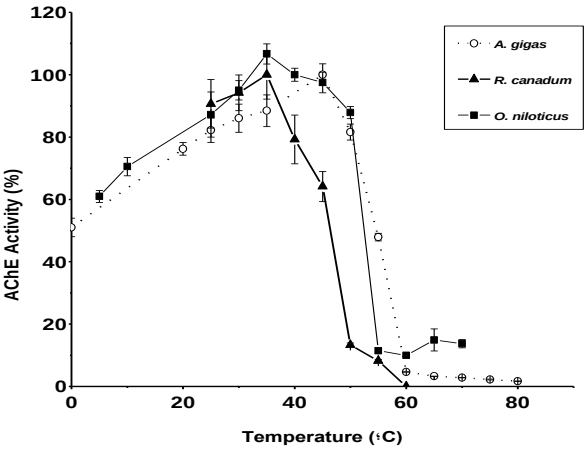

Figure 2 - Effect of temperature on the activity of brain AChE from three species. The activity was assayed in a range from 0 to 80ºC, during 180 s.

There is a safety margin where the enzyme can withstand against temperature fluctuations with minimum damage to its structure and operation. In the present study, optimum temperature was estimated around 45 ºC for *A. gigas* while for *O. niloticus* and *R. canadum* were 35 ºC as displayed in **Figure 2**. For the same parameter, Bocquené et al. [9] found 33 ºC for *Pleuronectes platessa*, Beauvais et al. [16] observed 25ºC for *Lepomis macrochirus* and Hazel [28] described 35 ºC for

Carassius auratus. Here, the enzymes from *A. gigas*, *R. canadum* and *O. niloticus* retained about 81, 21 and 66 % of the activity after incubation at 50 ºC for 30 min, respectively (**Fig. 3**). Zinckl et al. [29] reported for the coldwater fish *Oncorhynchus mykiss* brain cholinesterase absence of activity after 45 ºC.

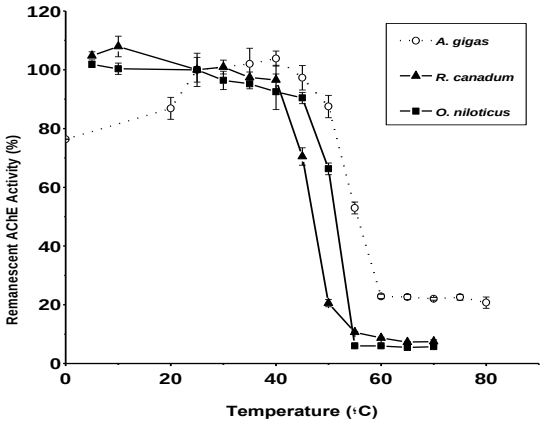

Figure 3 – AChE thermal stability assayed from 0 to 80ºC on the enzyme preparation for 30 min and after 15 min equilibrium at 25ºC.

The kinetic parameters found for the four species can be seen in **Table 1**. The results are close to those found in the literature for the Michaelis-Menten constant (Km) as well as for the Vmax. This last parameter is higher for the *E. electricus* AChE considering that this enzyme is purified whereas the other three enzymes under investigation are crude extract. In addition to the well known hyperbola model the *E. electricus* enzyme data were fitted according to two-site binding (Km = 0.08 mmol/L and Vmax = 52.29 U/mg protein) which was more appropriate. AChE from *Rachycentron canadum* and *E. electricus* (using the two-site binding model) showed the highest affinity for the substrate while *Oreochromis niloticus* Vmax value was lower than the one from Rodríguez-Fuentes and Gold-Bouchot [10], but they are in accordance to Chandrasekara and Pathiratne [30], who found a decrease in AChE activity during the *O. niloticus* growth following three developmental stages. BChE showed negligible activity in the brain of the species under analysis and this is in accordance with other results [10, 12-14].

The influence of pesticide toxicity on cholinesterases depends on the shape of the active site of the each enzyme. **Table 2** presents the IC50 of the OP and CB pesticides and their respective inhibition constant (Ki) for the interaction with the AChE from the four tropical species. In most of cases the AChE from *E. electricus* presented the lowest value of IC50, of the four species studied, excepting for TEPP (AChE from *A. gigas* – 0.009 while *E. electricus* AChE – 0.06 µmol/L). AChE inhibition by OP compounds followed different behaviour depending on the structure of the pesticides. There are two groups of organophosphorus pesticides, the phosphate group (oxon form; P=O) and the phosphorothioate group (thion form; P=S). The first one is the direct inhibiting form due to the greater electronegativity of oxygen in relation to sulphur when interacting in the active domain of the enzyme. The second group is less toxic and requires biotransformation to their oxo-analogues to become biologically active. This biotransformation occurs by oxidative desulfuration mediated by cytochrome P450 isoforms and flavin-containing mono-oxigenase enzymes, by N-oxidation and S-oxidation [3, 31]. The second group is synthesized in this form in order to resist the environmental factors and to increase the residual power of the compound since the OP's, in general, present a short half-life in the environment after the biotransformation.

OP's effect can be divided by the form of phosphorylation in the active site. Most of these pesticides contain two methyl or two ethyl ester groups bonded to the phosphorus atom. According to this structure they can dimethyl- or diethyl-phosphorylate the serine hydroxyl group in the active center. After the release of the leaving group, dimethyl-AChE can be spontaneously reactivated very slowly. On the other hand, diethyl-phosphoenzymes can recover their activity almost exclusively by using oximes or other reactivation agents [3, 31]. It means that diethyl-organophosphorus is able to inhibit the enzyme in long term. Dichlorvos and temephos present methyl ester groups and diazinon, chlorpyrifos and TEPP contain diethyl groups. In the present work, the effect of this characteristic cannot be observed on the enzyme activity due to the time of exposure (1 h) during the assays. Further studies are

Table 1 – Kinetics and physicochemical parameters of AChE from some freshwater and marine species

Species [References]	Km [mmol/L]	Vmax [U/mg protein]	Optimum pH	Optimum Temperature [ºC]	Source	Life stage
Oreochromis niloticus [10]	0.10 ± 0.03	0.229 ± 0.014	-	-	Brain	Juvenile 48.2 ± 3.9 g
Oreochromis niloticus [Present work]	0.39 ± 0.2	0.218 ± 0.007	8.0	35	Brain	Juvenile 9.0-15.0 cm
Pleuronectes vetulus [11]	1.69 ± 0.26	0.482 ± 0.034	-	-	Muscle	Juvenile 13.5-29.5 cm
Pleuronychtis verticalis [11]	0.30 ± 0.07* 0.23 ± 0.06**	0.524 ± 0.032* 0.120 ± 0.08**	-	-	Muscle	Juvenile
Solea solea [9]	-	-	7.5	-	Brain	-
Pleuronectes platessa [9]	-	-	8.5	33	Brain	-
Scomber scomber [9]	-	-	8.0	-	Brain	-
Colossoma macropomum [21]	0.43 ± 0.02	0.129 ± 0.05	7.5-8.0	45	Brain	Juvenile 30.0 ± 4.2 cm 512.5 ± 123.7 g
Arapaima gigas [present work]	0.42 ± 0.09	0.116 ± 0.002	8.0	45	Brain	Juvenile
Electrophorus electricus▲ [present work]	0.45 ± 0.11*** 0.08 ± 0.1****	67.82 ± 1.98*** 52.29 ± 6.6****	-	-	Electric organ	-
Rachycentron canadum [present work]	0.43 ± 0.14	0.243 ± 0.02	8.0	35	Brain	juvenile 51.67 ± 1.5 cm 1,575 ± 329.6 g

* Female specimens; ** Male specimens; - not determined; ***Hyperbolae model; ****Two-site binding model; ▲ Obtained from Sigma.

required to distinguish the reactivation conditions related to each compound.

Another feature of the interaction of OP compounds with the tissues is that most of them are lipophilic. According to Vale [31] they are rapidly absorbed and accumulated in fat, liver, kidneys and salivary glands. The phosphorothioates are more lipophilic than the phosphates. Some of the mentioned circumstances were observed in our laboratory where phosphorothioates lipophilic compounds (diazinon, chlorpyrifos, and temephos) provided a smooth fall in the AChE activity curves while the phosphate and less-lipophilic compounds (dichlorvos and TEPP) presented a sharp decline in the AChE activity (data not shown). This fact demonstrates the possible sequestration of the lipophilic compounds by the brain lipids in the extract and could explain its minor reactivity before biotransformation.

Carbamate pesticides are capable of readily inhibiting AChE without biotransformation, so they can induce acute toxicity effects faster than most of OP compounds (data not shown). The IC50 and Ki values for carbaryl and carbofuran are shown in **Table 2**.

The Ki results were calculated by the equation of Cheng and Prusoff [23] which is a function of the IC50 values and, as expected, were very low since

Table 2 – Pesticide* IC50 and Ki** values for *in vitro* AChE from freshwater and marine fish.

Species [Reference]	IC50 (µmol/L)	Ki (µmol/L)
Dichlorvos		
Alburnus alburnus [32]	0.63	-
Leuciscus idus [32]	0.31	-
Esox lucius [32]	0.31	-
Colossoma macropomum [19]***	0.36	-
Colossoma macropomum [21]	0.04	1.37×10^{-4}
Arapaima gigas [present work]	2.32	7.92×10^{-3}
Rachycentron canadum [present work]	6.9	1.36×10^{-2}
Oreochromis niloticus [present work]	5.4	3.26×10^{-2}
Electrophorus electricus [present work]▲	0.16	1.16×10^{-3}
Diazinon		
Oncorhynchus mykiss [33]	2.5	-
Danio rerio [33]	20.0	-
Poecilia reticulata [33]	7.5	-
Cyprinus carpio [33]	0.2	-
Pimephales promelas [34]	5.0×10^{3}	-
Colossoma macropomum [21]****	-	-
Arapaima gigas [present work]	1.5×10^{3}	5.13
Rachycentron canadum [present work]	-	-
Oreochromis niloticus [present work]	-	-
Electrophorus electricus [present work] ▲	0.3	2.18×10^{-3}
Chlorpyrifos		
Cyprinus carpio [35]	810	-
Colossoma macropomum [21]	7.6	2.61×10^{-2}
Arapaima gigas [present work]	7.87	2.69×10^{-2}
Rachycentron canadum [present work]	30.24	5.94×10^{-2}
Oreochromis niloticus [present work]	26.78	0.161
Electrophorus electricus [present work] ▲	0.03	2.18×10^{-4}
Temephos		
Oreochromis niloticus, Sarotherodon galilaea, Alestes nurse e *Schilbe mystus* [36]	NE	-
Colossoma macropomum [21]	NE	-
Arapaima gigas [present work]	NE	-
Rachycentron canadum [present work]	NE	-
Oreochromis niloticus [present work]	NE	-
Electrophorus electricus [present work] ▲	7.6	5.51×10^{-2}
TEPP		
Colossoma macropomum [21]	3.7	1.27×10^{-2}
Arapaima gigas [present work]	0.009	3.07×10^{-5}
Rachycentron canadum [present work]	8.1	1.59×10^{-2}
Oreochromis niloticus [present work]	20.75	0.125
Electrophorus electricus [present work] ▲	0.06	4.35×10^{-4}
Carbaryl		
Pimephales promelas [34]	10.0	-
Colossoma macropomum [21]	33.8	0.116
Arapaima gigas [present work]	12.25	4.18×10^{-2}
Rachycentron canadum [present work]	8.31	1.63×10^{-2}
Oreochromis niloticus [present work]	9.2	5.55×10^{-2}
Electrophorus electricus [present work] ▲	-	-
Carbofuran		
Cyprinus carpio [35]	0.45	-
Colossoma macropomum [21]	0.92	3.15×10^{-3}
Arapaima gigas [present work]	0.75	2.56×10^{-3}
Rachycentron canadum [present work]	0.082	1.61×10^{-4}
Oreochromis niloticus [present work]	0.19	1.15×10^{-3}
Electrophorus electricus [present work] ▲	0.005	3.63×10^{-5}

* - Purity degree varied from 97.4% to 99.9%; ** - According to Cheng & Prusoff [24] *** - Commercial formulation and; **** - up to 1.0 ppm. NE – Negligible effect; ▲Obtained from Sigma. *C. macropomum* Ki were calculated from data not shown of Assis et al., 2010.

decarbamoylation rates are slow and organophosphates are inhibitors that tend to irreversibility.

The enzyme from *E. electricus* was the most sensitive to pesticides as expected since it was extracted and purified from the electric organ. However, the enzymes from the other species were not far from this sensitivity despite being obtained by a faster and cheaper method. Among the other three tropical enzymes, AChE from *A. gigas* was extremely sensitive to TEPP while the enzyme from *R. canadum* was very sensitive to carbofuran.

All the enzymes were very sensitive to two of the analyzed pesticides (dichlorvos and carbofuran) showing their potential employment as biocomponents of pesticide sensors in environment and food samples.

Several physicochemical and kinetic features of brain acetylcholinesterase from juvenile of four species were investigated and these characteristics can provide more information regarding the use of AChE from sub-adult *A. Gigas, R. canadum, E. electricus* and *O. niloticus* as biocomponent of organophosphorus and carbamate biosensors in pesticide routine screening in the environment.

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Supplementary Figure 1

Effect of five organophosphorus (A, B, C, D and E) and two carbamate (F and G) pesticides on the activity of brain AChE from *A. gigas*. Concentrations ranged from 0.001 to 1000 ppm. Data are expressed as mean ± standard deviation. All the assays were performed at 25ºC.

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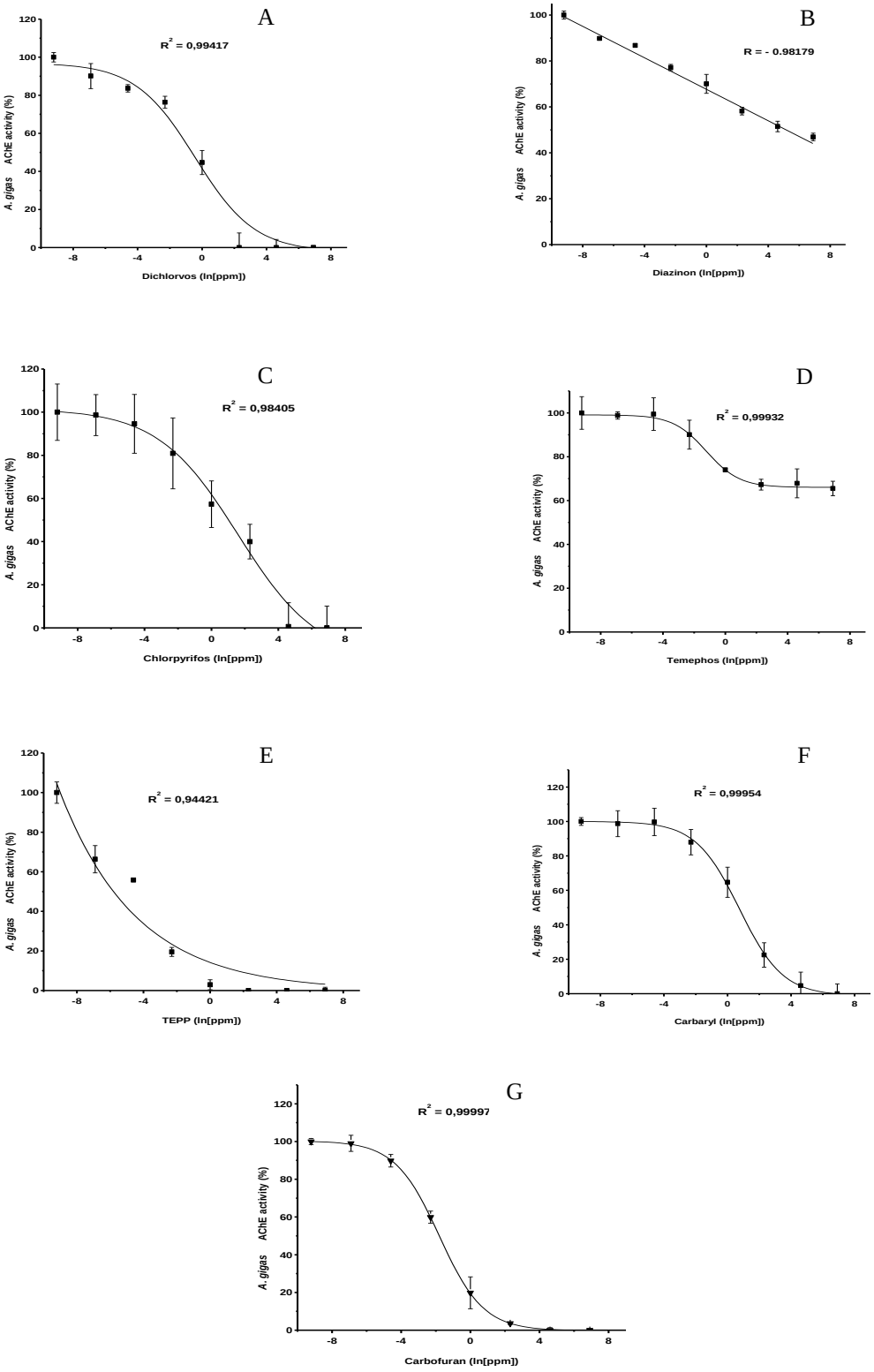

Supplementary Figure 2

Effect of five organophosphorus (A, B, C, D and E) and two carbamate (F and G) pesticides on the activity of brain AChE from *R. canadum*. Concentrations ranged from 0.001 to 1000 ppm. Data are expressed as mean ± standard deviation. All the assays were performed at 25ºC.

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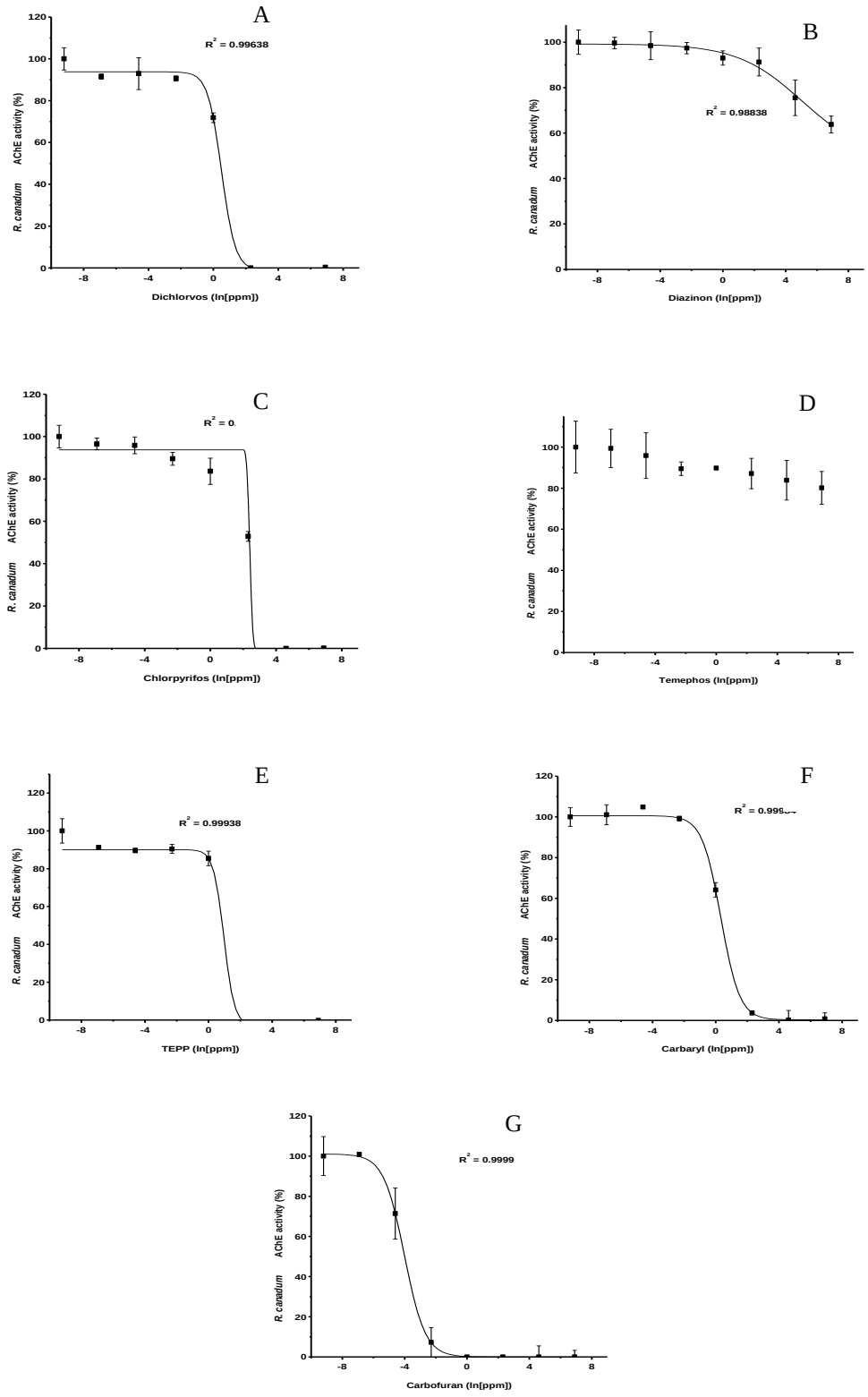

Supplementary Figure 3

Effect of five organophosphorus (A, B, C, D and E) and one carbamate (F) pesticides on the activity of brain AChE from *E. electricus*. Concentrations ranged from 0.001 to 1000 ppm. Data are expressed as mean ± standard deviation. All the assays were performed at 25ºC.

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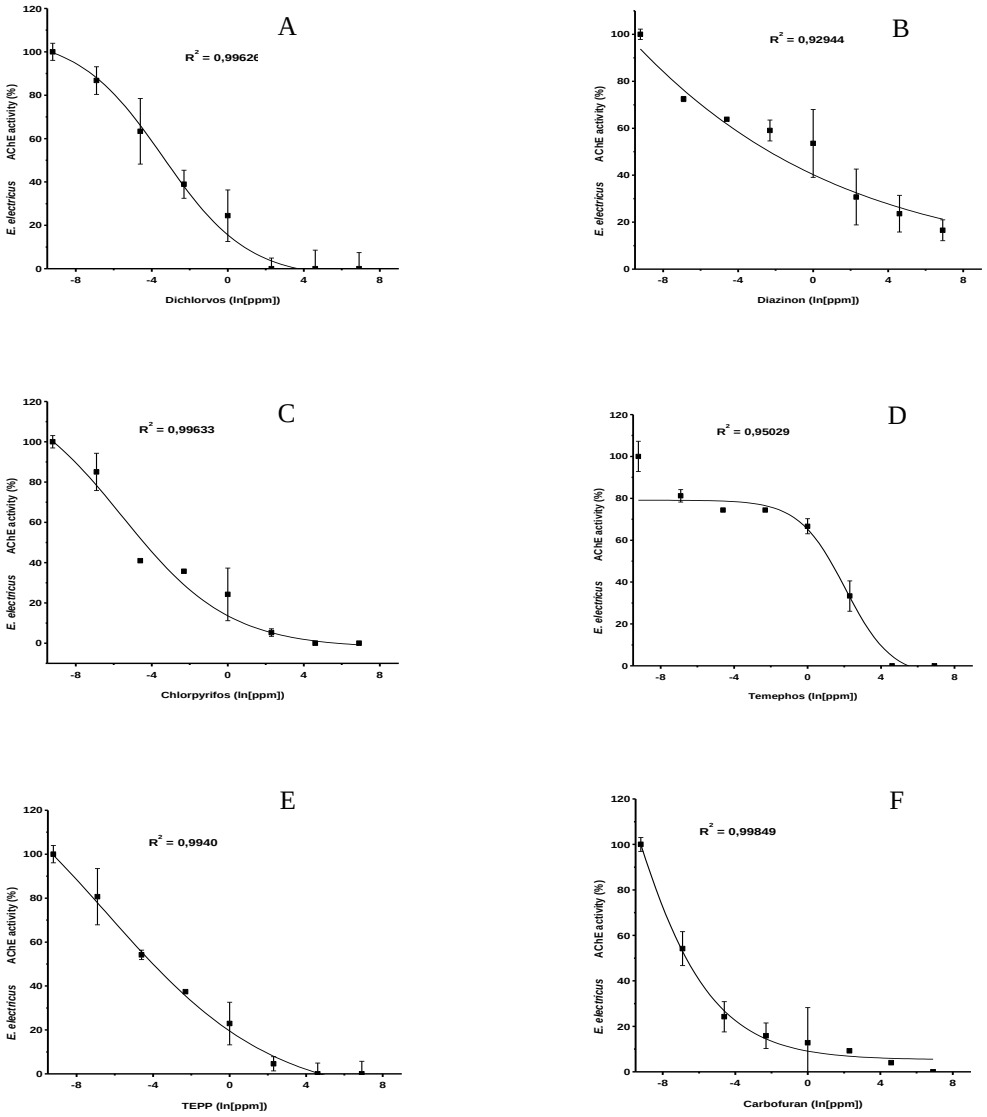

Supplementary Figure 4

Effect of five organophosphorus (A, B, C, D and E) and two carbamate (F and G) pesticides on the activity of brain AChE from *O. niloticus*. Concentrations ranged from 0.001 to 1000 ppm. Data are expressed as mean ± standard deviation. All the assays were performed at 25ºC.

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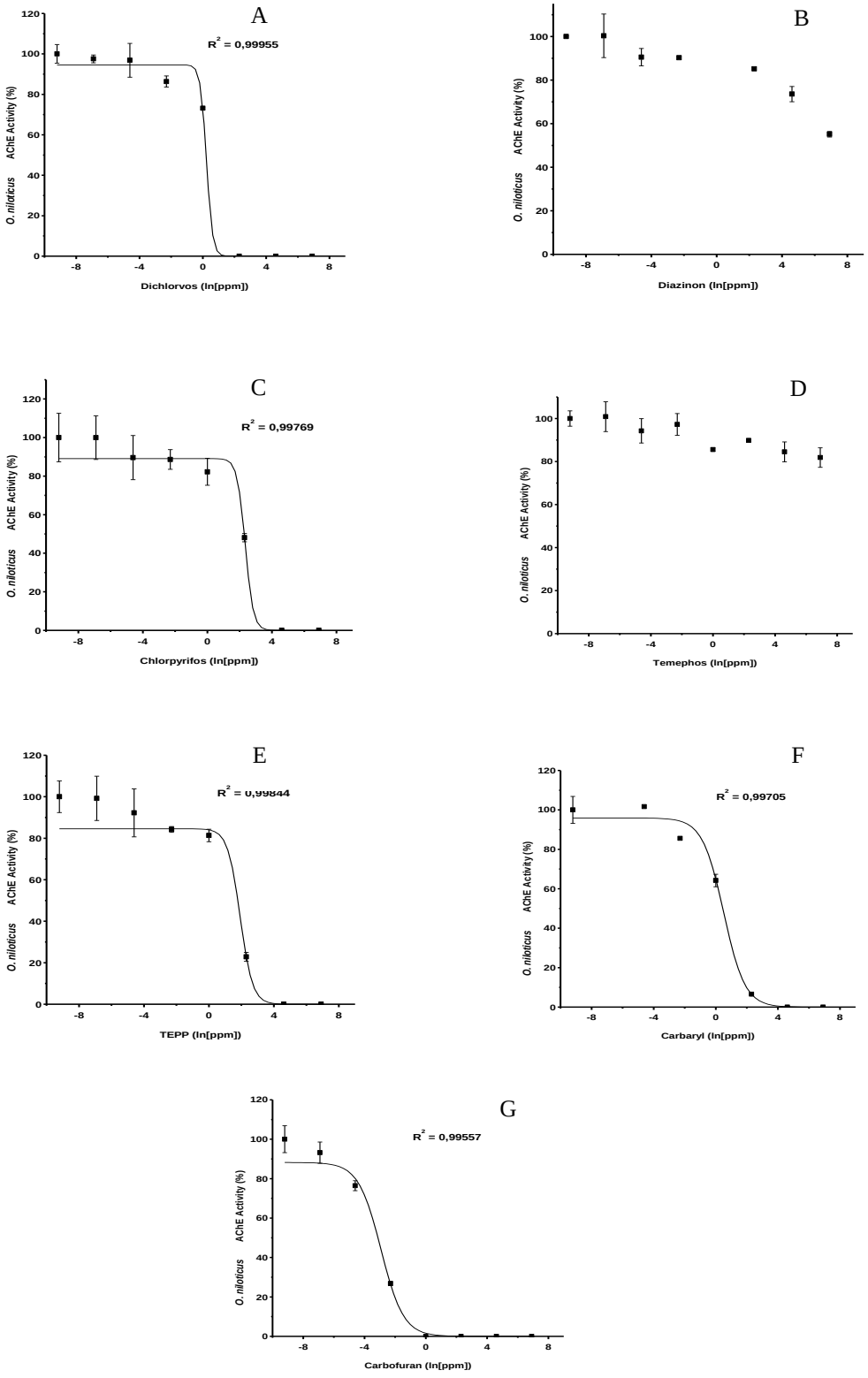

9 - CAPÍTULO V – IN VITRO EFFECT OF IONS ON THE ACTIVITY OF BRAIN ACETYLCHOLINESTERASE FROM FIVE TROPICAL FISH

ARTIGO ENVIADO À REVISTA JOURNAL OF ENVIRONMENTAL MONITORING

Running header: Effect of ions on acetylcholinesterase from five fish.

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In vitro effect of ions on the acetylcholinesterase from five fish

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Abstract

Brain acetylcholinesterase (AChE; EC 3.1.1.7) from five tropical fish (pirarucu, *Arapaima gigas*; tambaqui, *Colossoma macropomum*; cobia, *Rachycentron canadum*; electric eel, *Electrophorus electricus* and Nile tilapia, *Oreochromis niloticus*) were *in vitro* assayed in the presence of fifteen ions (Al^{3+}, As^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, $EDTA^{2-}$, Fe^{2+}, Hg^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}). The exposure was performed at concentrations ranging from 0.001 to 10 mmol L^{-1} (except for EDTA; up to 150 mmol L^{-1}). Hg^{2+}, As^{3+}, Cu^{2+}, Zn^{2+}, Cd^{2+} caused strong inhibition in the species under study. Al^{3+}, Ca^{2+}, K^{+}, Mg^{2+} and Mn^{2+} induced slight activation while Pb^{2+}, Ba^{2+}, Fe^{2+}, Li^{+} inhibited the AChE from some of the analyzed species. The lowest IC50 values were estimated for electric eel AChE in presence of Hg^{2+} (17.5 µmol L^{-1}), Pb^{2+} (14.2 µmol L^{-1}) and Zn^{2+} (16.1 µmol L^{-1}) showing that this enzyme could be susceptible to interference from these ions in environmental samples. The IC50 values estimated for these ions action on AChE activity from the other species were higher. Most of the inhibition was only detected at concentrations higher than 1 mM that is a far above those found in environmental samples not associated with mining or industrial enterprises. Therefore, excepting for *E. electricus* AChE, they constitute a minor interfering agent during the use of these enzymes as a biomarker of anticholinesterase compounds. In addition, AChE from these species showed a high potential to serve as a biomarker for the presence of mercury ion.

Key words: Ions, Acetylcholinesterase, Biomarkers, Fish

9.1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a crucial enzyme for the development and functioning of the nervous system and play an important role in hematopoietic differentiation (Silman and Sussman, 2005). Its classical function is to modulate the nerve impulse through the hydrolysis of the neurotransmitter acetylcholine in the synaptic cleft (Quinn, 1987). AChE inhibition is the mechanism of action of organophosphorus and carbamate pesticides, as well as the mode of action of the drugs used in treatment of Alzheimer's disease (Silman and Sussman, 2005). AChE have been also used for monitoring these pesticides and other compounds *in vivo* (Antwi, 1987; Rendón-von Osten et al., 2005) and *in vitro* (Beauvais et al., 2002; Shaoguo et al., 2003; Rodríguez-Fuentes and Gold-Bouchot, 2004), including as a biocomponent of electrochemical devices (Marco and Barceló, 1996; Amine et al., 2006).

The investigation of AChE inhibitors and interfering substances are relevant to identify the usefulness of this enzyme as a tool in environmental and food monitoring (Fairbrother and Bennett, 1988; Bocquené et al., 1990; Payne et al., 1996; Rodríguez-Fuentes and Gold-Bouchot, 2004; Rodríguez-Fuentes et al., 2008). Monitoring at biochemical level can specifically detect the presence of contaminants in the environment before they reach higher organizational levels (Monserrat et al., 2003).

Several studies reported the influence of ions on the activity of AChE (Abou-Donia and Menzel, 1967; Mukherjee and Bhattacharya, 1974; Olson and Christensen, 1980; Tomlinson et al., 1981; Hughes and Bennett, 1985; Gill et al., 1990; 1991; Devi et al., 1995; Najimi, 1997; Reddy et al., 2003). Conformational inhibitory changes in cholinesterases ascribed to zinc were responsible for its protective effect against the organophosphate chlorpyrifos in serum and liver from rats (Goel et al., 2000). The interaction between these enzymes and lithium presented two contrasting behaviours: relieving action against AChE inhibition by lead (Hughes and Bennett, 1985) and lethal synergism with physostigmine (eserine) and neostigmine (Davis and Hatoum, 1980). Fish AChE activation by Ca^{2+}, Mg^{2+}, Al^{3+} has been reported (Tomlinson et al., 1981; Hughes and Bennett, 1985). Therefore, high content of these ions in water samples from rivers can influence the detection of anticholinesterasic pesticides. These findings must be

taken into account when biosensors based on AChE activity are proposed to analyze pesticide presence in some environment conditions. This fact can lead to false positives or negatives and misinterpretations in the analysis of results.

There are high inter and intra-specific AChE polymorphism that can cause varied responses to the same compounds, thereby hindering the evaluation and comparison of results from different studies (Tõugu, 2001). Therefore, studies on the enzyme behaviour in different species are recommendable (Weill et al., 2003; Wiesner et al., 2007). Cholinesterase inhibition has been assayed in several species including aquatic organisms due to its ability to assess the environmental impact even when these compounds are not present in the water (Sturm et al., 1999a).

This study aimed to investigate the effect of different ions (Al^{3+}, As^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, $EDTA^{2-}$, Hg^{2+}, K^{+}, Li^{+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}) that could influence the activity of brain AChE from five fish, providing information to support its potential use as a biomarker for the presence of anticholinesterase agents.

9.2. Materials and Methods

9.2.1. Materials

AChE from electric eel *Electrophorus electricus* type VI-S, Acetylthiocholine iodide, bovine serum albumin, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), tris (hydroxymethyl) aminomethane e magnesium sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen chloride, aluminium chloride, barium chloride, calcium chloride, lithium chloride and sodium arsenite were obtained from Merck (Darmstadt, Germany). Cadmium chloride, copper chloride, ferrous chloride, manganese chloride, lead chloride and zinc chloride were acquired from Vetec (Rio de Janeiro, RJ, Brazil). Disodium EDTA, mercuric chloride and potassium chloride were from Reagen (Rio de Janeiro, RJ, Brazil). The microplate spectrophotometer used was Bio-Rad xMark™ (Hercules, CA, USA) whereas the tissue disrupter was IKA RW-20 digital (Staufen, Germany). The juvenile specimens of *C. macropomum* (30.0 ± 4.2 cm; 512.5 ± 123.7 g), *A. gigas* (76.8 ± 8.7 cm; 4,118 ± 207.9 g) and *O. niloticus* (12.0 ± 3.0 cm; 7.9 ± 1.2 g) were supplied by the Department of Fisheries and Aquaculture of the Universidade Federal Rural de Pernambuco (Recife, PE, Brazil). *R. canadum* (51.67 ± 1.5 cm; 1,575 ± 329.6 g) was supplied by Aqualider Ltda. (Recife, PE, Brazil).

9.2.2. Methods

9.2.2.1. Enzyme extraction

The juvenile fishes were sacrificed in an ice bath (0ºC). The brains were immediately removed, pooled and homogenized in 0.5 M Tris-HCl buffer, pH 8.0 (from now on abbreviated as the buffer), maintaining a ratio of 20 mg of tissue per ml of buffer. The homogenates were centrifuged for 10 min at 1000 x *g* (4ºC) and the supernatants (crude extracts) were frozen at -20ºC for further assays.

9.2.2.2. Enzyme activity and protein determination.

Enzyme activity was modified from Assis et al. (2010). Briefly: 0.25 mM DTNB (200 μL) prepared in 0.5 M Tris-HCl buffer, pH 7.4 was added to the crude extract (20 μL), and the reaction started by the addition of 62 mM acetylthiocholine iodide (20 μL) except for the C. *Macropomum* assay (125 mM). Enzyme activity was determined by reading the absorbance increase at 405 nm during 180 s. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 μM of substrate per minute. A blank was prepared with the buffer instead crude extract sample. Protein content was estimated according to Sedmak and Grossberg (1978), using bovine serum albumin as the standard.

9.2.2.3. Activity in presence of ions

AChE activity was assayed in presence of fifteen ions: Al^{3+} ($AlCl_3$), Ba^{2+} ($BaCl_2$), Ca^{2+} ($CaCl_2$), Cd^{2+} ($CdCl_2$), Cu^{2+} ($CuCl_2$ and $CuSO_4$), Fe^{3+} ($FeCl_3$), Hg^{2+} ($HgCl_2$), K^{+} (KCl), Li^{+} (LiCl), Mg^{2+} ($MgSO_4$), Mn^{2+} ($MnCl_2$), As^{3+} ($NaAsO_2$), Pb^{2+} ($PbCl_2$ and $Pb(C_2H_3O_2)_2$), Zn^{2+} ($ZnCl_2$) and the complex chelating ion $EDTA^{2-}$ as $C_{10}H_{14}N_2Na_2O_8$. The ions were diluted in distilled water to five final concentrations ranging from 0.001 to 10 mM (excepting $EDTA^{2-}$ up to 150 mM), being each concentration 10-fold higher than the previous one. The ions solutions (10 μL) were incubated with crude extract (10 μL) for 40 min (Bocquené et al., 1990) at 25ºC and the residual activity was determined according to 9.2.2.2. The activity in the absence of the ions were considered as 100%. DTNB (200 μL) was then added to each of the incubated solutions. Data were statistically analyzed using one-way ANOVA and Tukey test and were fitted to linear and non-linear regression through sigmoidal (Boltzmann) or exponential decay ($\rho < 0.05$) modeling

using MicroCal® Origin® Version 8.0 in order to estimate the concentration capable to inhibit enzyme activity in 50% (IC_{50}). The bimolecular constant Ki was calculated using the Cheng and Prusoff equation (1973).

Control assays were carried out with ions that activated the enzymes in order to verify false positive occurrence by a possible influence in extent of DTNB binding: before reading, 10 µL of the samples were incubated with 10 µL of neostigmine bromide 10 mM (which completely inhibited the enzyme of all species under study) and with 10 µL of each of these ions (10 mM). Blanks were performed replacing the samples by buffer and following the same procedure.

9.3. Results and discussion

Some studies pointed to the influence of ions on the AChE activity by binding to peripheral sites (Tomlinson et al., 1980; Olson and Christensen, 1980). The main peripheral anionic site in AChE is described as a region near the rim of the gorge where is located the active center (Eastman et al., 1995). Nevertheless there are binding sites for positively charged activators and inactivators far from the active site of the enzyme which are different for organic and inorganic molecules (Tomlinson et al., 1981). AChE from several sources are under allosteric control and such allosteric sites bind depolarizing, nondepolarizing neuromuscular blocking agents, divalent cations, quaternary ammonium activators and inhibitors (Roufogalis and Wickson, 1973). Moreover, some organic and inorganic ions are suggested to change the hydration state of the active center, modifying the rate of hydrolysis by AChE (Hughes and Bennet, 1985). In the present study, were used five concentrations of each ion and due to the high amount of graphic data were chosen to report the results referring to 1 mM concentration (**Table 1)** and to the IC_{50} and Ki values (**Table 2** and **Table 3**).

Five ions caused increase in enzyme activity at 1 mM: Al^{3+}, Ca^{2+}, K^{+}, Mg^{2+} and Mn^{2+}. The control assays with these ions showed no statistical difference between them and the respective blanks (Fig. 1).

In some works aluminium inhibited AChE from bovine brain and from electric organ of *E. electricus* and this inhibition occurs in an ionic strength dependent manner (Marquis and Lerrick, 1982; Sharp and Rosenberry, 1985; Moraes and Leite, 1994). They defend an interaction between this ion and the residue Glu in the

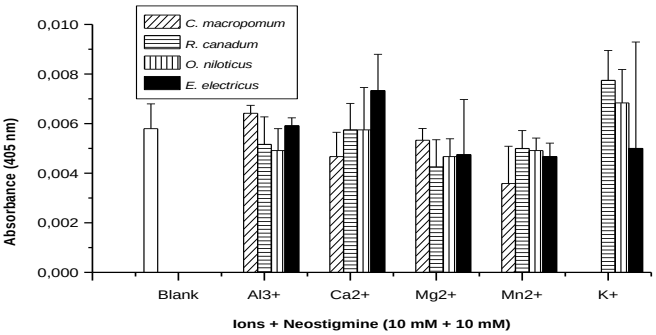

Fig.1 Control assays with ions that activated the enzymes to verify false positive occurrence by a possible DTNB binding with other proteins. No statistical differences were observed by using ANOVA and Tukey test ($\rho < 0.05$).

active site of the enzyme. However, such residue can only provide a weak interaction confirmed by the findings for the active site of BChE (which also present this residue in its catalytic triad) by Sarkarati et al. (1999) and Szilágyi et al. (1994). Here, the ionic strength conditions are different and aluminium III activated *E. electricus* AChE in 4% while for the same species Hughes and Bennet (1985) described an increase about 30%. These results are consistent with data about the role played by aluminium in Alzheimer's disease where it appear to increase the ChE activity in brain of human and rat by binding to peripheral site or altering the neuron membrane integrity (Zatta et al., 2002).

In the present work, Ca^{2+} increased the activity of AChE from *C. macropomum* (20%), *R. canadum* (10%) and *E. electricus* (6%). In contrast to the value of Ca^{2+} on *E. electricus* activity, Tomlinson et al. (1980) and Hughes and Bennet (1985) also worked with this species and found activations of 60% and 40%, respectively. Mg^{2+} promoted an increase in *R. canadum* (15%) and *E. electricus* (10%) enzymic activity while Tomlinson et al. (1980) reported about 60% in their work. The activation reported for Ca^{2+} and Mg^{2+} by these lasts authors occurred at high concentrations of substrate. The ion K^{+} only activated *C. macropomum* AChE and in approximately 20%. The activation values of Mn^{2+} on *R. canadum* and *E. electricus* activities were 38 and 5%, respectively.

Copper and zinc are known as strong inhibitors of AChE. The inhibitions found here, respectively, for copper and zinc were 23 and 15% (*A. gigas*), 17 and 35% (*C. macropomum*), 75 and 23% (*R. canadum*), 75 and 78% (*E. electricus*), 18 and 29% (*O. niloticus*). The findings by Tomlinson et al. (1980) of *E. electricus*

Table 1 – Inhibition or activation (a) of AChE activity from several freshwater and marine species at 1mM by fifteen ions and heavy metals.

Species	Al^{3+}	As^{3+}	Ba^{2+}	Ca^{2+}	Cd^{2+}	Cu^{2+}	$EDTA^{2-}$	Fe^{2+}	Hg^{2+}	K^{+}	Li^{+}	Mg^{2+}	Mn^{2+}	Pb^{2+}	Zn^{2+}
Oreochromis niloticus [Present work]	ns	61%	ns	ns	35%	18%	ns	ns	100%	ns	ns	ns	ns	18%	29%
Colossoma macropomum [present work]	ns	57%	25%	20% (a)	18%	ns	ns	20%	100%	20% (a)	14%	ns	ns	18%	35%
Arapaima gigas [present work]	ns	10%	ns	ns	ns	23%	ns	15%	71%	ns	ns	ns	ns	32%	15%
Electrophorus electricus [present work][e]	4% (a)	57%	60%	6% (a)	49%	75%	28%	43%	100%	ns	57%	10% (a)	5% (a)	71%	78%
Rachycentron canadum [present work]	ns	63%	14%	10% (a)	33%	75%	6%	ns	100%	ns	10%	15% (a)	38% (a)	15%	23%
Electrophorus electricus [34][f]	-	-	-	± 60% (a)	-	± 100%	-	-	-	-	-	± 60% (a)	-	-	± 100%
Electrophorus electricus [19]	± 30% (a)	-	-	± 40% (a)	-	± 20%	-	-	-	-	NE	-	-	± 100%	-
Cyprinus carpio [44]	-	-	-	-	-	± 69%[b]	-	-	-	-	-	-	-	-	ns[c]
Danio rerio [45]	-	-	-	-	ns	-	-	-	-	-	-	-	-	-	ns
Puntius conchonius [20,21]	-	-	-	-	ns[d]	-	-	-	67%	-	-	-	-	-	-
Pimephales promelas [17]	-	50% [g]	ns	ns	50%[h]	50%[i]	ns	ns	50% [j]	-	-	ns	-	50%[k]	50% [l]

ns – not significant; - not determined; the symbol ± is related to data obtained by graphs in the articles; [b] at 0.36 mM; [c] at 0.31 mM; [d] at 0.001 mM; [e] Obtained from Sigma; [f] Obtained from Sigma and further purified by affinity chromatography; [g] at 0.03 mM; [h] at 0.57 mM; [i] at 0.16 mM; [j] at 1.6 mM; [k] at 7.1 mM; [l] at 10 mM.

AChE for the same ions were both about 100% inhibition at 1 mM, which is in contrast to Hughes and Bennett (1985) who found 20% inhibition of enzyme activity in the same species. Bocquené et al. (1990) reported an inhibition of 100% in two marine species (*Scomber scomber* and *Pleuronectes platessa*) under copper exposition at 1 mM. For zinc the values for the same species were, respectively, 57.4 and 70% at 1 mM.

According to Valle and Ulmer (1972), mercury, lead and cadmium inhibit a large number of enzymes by strongly interacting with functional sulfhydryl groups and AChE is one of such enzymes. Moreover, they described that mercury was the most inhibitory while lead was the least one. Thus, in the present work, among the fifteen ions analyzed, the most inhibitory ion was the Hg^{2+} ion which, completely inactivated AChE from *C. macropomum*, *R. canadum*, *E. electricus* and *O. Niloticus at* 1 mM. The enzyme from *A. gigas* was inhibited in 71% of its activity. Values not too discrepant than that by Olson and Christensen (1980) who found for *Pimephales promelas* 50% inhibition at 1.6 mM. Gill et al. (1990) using AChE from *Puntius conchonius* observed 67% of inhibition at 0.001 mM. Tomlinson et al. (1981) working with AChE from *E. electricus* reported that Hg^{2+} and Pb^{2+}

Table 2 – IC_{50}[a] (mmol L^{-1}) of AChE activity from several freshwater and marine species exposed to fifteen ions and heavy metals.

Species	Al^{3+}	As^{3+}	Ba^{2+}	Ca^{2+}	Cd^{2+}	Cu^{2+}	$EDTA^{2-}$	Fe^{2+}	Hg^{2+}	K^{+}	Li^{+}	Mg^{2+}	Mn^{2+}	Pb^{2+}	Zn^{2+}
Oreochromis niloticus [Present work]	-	0.58	-	-	-	-	-	-	0.24	-	-	-	-	-	6.11
Colossoma macropomum [present work]	-	0.32	-	-	6.30	4.13	-	-	0.13	-	-	-	-	-	3.92
Arapaima gigas [present work]	-	-	-	-	-	5.77	-	-	0.38	-	-	-	-	-	-
Electrophorus electricus [present work][b]	-	0.98	0.05	-	1.26	0.05	21.25	1.16	0.01	-	0.38	-	-	0.01	0.01
Rachycentron canadum [present work]	-	0.21	-	-	1.10	0.37	-	-	0.12	-	-	-	-	-	6.29
Cyprinus carpio [44]	-	-	-	-	-	0.06	-	-	-	-	-	-	-	-	-
Pimephales promelas [17]	18.0	0.03	-	-	0.57	0.16	-	-	1.60	-	-	-	-	7.10	10.0

[a] Concentration capable to inhibit 50% of enzyme activity; [b] Obtained from Sigma

Table 3 – Ki (µmol L^{-1}) of AChE activity from several freshwater and marine species exposed to fifteen ions and heavy metals.

Species	Al^{3+}	As^{3+}	Ba^{2+}	Ca^{2+}	Cd^{2+}	Cu^{2+}	$EDTA^{2-}$	Fe^{2+}	Hg^{2+}	K^{+}	Li^{+}	Mg^{2+}	Mn^{2+}	Pb^{2+}	Zn^{2+}
Oreochromis niloticus [Present work]	-	3.44	-	-	-	-	-	-	1.42	-	-	-	-	-	36.25
Colossoma macropomum [present work]	-	2.20	-	-	43.39	28.45	-	-	0.90	-	-	-	-	-	27.00
Arapaima gigas [present work]	-	-	-	-	-	38.82	-	-	2.56	-	-	-	-	-	-
Electrophorus electricus [present work][a]	-	7.06	0.36	-	9.08	0.36	153.12	8.36	0.07	-	2.74	-	-	0.07	0.07
Rachycentron canadum [present work]	-	2.38	-	-	12.45	4.19	-	-	1.36	-	-	-	-	-	71.22

[a] Obtained from Sigma

complex with the product of Ellman method thiocholine interfering in the assay. However in the same work was found that Hg^{2+} strongly inhibited the enzyme when using *p*-nitrophenyl acetate as substrate and this ion decreased the rate of carbamoylation of the enzyme active site by MC7 which proves the tight binding of Hg^{2+} to the peripheral sites of AChE.

At 1 mM lead was capable to inhibit the enzyme from *A. gigas* (32%), *C. macropomum* (18%), *R. canadum* (15%), *E. electricus* (71%) and *O. niloticus* (18%). Hughes and Bennet (1985) observed an inhibition of about 100% with *E. electricus* and Olson and Christensen (1980) reported 50% inhibition at 7.1 mM for *Pimephales promelas*. In this work, cadmium induced inhibitions of 18 (*C. macropomum*), 33 (*R. canadum*), 49

(*E. electricus*) and 35% (*O. niloticus*) while inhibited *P. promelas* AChE in 50% of its activity at 0.57 mM (Olson and Christensen, 1980).

According to Olson and Christensen (1980), the ion As^{3+} (from AsO^{2-}) is much more inhibitory than As^{5+}. Their findings with the first one were 50% of inhibition at 0.03 mM using *P. promelas*. Here, we used As^{3+} which, inhibited *A. gigas* (10%), *C. macropomum* (57%), *R. canadum* (63%), *E. electricus* (57%) and *O. niloticus* (61%) at 1 mM. Other report about exposition to arsenic in *Scomber scomber* and *Pleuronectes platessa* describes 33 and 31% of inhibition, respectively at 1 mM (Bocquené et al., 1990).

Ba^{2+} and Li^{+} induced, in our experimental conditions, a similar pattern of inhibition. The values found for its exposition were, respectively 25 and 14% (*C. macropomum*), 14 and 10% (*R. canadum*) and 60 and 57% (*E. electricus*). Fe^{2+} caused a decrease in the activity of *A. gigas* (15%), *C. macropomum* (20%) and *E. electricus* (43%).

The chelating ion $EDTA^{2-}$ only inhibited *R. canadum* (6%) and *E. electricus* (28%) at 1 mM. The enzymes from the other species in study were significantly inhibited only after 100 mM by this ion. Such results (excepting for *E. electricus*) are in accordance with Tomlinson et al. (1981) and enables this chelate to be a protective agent against divalent metalic interferents.

9.4. Conclusions

The commercial enzyme from *E. electricus* was strongly influenced by the majority of the ions analyzed (unlike the other enzymes proposed in this paper) which is an undesirable feature for a biosensor. Nevertheless, EDTA can be used to protect this enzyme against divalent metalic cations.

In contrast with *E. electricus* enzyme, AChE from *A. gigas* was the most insensitive to the ions.

The most reactive ion was Hg^{2+} which strongly inhibited the AChE from the five species.

Ions and heavy metals may arise as probable contaminants in samples from different sources and can cause false positives or negatives in pesticides or another anticholinesterasic agent analysis (Payne et al., 1996). In the other hand, analyzing the inhibition produced by these substances along with other methods, it is possible to use the enzyme also as a biomarker for the presence of some heavy metals, according to the waste composition from a given area. Bocquené et al. (1990) and Payne et al. (1996) suggested that in most of cases heavy metals have little potential to inhibit the enzyme in samples not associated with mine and industrial effluents.

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9.5. References

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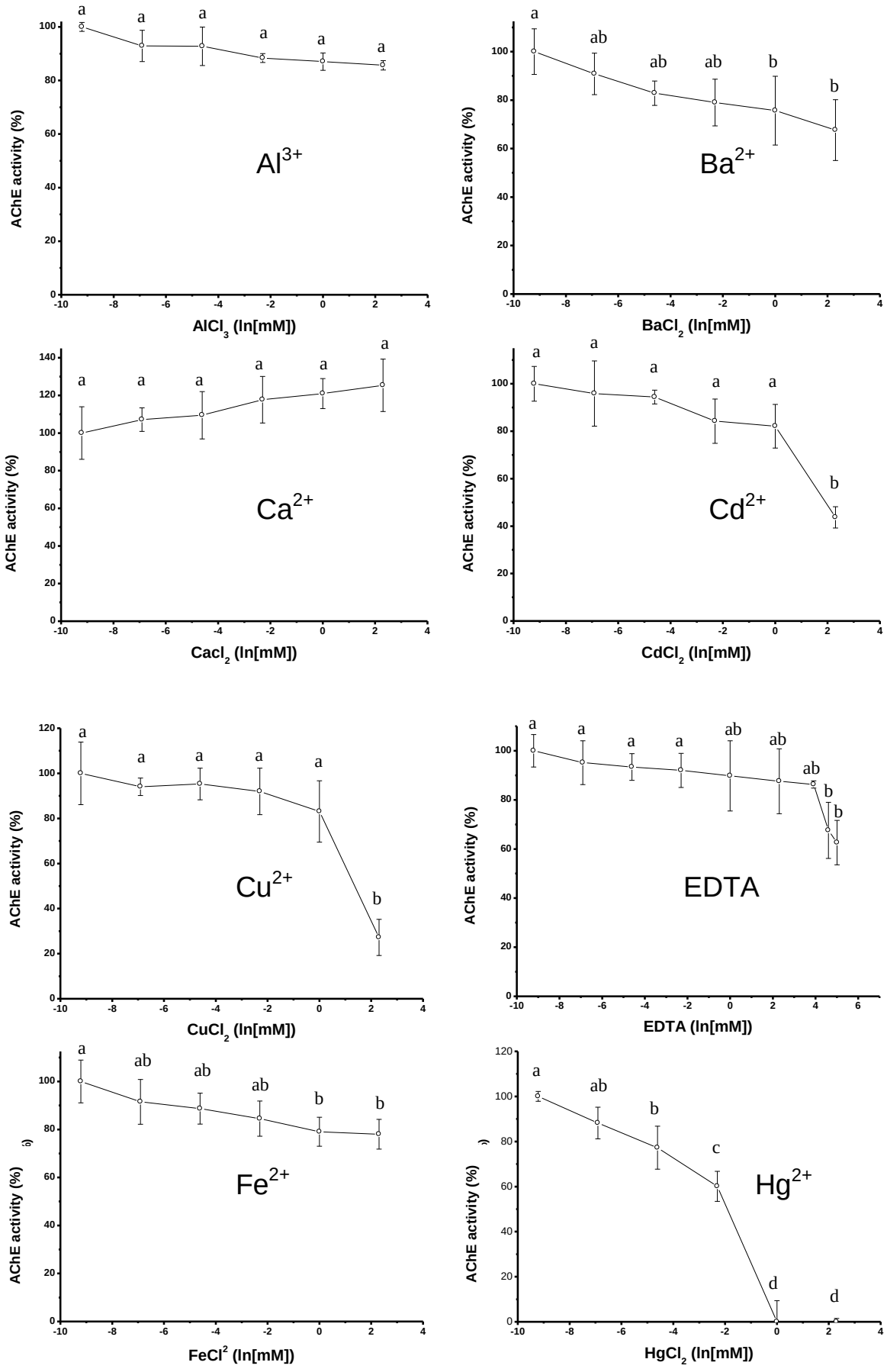
Supplementary data



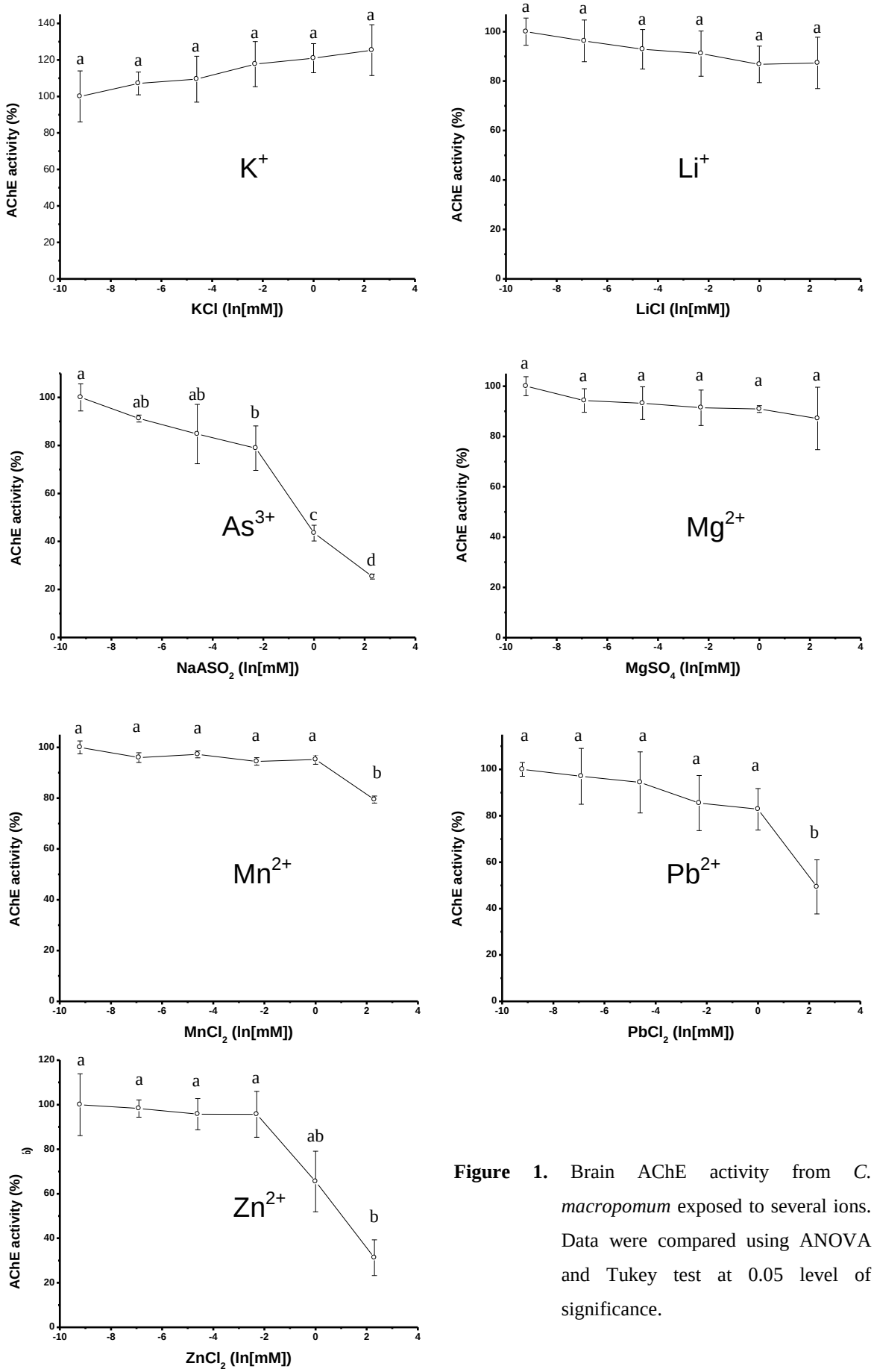
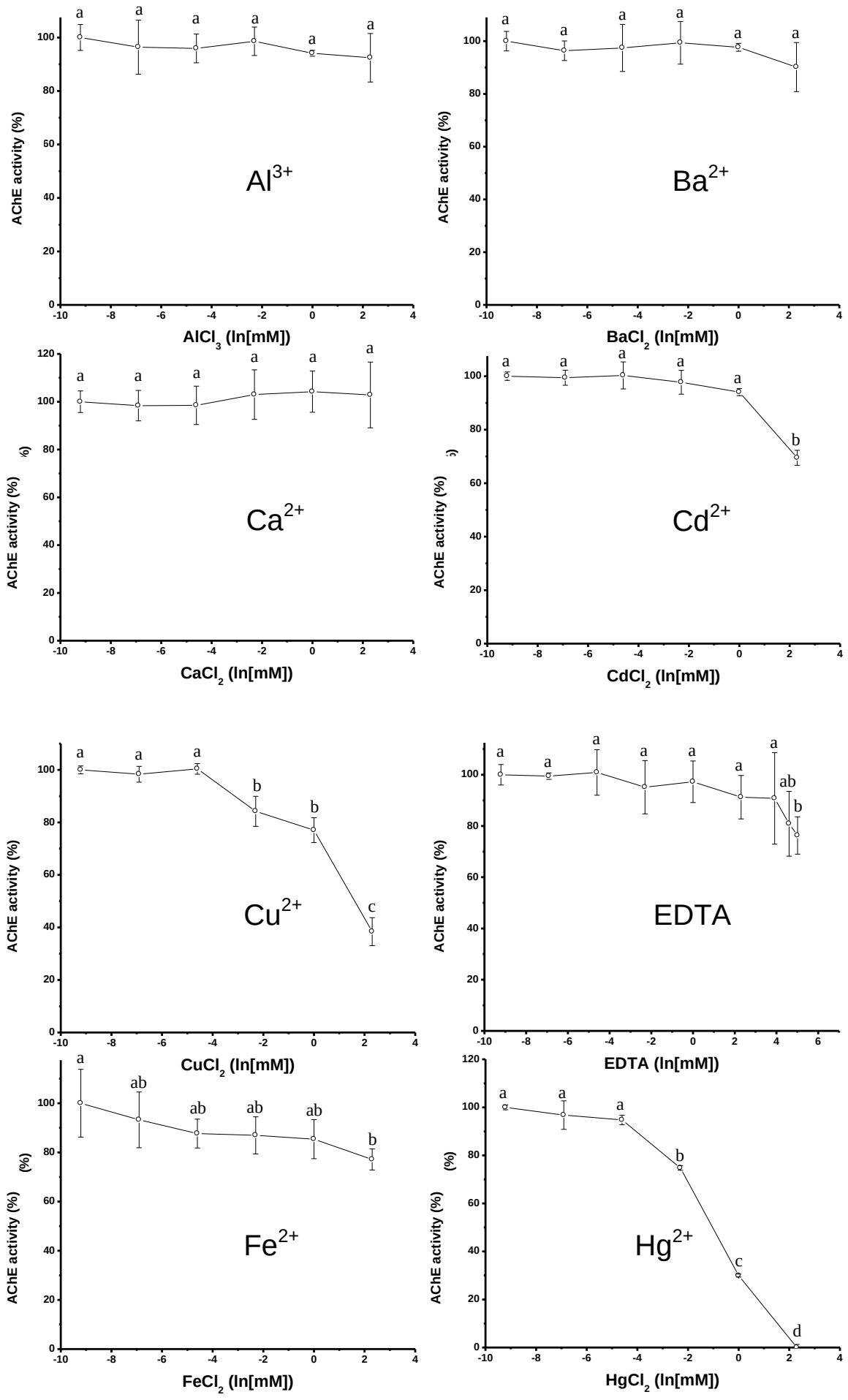


Figure 1. Brain AChE activity from *C. macropomum* exposed to several ions. Data were compared using ANOVA and Tukey test at 0.05 level of significance.


AChE activity (%)
Al3+
AlCl3 (ln[mM])
Ba2+
BaCl2 (ln[mM])
Ca2+
CaCl2 (ln[mM])
Cd2+
CdCl2 (ln[mM])
Cu2+
CuCl2 (ln[mM])
EDTA
EDTA (ln[mM])
Fe2+
FeCl2 (ln[mM])
Hg2+
HgCl2 (ln[mM])

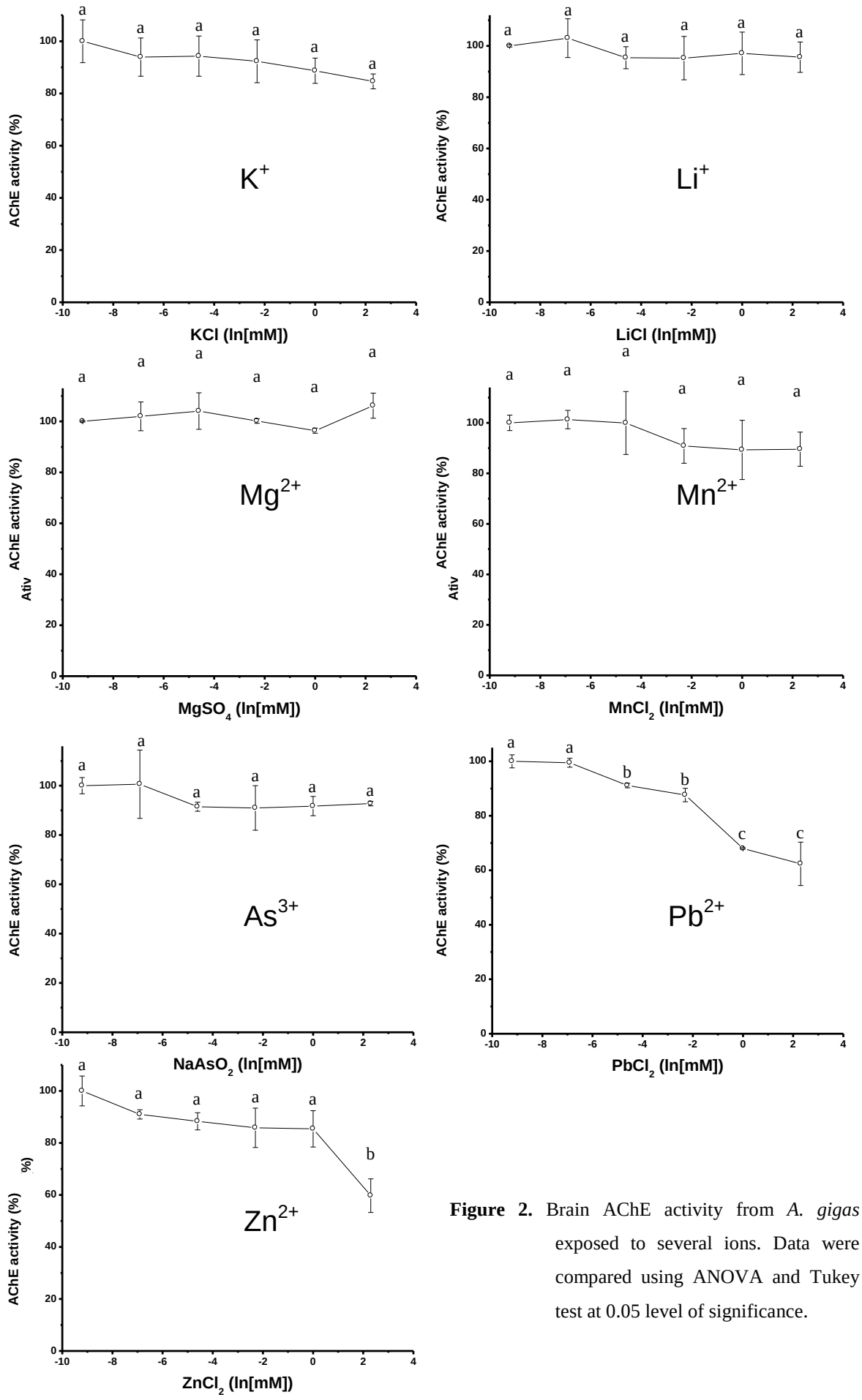
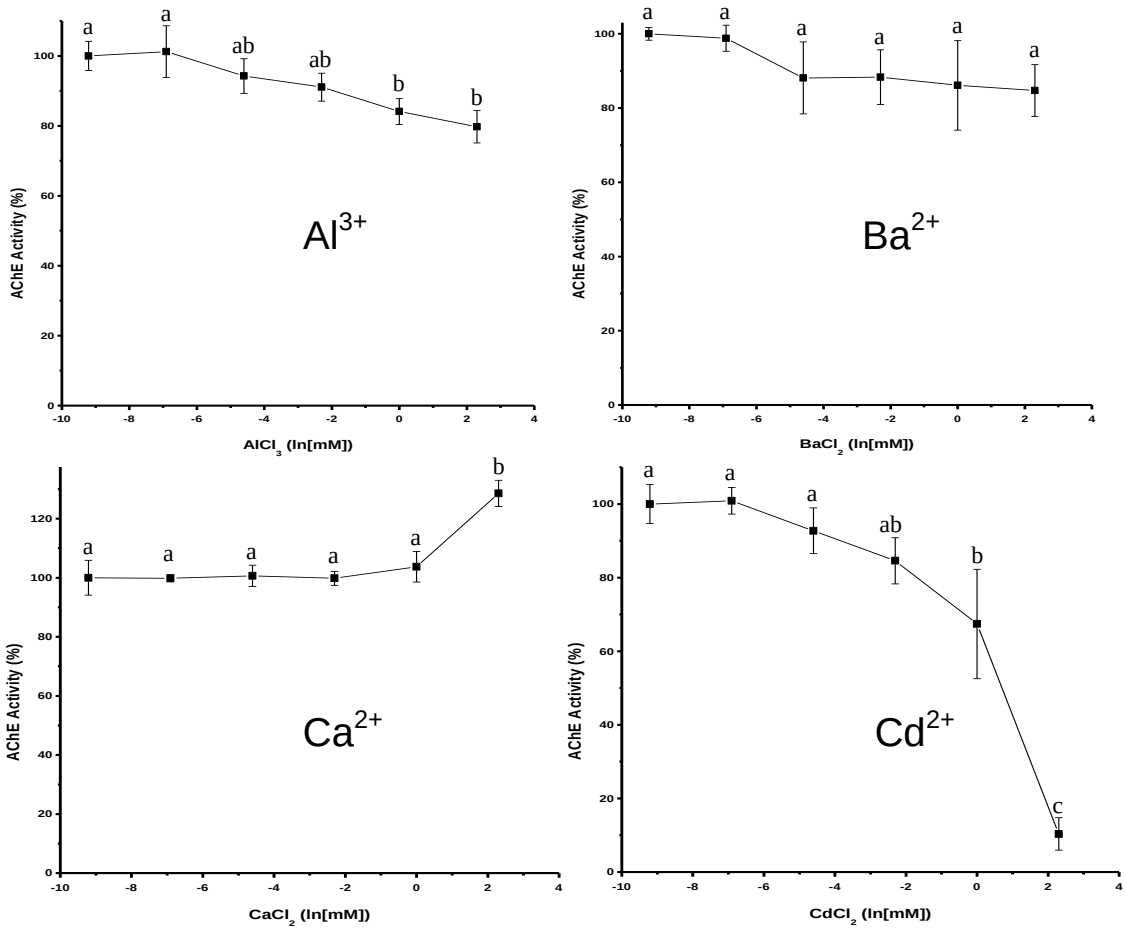
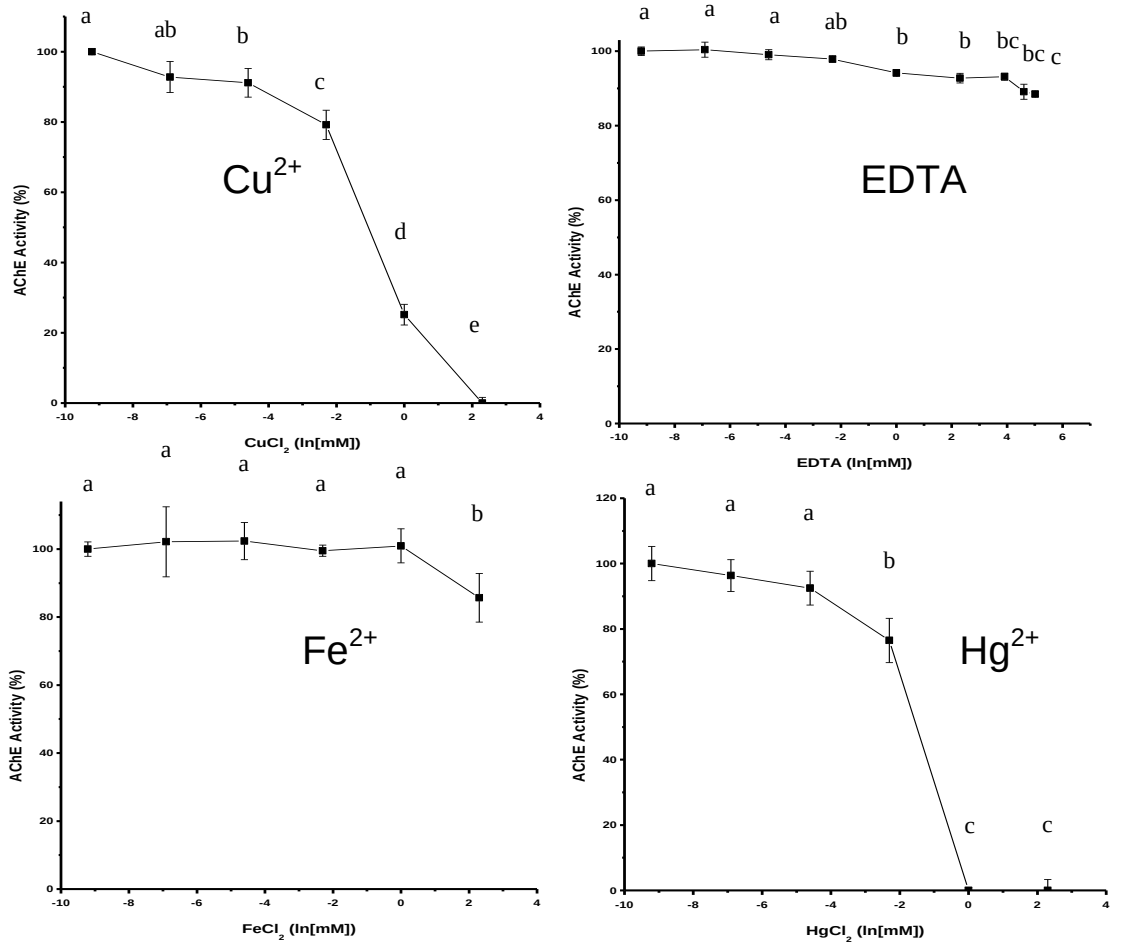


Figure 2. Brain AChE activity from *A. gigas* exposed to several ions. Data were compared using ANOVA and Tukey test at 0.05 level of significance.


Al3+
AlCl3 (ln[mM])
Ba2+
BaCl2 (ln[mM])
Ca2+
CaCl2 (ln[mM])
Cd2+
CdCl2 (ln[mM])
AChE Activity (%)


Cu2+
CuCl2 (ln[mM])
EDTA
EDTA (ln[mM])
Fe2+
FeCl2 (ln[mM])
Hg2+
HgCl2 (ln[mM])
AChE Activity (%)

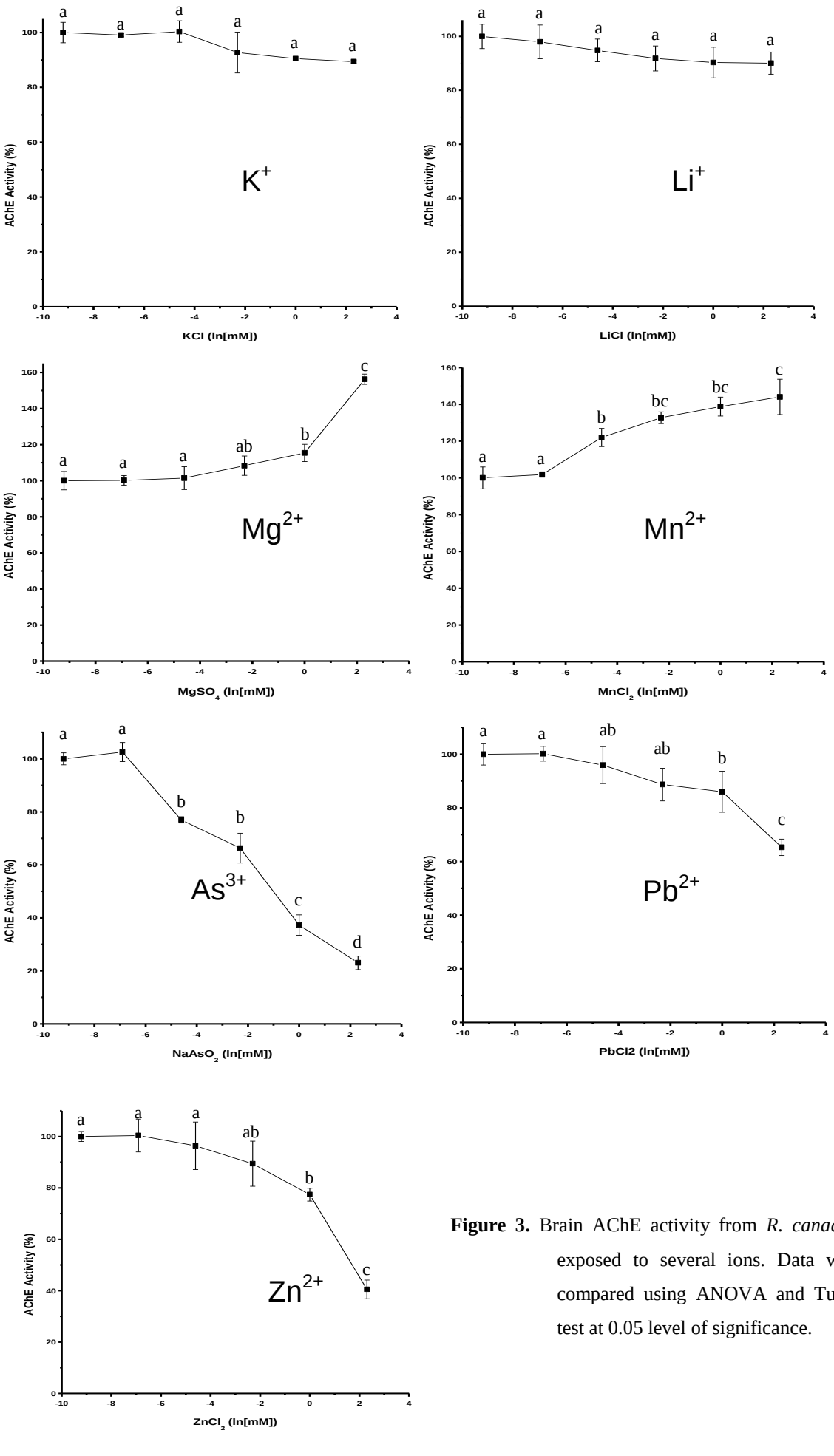
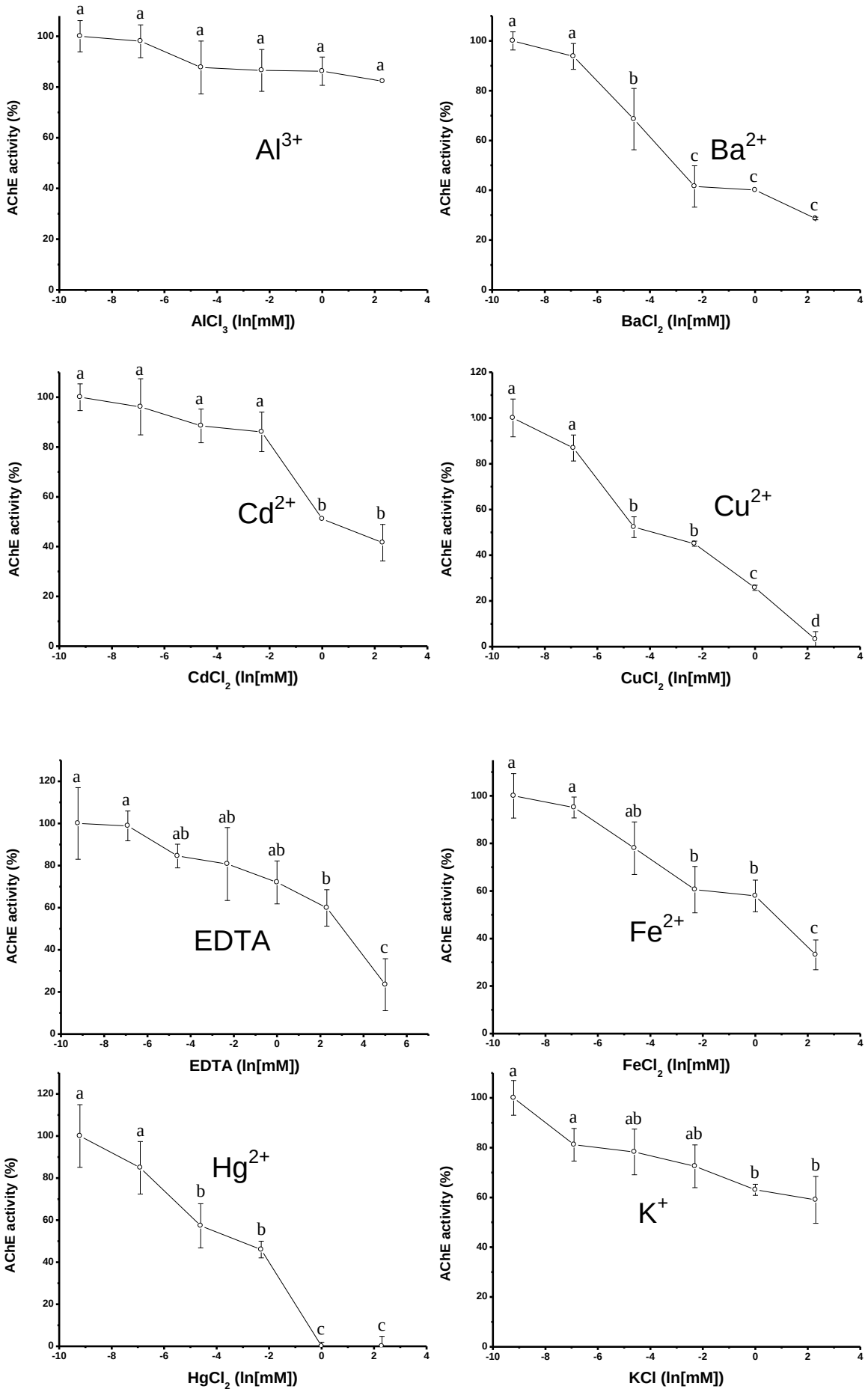


Figure 3. Brain AChE activity from *R. canadum* exposed to several ions. Data were compared using ANOVA and Tukey test at 0.05 level of significance.


AChE activity (%)
Al3+
AlCl3 (ln[mM])
Ba2+
BaCl2 (ln[mM])
Cd2+
CdCl2 (ln[mM])
Cu2+
CuCl2 (ln[mM])
EDTA
EDTA (ln[mM])
Fe2+
FeCl2 (ln[mM])
Hg2+
HgCl2 (ln[mM])
K+
KCl (ln[mM])

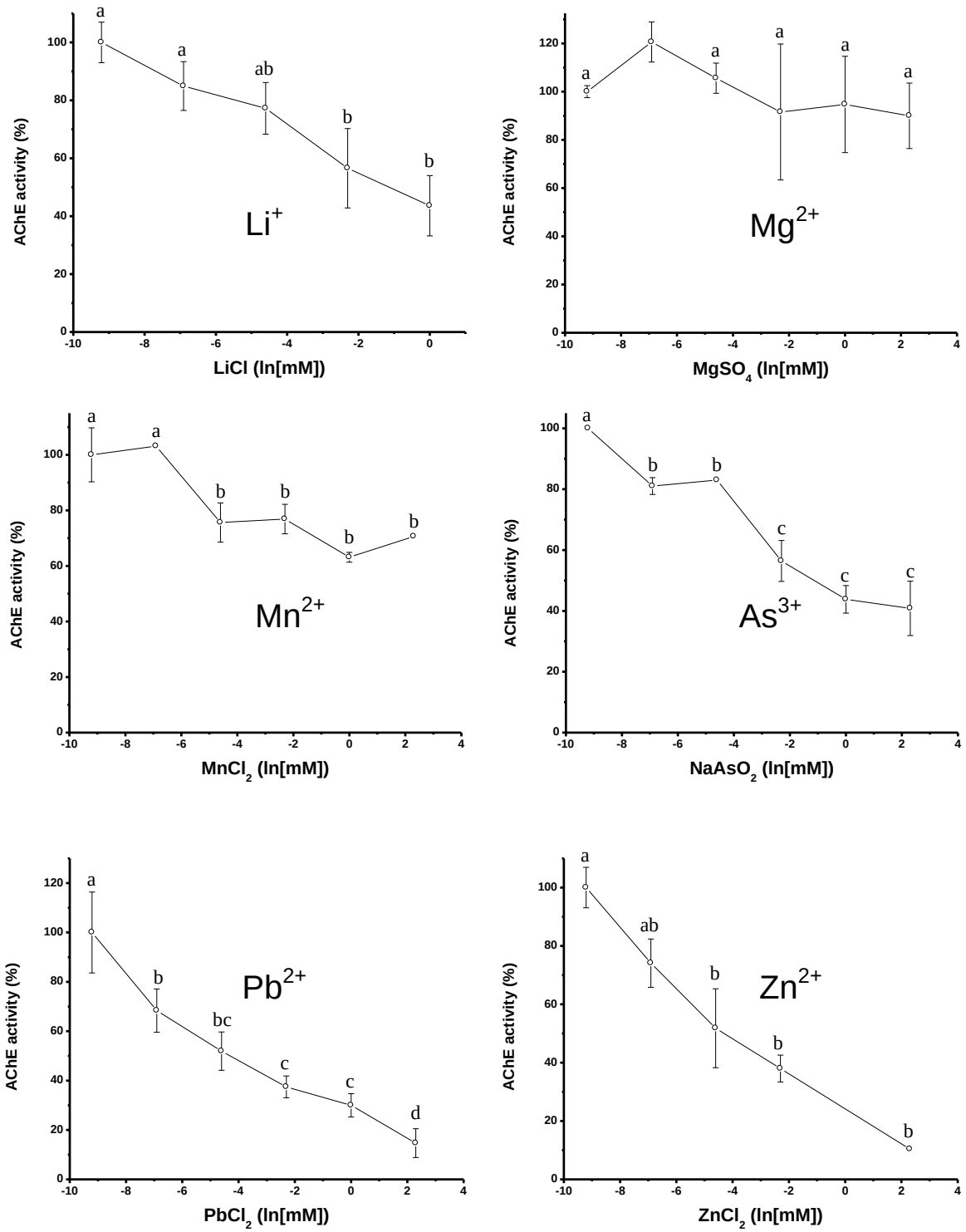
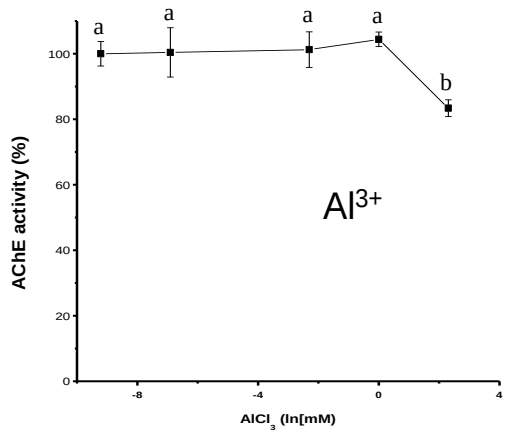
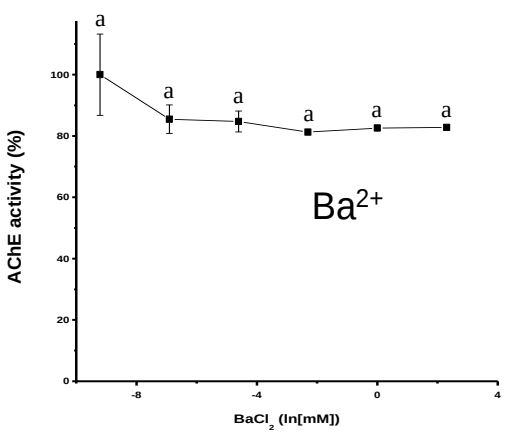
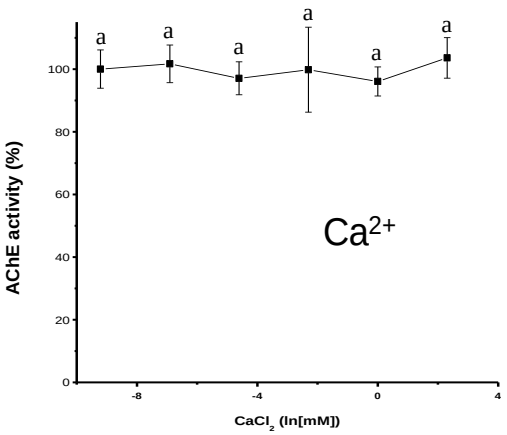
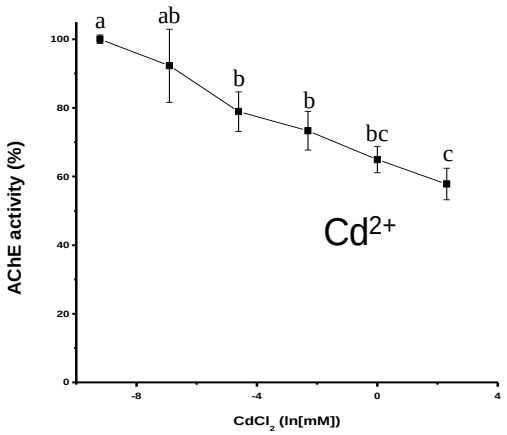


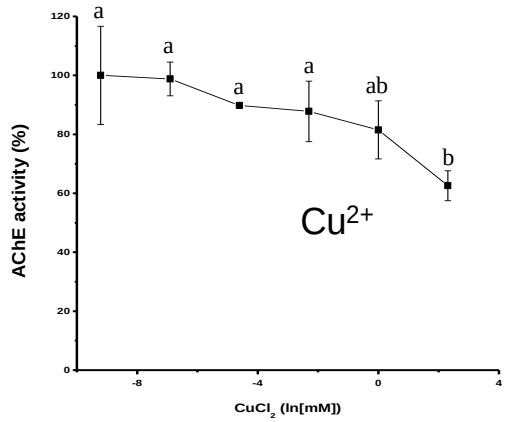
Figure 4. Brain AChE activity from *E. electricus* exposed to several ions. Data were compared using ANOVA and Tukey test at 0.05 level of significance.

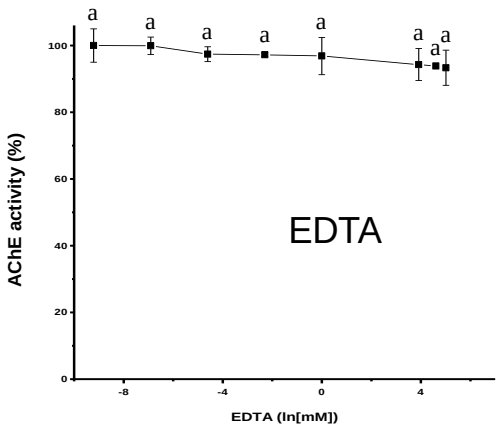

a a a a b
AChE activity (%)
Al3+
AlCl3 (ln[mM)

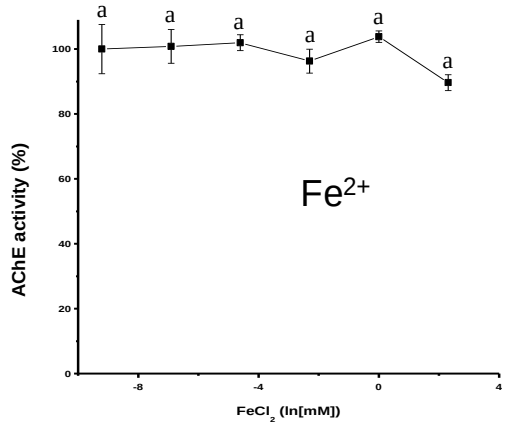

a a a a a a
AChE activity (%)
Ba2+
BaCl2 (ln[mM])

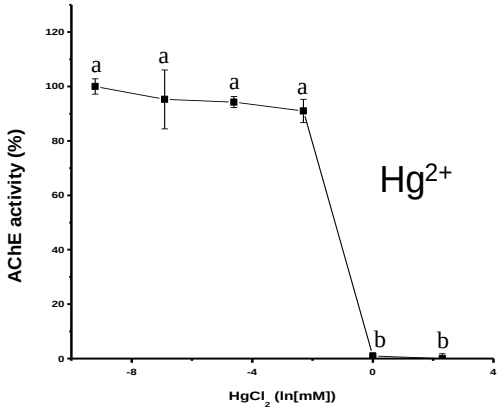

a a a a a a
AChE activity (%)
Ca2+
CaCl2 (ln[mM])


a ab b b bc c
AChE activity (%)
Cd2+
CdCl2 (ln[mM])


a a a a ab b
AChE activity (%)
Cu2+
CuCl2 (ln[mM])


a a a a a a a a
AChE activity (%)
EDTA
EDTA (ln[mM])


a a a a a a
AChE activity (%)
Fe2+
FeCl2 (ln[mM])


a a a a b b
AChE activity (%)
Hg2+
HgCl2 (ln[mM])

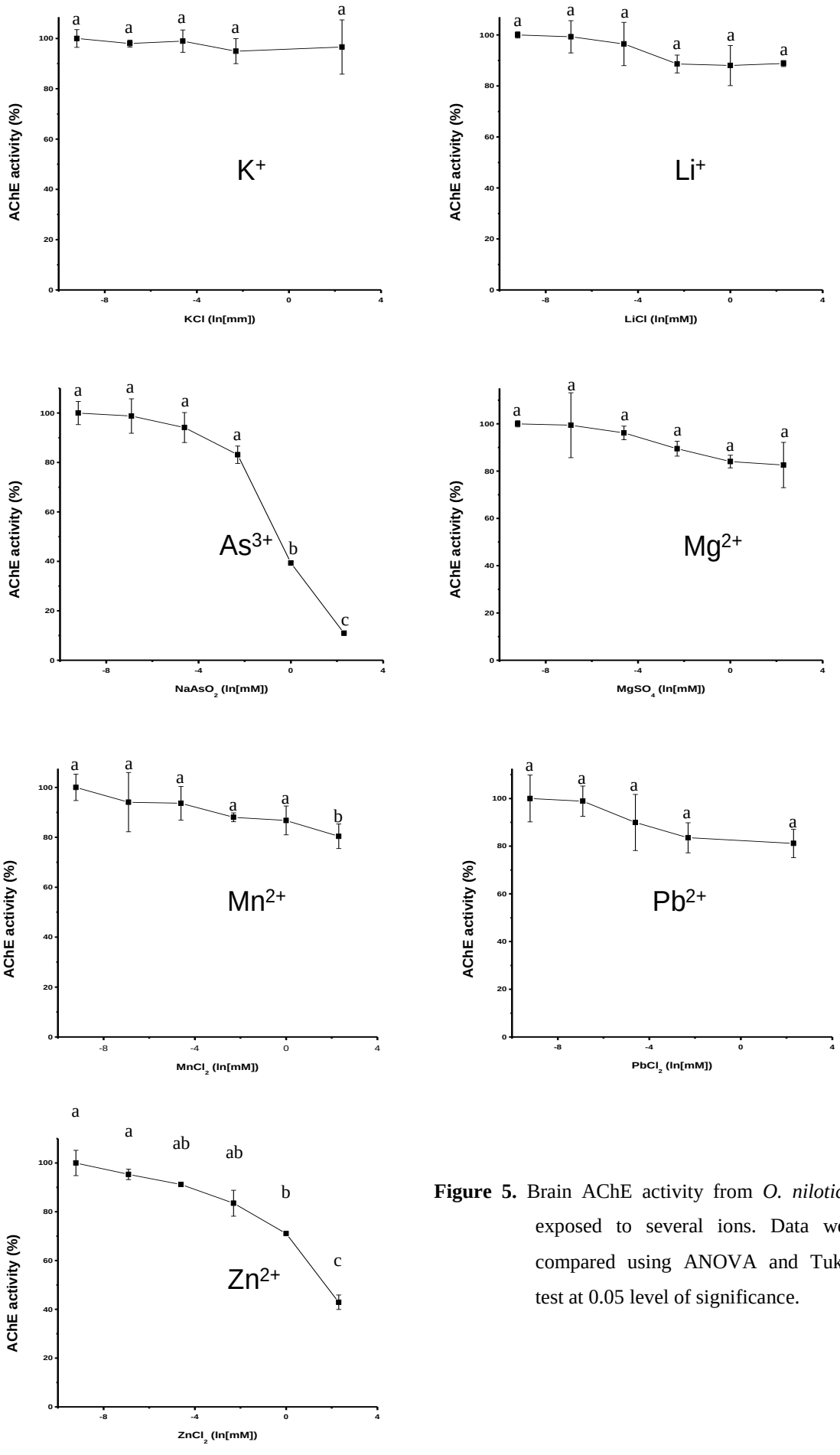
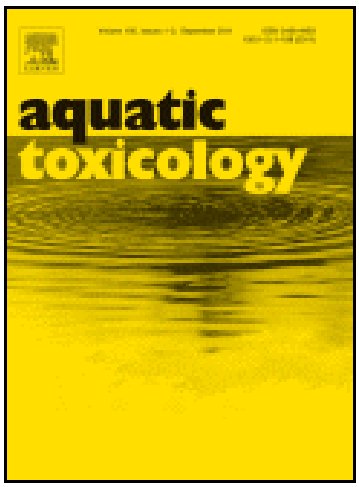


Figure 5. Brain AChE activity from *O. niloticus* exposed to several ions. Data were compared using ANOVA and Tukey test at 0.05 level of significance.

10 - CAPÍTULO VI – KINETIC AND PHYSICOCHEMICAL PROPERTIES OF BRAIN ACETYLCHOLINESTERASE FROM THE PEACOCK BASS (*Cichla ocellaris*) AND IN VITRO EFFECT OF PESTICIDES AND METAL IONS

ARTIGO ENVIADO À REVISTA AQUATIC TOXICOLOGY



Running header: Acetylcholinesterase from *Cichla ocellaris*.

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Total number of words (text and references): 5,271 words

Kinetic and physicochemical properties of brain acetylcholinesterase from the peacock bass (*Cichla ocellaris*) and *in vitro* effect of pesticides and metal ions

Kaline Catiely Campos Silva, Caio Rodrigo Dias Assis, Amanda Guedes Linhares, Vagne Melo Oliveira, Renata Cristina Penha França, Elba Veronica Matoso Maciel de Carvalho, Luiz Bezerra Carvalho Jr and Ranilson Souza Bezerra

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Abstract

Brain acetylcholinesterase (AChE; EC 3.1.1.7) from peacock bass (*Cichla ocellaris*) was characterized and its activity was *in vitro* assayed in the presence of seven pesticides (five organophosphates: dichlorvos, diazinon, chlorpyrifos, temephos, tetraethyl pyrophosphate - TEPP and two carbamates: carbaryl and carbofuran) and fourteen metal ions (Al^{3+}; As^{3+}; Ba^{2+}; Ca^{2+}; Cd^{2+}; Cu^{2+}; Fe^{2+}; Hg^{2+}; K^{+}; Li^{+}; Mg^{2+}; Mn^{2+}; Pb^{2+}; Zn^{2+}) and $EDTA^{2-}$. The kinetic parameters Km and Vmax were determined as 0.769 mM and 0.189 U/mg protein, respectively. Moreover, characters of catalytic power of the enzyme as turnover number (Kcat) and catalytic efficiency (Kcat/Km) were estimated. Selective inhibitors as BW284c51, Iso-OMPA, neostigmine and eserine have confirmed AChE as responsible for the analyzed activity. Optimum pH was found to be 8.0 and optimum temperature was 45ºC. The enzyme retained approximately 45 % of the activity after incubation at 50ºC for 30 min. All the employed pesticides showed inhibitory effects on *C. ocellaris* AChE. However, the strongest effects were observed with carbofuran (IC_{50} = 0.21 µM and Ki = 2.57 x 10^{-3} µM). The enzyme was inhibited by As^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+} whereas its activity was resistant to EDTA until at least 10 mM. The present study provides assay conditions and data about AChE from *C. ocellaris* in relation to its use as *in vitro* biomarker of organophosphorus and carbamate pesticide in routine environmental screening programs.

Key words: Organophosphorus, carbamates, Acetylcholinesterase, Biomarkers, *Cichla ocellaris*

10.1. Introduction

Organophosphate (OP) and carbamate (CB) compounds are the most widely used insecticides in the world. In 2007, only organophosphates accounted for 35% of all insecticides used in the United States (USEPA, 2011). Its toxicity lies in an inhibitory action on cholinesterases enzymes such as acetylcholinesterase (AChE; EC 3.1.1.7) that participates in neuronal communication in most invertebrates and vertebrates, through the hydrolysis of the neurotransmitter acetylcholine in the synaptic cleft (Quinn, 1987) and butyrylcholinesterase (BChE; EC 3.1.1.8) whose physiological function are not elucidated and is commonly considered a detoxifying enzyme (Soreq and Zakut, 1990; Çokugras, 2003; Nicolet *et al*., 2003).

This inhibitory action results in the accumulation of acetylcholine in the synapses of the central nervous system, neuromuscular junctions, sympathetic and parasympathetic nerve endings. High concentration of this neurotransmitter is then released to its receptors (Tõugu, 2001). This inhibition is a specific reaction, considered the main effect of exposure to organophosphorus pesticides (Taylor *et al*., 1995) and carbamates (Jarrard *et al*., 2004). Their mechanism of action occurs through binding to the esteratic site of AChE, with phosphorylation to organophosphates and carbamoylation in the case of carbamates (Quinn, 1987).

AChE has been also used for monitoring these pesticides and other compounds *in vivo* (Antwi, 1987; Rendón-von Osten *et al*., 2005) and *in vitro* (Beauvais *et al*., 2002; Shaoguo *et al*., 2003; Rodríguez-Fuentes and Gold-Bouchot, 2004). The investigation of AChE inhibitors is relevant to identify the usefulness of this enzyme as a tool in environmental and food monitoring (Fairbrother and Bennett, 1988; Bocquené *et al*., 1990; Payne *et al*., 1996; Rodríguez-Fuentes and Gold-Bouchot, 2004; Rodríguez-Fuentes *et al*., 2008). Monitoring at biochemical level can specifically detect the presence of contaminants in the environment before

they reach higher organizational levels (Monserrat et al., 2003).

Studies using fish such as Nile tilapia, *Oreochromis niloticus* (Rodríguez-Fuentes and Gold-Bouchot, 2000), the North-American centrarchid Bluegill, *Lepomis macrochirus* (Beauvais *et al*., 2002), the silver salmon *Oncorhynchus kisutch* (Jarrard et al., 2004), common carp *Cyprinus carpio* (Chandrasekara and Pathiratne, 2005) and correlation between behavioral and physiological changes in indicators of several species (Scott and Sloman, 2004) have confirmed fish as a practical and economically viable source of acetylcholinesterase, able to make routine procedures for biomonitoring of water resources (Bocquené, Galgani and Truquet, 1990). The species chosen in this work was the peacock bass (*Cichla ocellaris*) which is an important native fish. Originally from the Amazon basin (artificially dispersed in several basins of South America) and presenting carnivorous feeding habits, it has demonstrated considerable efficiency in controlling invasive fish in reservoirs. These features, coupled with the excellent quality of its meat, make this species an alternative of high potential to be used in intensive fish farming in the future.

There is still a big gap to be filled for the diagnosis of aquatic environments contaminated by pesticides and, in addition to this fact, there are different results reported in the literature as the correlations between concentrations of pesticides used and the inhibitions rates of absorption and degradation. This study aims to investigate physicochemical and kinetic features of the brain AChE from *C. ocellaris* as well as its behavior in presence of anticholinesterasic pesticides and metal ions in order to identify it as a possible tool for use in environmental monitoring.

10.2. Materials and Methods

10.2.1. Materials

Acetylthiocholine iodide, S-butyrylthiocholine iodide, tetraisopropyl pyrophosphoramide (Iso-OMPA), 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51), neostigmine bromide, eserine, bovine serum albumin, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), tris (hydroxymethyl) aminomethane, dimethyl sulfoxide (DMSO) and magnesium sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade dichlorvos (98.8%), diazinon (99.0%), chlorpyrifos (99.5%), temephos (97.5%), TEPP (97.4%) (Tetraethyl pyrophosphate), carbofuran (99.9%) and carbaryl (99.8%) were obtained from Riedel-de-Haën, Pestanal® (Seelze, Germany). The other reagents were of analytical grade. The juvenile (sub-adults) specimens of *C. ocellaris* (31.17 ± 2.47 cm; 322 ± 14.28 g) were captured in São Francisco River, near the city of Petrolândia, Pernambuco State, Brazil.

10.2.2. Methods

10.2.2.1. Enzyme extraction

The fishes were sacrificed in an ice bath (0ºC). The brains were immediately removed, pooled and homogenized (tissue disrupter IKA RW-20 digital, Staufen, Germany) in 0.5 M Tris-HCl buffer, pH 8.0, until reach a ratio of 20 mg of tissue per mL of buffer. The homogenates were centrifuged for 10 min at 1,000 x *g* (4ºC) and the supernatants (crude extracts) were frozen at -20ºC for further assays.

10.2.2.2. Enzyme activity and protein determination.

Enzyme activity was performed according to Assis *et al*. (2010) as follows: 0.25 mM DTNB (200 μL) prepared in 0.5 M Tris-HCl buffer pH 7.4 was added to the crude extract (20 μL), and the reaction started by the addition of 62 mM of acetylthiocholine or S-butyrylthiocholine iodide (20 μL). Enzyme activity was determined by following the absorbance increase at 405 nm for 180 s using a microplate spectrophotometer Bio-Rad xMark™ (Hercules, CA, USA). A unit of activity (U) was defined as the amount of enzyme capable of converting 1 μM of substrate per minute. The blanks were prepared with the buffer instead of crude extract sample. Protein content was estimated according to Sedmak and Grossberg (1978), using bovine serum albumin as the standard.

10.2.2.3. Kinetic parameters

The kinetic parameters Michaelis-Mentem constant (Km) and maximum velocity (Vmax), were estimated with increasing acetylthiocholine concentrations from 0.8 to 20.8 mM final concentration and fitting to non-linear regression using the software MicroCal™ Origin® Version 8.0 (MicroCal, Northampton, MA, USA). The parameters of enzyme catalytic power as the turnover number (Kcat) and the catalytic efficiency (Kcat/Km) were determined by the estimation of the total amount of AChE (Et) present in brain extracts of the species under analysis according to Assis et al. (2011b).

10.2.2.4. Optimal pH and temperature

Assays were performed with DTNB solutions in a pH range from 4.0 to 9.0 by using citrate-phosphate (4.0 – 7.5), tris-HCl (7.2 – 9.0) buffers. Substrate non-enzymatic hydrolysis (in basic pH) was corrected by subtracting their values from the activities. Optimum temperature was established by assaying the enzyme activity at temperatures ranging from 0 to 80ºC for 180 s. Thermal stability of fish AChE was evaluated by exposing crude extract samples for 30 min at temperatures ranging from 25 to 80ºC and assaying the remaining activity after 15 minutes of 25ºC (room temperature) equilibration.

10.2.2.5. Selective inhibitors assays

The samples were subjected to selective inhibitors BW284c51 (AChE inhibitor), Iso-OMPA (BChE inhibitor), neostigmine bromide and eserine (total cholinesterases inhibitor) in order to identify which cholinesterases are present in the brain of *C. ocellaris*. The inhibitors were diluted at concentrations from 0.001 to 10 mM with each subsequent concentration 10-fold higher than the previous concentration. They were incubated (10 μL) with the crude extract (10 μL) for 1 h. Then, DTNB 0.25 mM were added (200 μL) and the reaction started with the addition of 62 mM substrate (20 μL). The reading was followed at 405 nm for 180s under the same conditions of 6.2.2.2. The respective residual activities were determined, considering the absence of inhibitors as 100% activity.

10.2.2.6. Activity in presence of metal ions

AChE activity was assayed in presence of fifteen ions: Al^{3+} ($AlCl_3$), Ba^{2+} ($BaCl_2$), Ca^{2+} ($CaCl_2$), Cd^{2+} ($CdCl_2$), Cu^{2+} ($CuCl_2$ and $CuSO_4$), Fe^{3+} ($FeCl_3$), Hg^{2+} ($HgCl_2$), K^+ (KCl), Li^+ (LiCl), Mg^{2+} ($MgSO_4$), Mn^{2+} ($MnCl_2$), As^{3+} ($NaAsO_2$), Pb^{2+} ($PbCl_2$ and $Pb(C_2H_3O_2)_2$), Zn2+ ($ZnCl_2$) and the complex chelating ion $EDTA^{2-}$ as $C_{10}H_{14}N_2Na_2O_8$. The ions were diluted to concentrations ranging from 0.001 to 10 mM (excepting $EDTA^{2-}$ up to 150 mM), being each concentration 10-fold higher than the previous one as described for selective inhibitors. The ions solutions (10 μL) were incubated with crude extract (10 μL) for 40 min (Bocquené et al., 1990) at 25ºC and the residual activity was determined according to 2.2.2 and 2.2.5.

10.2.2.7. Inhibition assay by pesticides

AChE inhibition assays were carried out using the organophosphates dichlorvos, diazinon, chlorpyrifos, temephos and TEPP and the carbamates carbaryl and carbofuran as inhibitors. The insecticides were diluted to seven concentrations ranging from 0.001 to 1000 ppm (μg/mL). These concentrations respectively corresponded (in μM) to: 0.0045 to 4520 (dichlorvos); 0.0032 to 3280 (diazinon); 0.0028 to 2850 (chlorpyrifos); 0.0021 to 2140 (temephos); 0.0034 to 3450 (TEPP); 0.0061 to 6130 (carbaryl); and 0.0045 to 4520 (carbofuran). The incubation was performed in accordance to Assis *et al*. (2007) and the residual activity was determined according to 6.2.2.5. All assays were carried out at room temperature (25ºC).

10.2.2.8. Estimation of IC_{50}, IC_{20} and Ki

Data from curves generated in the inhibition assays were statistically analyzed by linear and non-linear regression fitted to sigmoidal (Boltzmann) or exponential decay ($\rho < 0.05$) modeling using MicroCal™ Origin® Version 8.0. Then, were estimated the IC_{50} and IC_{20} (concentration able to inhibit the enzyme in 50 and 20 % of its activity, respectively) corresponding to each inhibitor, pesticide or ion. These data were required to calculate the inhibition constant (Ki) using the equation of Cheng and Prussoff (1973).

10.3. Results

The kinetic parameters Km and Vmax found for *C. ocellaris* were 0.77 mM and 0.189 U/mg protein, respectively using the substrate acetylthiocholine. **Table 1** compares these parameters from several species, where can be seen the variation for Km from 0.1 (*O. niloticus*) to 1.69 mM (*P. Vetulus*) while for Vmax values ranged from 0.129 (*C. macropomum*) to 0.482 U/mg protein (*P. Vetulus*). **Table 1** also displays parameters of catalytic power Kcat and Kcat/Km of *C. ocellaris* AChE which were found to be 1420 s^{-1} and 1.85x106 $M^{-1}s^{-1}$, respectively. They were compared with unpublished data from *C. macropomum* and *P. Squamipinnis*.

Optimum pH for *C. ocellaris* enzyme was found to be 8.0 (**Fig. 1A**). These results are next to the values found for some studies showed in **Table 2**: *Solea solea* (7.0), *Scomber scomber* (8.0) and *Pleuronectes platessa* (8.5).

Figure 1B displays the optimum temperature for *C. ocellaris* AChE estimated as 45 ºC, the same for *C. macropomum*, excepting *P. squamipinnis* (35 ºC) (**Table 2**). For the same parameter, was found 33 ºC for

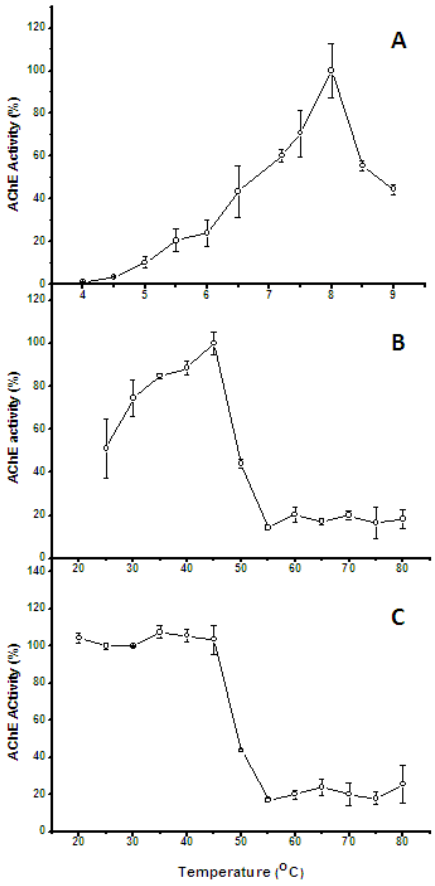

Figure 1 – (A) Effect of pH on the AChE activity from brain of C. ocellaris. The pH range was attained by using citrate-HCl, citrate-phosphate and tris-HCl buffers; (B) Effect of temperature on the activity of brain AChE from C. ocellaris. The activity was assayed in a range from 25 to 80ºC; (C) AChE thermal stability assayed from 20 to 80ºC on the enzyme preparation for 30 min and after 15 min equilibrium at 25ºC.

Pleuronectes platessa, while 25 and 35ºC for *Lepomis macrochirus* and *Carassius auratus*, respectively. The enzyme retained about 45 % of the activity after incubation at 50ºC for 30 min and 15 min equilibration in room temperature (**Fig. 1C**).

Figure 2 shows the *C. ocellaris* AChE activity under exposure to selective inhibitors. It can be observed higher residual activity of brain AChE from *C. ocellaris* which retained almost 100% activity even at 10 mM of Iso-OMPA (**Fig. 1A**), while the results for exposition to BW284c51 was a sudden drop in its activity after 0.001 mM of this inhibitor (**Fig. 1B**). Under neostigmine and eserine exposure, activity decreased sharply at 0.001 mM (**Figs. 1C, 1D**). The IC_{50} and Ki related to each selective inhibitor are presented in **Table 3**.

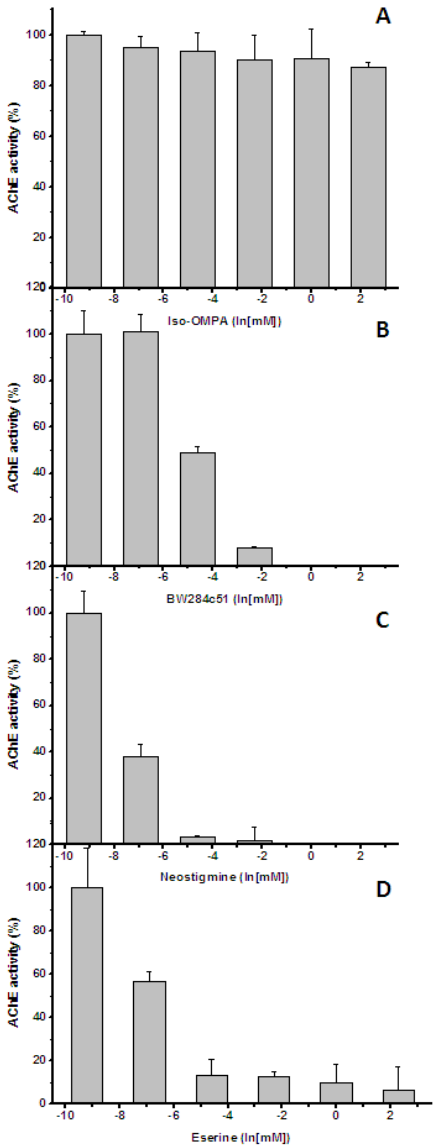

Figure 2 – Activity of C. ocellaris brain AChE in presence of increasing concentrations (0-10 mM) of the selective inhibitors: (A) Iso-OMPA; (B) BW284c51; and the total ChEs inhibitors: (C) neostigmine; (D) eserine.

In relation to heavy metals and other ions, ten cations caused no significant effect on enzyme activity in the concentration range until 1 mM: Al^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Fe^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Pb^{2+}. $EDTA^{2-}$ also did not affect *C. ocellaris* AChE activity in this concentration. On the other hand, some ions caused inhibition As^{3+} (75%), Cu^{2+} (35%), Hg^{2+} (100%) and Zn^{2+} (18%) (**Table 4**). Their IC_{50} and Ki are displayed in **Table 5**. The inhibition by Cd^{2+}, Fe^{2+}, Li^{+} and Pb^{2+}

Table 1. Kinetic and catalytic efficiency parameters of brain AChE from *C. ocellaris* and other species.

Species [References]	**K_m [mM]**	**V_{max} [U/mg protein]**	**K_{cat} [s^{-1}]**	**K_{cat}/K_m [$M^{-1}s^{-1}$]**
Cichla ocellaris [present work]	0.769 ± 0.27	0.189 ± 0.04	1420	1.85 x 10^6
Colossoma macropomum [Assis et al., 2010]	0.430 ± 0.02	0.129 ± 0.05	-	-
Colossoma macropomum [unpublished data]	-	-	6500	1.51 x 10^7
Pachyurus squamipinnis [unpublished data]	0.454 ± 1.58	0.218 ± 0.023	1590	3.5 x 10^6
Oreochromis niloticus [Rodríguez-Fuentes and Gold-Boucht, 2004]	0.10 ± 0.03	0.229 ± 0.014	-	-
Pleuronectes vetulus [Rodríguez-Fuentes et al., 2008]	1.69 ± 0.26	0.482 ± 0.034	-	-

- not determined

occurred only after 1 mM while for $EDTA^{2-}$, inhibition took place after 10 mM (data not shown).

The degree of AChE inhibition by pesticides is represented by the IC_{50} reached for each pesticides and their respective inhibition constant (Ki). The most inhibitory pesticides in contact with *C. ocellaris* AChE were carbofuran, TEPP and carbaryl whose IC_{50} were, respectively, 0.21 µM (Ki = 2.57 x 10^{-3} µM), 0.37 µM (Ki = 4.53 x 10^{-3} µM) and 4.41 µM (Ki = 5.4 x 10^{-2} µM). Temephos did not reach IC_{50} in the concentration range analyzed. **Table 6** also shows the IC_{20} of the pesticides considering that 20% of AChE inhibition is an important point to develop threshold limits by several international regulations.

10.4. Discussion

Before investigating the use of a biomolecule as a biomarker it is necessary to know its normal behaviour through characterization of important features for such role. In the case of enzymes, this characterization is the determination of kinetic and physicochemical parameters of their activity. The km and Vmax found for *C. ocellaris* brain AChE are close to those present in the literature for the same parameters. Among other species, *C. ocellaris* values were compared with *Colossoma macropomum* data from Assis *et al*. (2010) and unpublished data of *Pachyurus squamipinnis*. Moreover, they are in the range value for these parameters according to a recent review (Assis, 2011a) and not far from the values for another Cichlid (Nile tilapia) reported by Rodríguez-Fuentes and Gold-Bouchot (2004). The turnover number Kcat estimated for *C. ocellaris* are in the same order of magnitude as *C. macropomum* and *P. squamipinnis* AChE. However, the values for catalytic efficiency Kcat/Km represent approximately 10-fold less than results for purified enzymes from other species reported by Fersht (1999) probably by the lower concentration in brain extracts besides their higher viscosity, since AChE rate of catalysis is controlled by substrate diffusion limit in the medium (Fersht, 1999; Miller and Wolfenden, 2002).

Optimum temperature of enzymes is not the same as the temperatures commonly found in habitat of the species. They must work in a safe range around the optimum temperature, since after that enzymatic activity does not respond proportionally to increases in temperature and is at denaturation risk. Some enzymes may be damaged, even when long exposed to its optimum temperature. In our experience with other species, the stability peak occurs before optimum temperature. As we can see in the results session, the activity of *C. ocellaris* AChE presents

Table 2. Physicochemical parameters of brain AChE from *C. ocellaris* and other species.

Species [References]	pH optimum	Optimum Temperature [ºC]
Cichla ocellaris [present work]	8.0	45
Colossoma macropomum [Assis et al., 2010]	7.5-8.0	45
Pachyurus squamipinnis [unpublished data]	8.0	35
Solea solea [Bocquene, Galgani e Truquet, 1990]	7,5	-
Pleuronectes platessa [Bocquene, Galgani e Truquet, 1990]	8,5	33
Scomber scomber [Bocquene, Galgani e Truquet, 1990]	8,0	-

- not determined

low thermal stability immediately above of its optimum temperature.

No activity was found using the substrate S-butyrylthiocholine iodide and analyzing the behaviour of the enzyme in presence of selective inhibitors, the cholinesterase under study here can be confirmed as

Table 3. IC_{50} and K_i *in vitro* estimated for *C. ocellaris* in presence of selective inhibitors.

Inhibitor	IC_{50} (µM)	K_i (µM)
BW284c51	9.00	0.11
Iso-OMPA	-	-
Neostigmine	0.69	0.0084
Eserine	1.4	0.0172

– No effect

acetylcholinesterase. Rodríguez-Fuentes and Gold-Bouchot (2004), Jung *et al*. (2007), Pezzementi and Chatonnet (2010), reported absence of BChE activity in some fish species, mainly in brain.

The investigation of AChE inhibitors and interfering substances are relevant to identify the usefulness of this enzyme as a tool in environmental monitoring. Several studies reported the influence of ions and heavy metals on the activity of AChE (Abou-Donia and Menzel, 1967; Tomlinson *et al*., 1980; Olson and Christensen, 1980; Bocquené *et al*., 1990; Reddy *et al*., 2003). Therefore, high content of these ions in water samples from rivers, lakes and reservoirs can influence the detection of anticholinesterasic pesticides. These findings must be taken into account when biomarkers and biosensors based on AChE activity are proposed to analyze pesticide presence or other anticholinesterasic compound in some environment conditions. This fact can lead to false positives or negatives and misinterpretations in the analysis of results.

Table 4. Inhibition of AChE activity from *C. ocellaris* by metal ions at 1 mM ($\rho < 0.05$).

Al^{3+}	As^{3+}	Ba^{2+}	Ca^{2+}	Cd^{2+}	Cu^{2+}	$EDTA^{2-}$
NE	75%	-	-	-	35%	-

Hg^{2+}	K^{+}	Li^{+}	Mg^{2+}	Mn^{2+}	Pb^{2+}	Zn^{2+}	Fe^{2+}
100%	-	-	-	-	-	18%	-

– No effect at 1 mM

Several studies pointed to the influence of ions on the AChE activity by binding to peripheral sites (Tomlinson *et al*., 1980; Olson and Christensen, 1980). Moreover, some organic and inorganic ions are suggested to change the hydration state of the active center, modifying the rate of hydrolysis by AChE (Hughes and Bennet, 1985).

Among the fifteen ions under study no one caused significant increase in *C. ocellaris* enzyme activity at 1 mM while five ions presented inhibitory effect. Copper and zinc are known as inhibitors of AChE (Tomlinson et al., 1980; Olson and Christensen, 1980; Bocquené *et al*., 1990). The inhibitions found here,

Table 5. IC_{50} and Ki values estimated for AChE from *C. ocellaris* and IC_{50} for *Pimephales promelas** in presence of some metal ions.

Species	***C. ocellaris***		***P. promelas***
Ions	**IC_{50} (mM)**	**K_i (mM)**	**IC_{50} (mM)**
As^{3+}	0.1	0.59×10^{-3}	0.03
Cd^{2+}	6.14	36.4×10^{-3}	0.57
Cu^{2+}	2.1	12.5×10^{-3}	0.16
Hg^{2+}	0.22	1.34×10^{-3}	1.60
Zn^{2+}	2.57	15.3×10^{-3}	10.0

*From Olson and Christensen (1980)

Table 6. IC_{50} and K_i *in vitro* estimated for *C. ocellaris* and *C. macropomum* in presence of some organophosphorus and carbamate pesticides.

Species [Reference]	**IC_{20} (µM)**	**IC_{50} (µM)**	**K_i (µM)**
Dichlorvos			
Cichla ocellaris [present work]	4.02	5.52	6.76×10^{-2}
Colossoma macropomum [Assis et al., 2010]	-	0.04	1.37×10^{-4}
Diazinon			
Cichla ocellaris [present work]	182.57	2.9×10^{3}	36.3
Colossoma macropomum [Assis et al., 2010]	ns	ns	ns
Chlorpyrifos			
Cichla ocellaris [present work]	2.17	10.13	1.21×10^{-1}
Colossoma macropomum [Assis et al., 2010]	-	7.6	2.61×10^{-2}
Temephos			
Cichla ocellaris [present work]	ns	ns	ns
Colossoma macropomum [Assis et al., 2010]	ns	ns	ns
TEPP			
Cichla ocellaris [present work]	0.32	0.37	4.53×10^{-3}
Colossoma macropomum [Assis et al., 2010]	-	3.7	1.27×10^{-2}
Carbaryl			
Cichla ocellaris [present work]	1.18	4.41	5.4×10^{-2}
Colossoma macropomum [Assis et al., 2010]	-	33.8	1.16×10^{-1}
Carbofuran			
Cichla ocellaris [present work]	0.082	0.21	2.57×10^{-3}
Colossoma macropomum [Assis et al., 2010]	-	0.92	3.15×10^{-3}

- not determined; ns – not significant in the concentration range analyzed.

respectively, for copper and zinc were 35 and 18%. The findings by Tomlinson et al. (1980) with partially purified *Electrophorus electricus* AChE for the same ions were both about 100% inhibition at 1 mM. Bocquené *et al*. (1990) also reported an inhibition of 100% in two marine species (*Scomber scomber* and *Pleuronectes platessa*) under copper exposition at 1 mM and for zinc the values for the same species were, respectively, 57.4 and 70% at 1 mM.

According to Valle and Ulmer (1972), mercury, lead and cadmium inhibit a large number of enzymes by strongly interacting with functional sulfhydryl groups of them and AChE is one of such enzymes. Moreover, they described that mercury was the most inhibitory while lead was the least one. In the present work, the most reactive was the Hg^{2+} ion which, completely inactivated *C. ocellaris* AChE at 1 mM. Values not too discrepant than that by Olson and Christensen (1980) who found for *Pimephales promelas* 50% inhibition at 1.6 mM. Gill *et al*. (1990) using AChE from *Puntius conchonius* observed 67% of inhibition at 0.001 mM. Tomlinson et al. (1981) working with AChE from *E. electricus* reported that Hg^{2+} and Pb^{2+} complex with the product of Ellman method thiocholine interfering in the assay, however in the same work was found that Hg^{2+} strongly inhibited the enzyme when using *p*-nitrophenyl acetate as substrate and this ion decreased the rate of carbamoylation of the enzyme active site by MC7 which proves the tight binding of Hg^{2+} to the peripheral sites of AChE. In this work, lead and cadmium only decreased AChE activity after 1 mM.

According to Olson and Christensen (1980), the ion As^{3+} (from AsO_2-) is much more inhibitory than As^{5+}. Their findings with the first one were 50% of inhibition at 0.03 mmol/L using *P. promelas*. Here, we used As^{3+} which, inhibited the enzymatic activity in 75% at 1 mM. Other report about exposition to arsenic in *Scomber scomber* and *Pleuronectes platessa* describes 33 and 31% of inhibition, respectively at 1 mM (Bocquené *et al*., 1990).

The chelating ion $EDTA^{2-}$ only inhibited *C. ocellaris* AChE after 10 mM. Such results are in accordance with Tomlinson et al. (1981) and enable this chelate to be a protective agent against divalent metallic interferents.

OP compounds follow different behaviours in its interaction with the active site of cholinesterases depending on the chemical structure of these pesticides. The characteristics of the two groups of organophosphorus pesticides represented by the phosphates (oxon form; P=O) and the phosphorothioates (thion form; P=S) implies in important differences in the power of inhibition. The first group directly inhibits the cholinesterases since the higher electronegativity of the double-bonded oxygene in the phosphoester allows them to strongly interact with the hydroxyl serine group of the enzyme active site. The second one requires bioactivation to achieve their full toxic potential. This biotransformation occurs mainly by environmental factors and oxidative desulfuration mediated by cytochrome P450 isoforms which are found in several tissues, including brain (WHO, 1986; Vale, 1998; Cunha Bastos *et al*., 1999). This may be the reason why TEPP and dichlorvos (P=O) inhibited more intensely the AChE from *C. ocellaris* (and *C. macropomum* for comparison) than chlorpyrifos and diazinon (P=S). In addition, some of the organophosphorus compounds are lipophilic and they are absorbed and accumulated in fat, liver, kidneys and salivary glands. In general, the phosphorothioates are more lipophilic than the phosphates (Vale, 1998). It implies in sequestration of the lipophilic compounds by the brain lipids in the extract and the consecutive minor reactivity by such compounds.

Carbamate insecticides are direct inhibitors of AChE by carbamoylation of the active site and do not require biotransformation, so they can induce acute toxic effects faster than most of OP compounds. AChE can recover its activity in 24 h or less. However, the symptoms of CB inhibition can be more severe.

The importance of investigating the responses from AChE of several species is linked to the fact that different species present different susceptibility to the anticholinesterasic compounds (Assis et al., 2011a). The enzyme of a given species can provide the best monitoring of a compound and another species may be more sensitive to another compound. This monitoring should not be restricted to the environmental health. The enzyme sensitivity can be compared with parameters for human health (Maximum Concentration Levels – MCL's and Acceptable Daily Intakes - ADI's in natural or drinking waters, for example), since their threshold limits are below the limits for animals. According to *Food and Agriculture Organization* (2007) 20% inhibition of AChE activity is the point from which can be considered the

presence of an anticholinesterasic agent. 20% of inhibition in mammals is also the limit to estimate ADI's of anticholinesterasic compounds. Signals and symptoms appear from 50% inhibition and death occurs after 90%.

In the exposure to pesticides, the strongest inhibitory effect on *C. ocellaris* AChE was achieved by the carbamate carbofuran which is known to be a potent anticholinesterasic agent (Tham *et al*., 2009). This carbamate IC_{20} and IC_{50} values (0.082 µM ~ 18 µg/L and 0.21 µM ~ 46.46 µg/L, respectively) for *C. ocellaris* AChE is below or next to the recommended limits of tolerance in some regulations. Brazilian regulations about Maximum Concentration Levels (MCL's) *Resolução CONAMA no. 20/1986* advocate 100 µg/L of organophosphates and carbamate compounds in natural waters of class 3 (water for domestic supply after conventional treatment; irrigation of tree crops, cereals and forage; watering of animals) while the *USEPA National Primary Drinking Water Standards* provides a Maximum Contaminant Level (MCL) of 40 µg/L for carbofuran.

In comparation with national and international institutions (USEPA, 1984; WHO/FAO, 2004; EFSA, 2004 and ANVISA, 2006), the ADI's for carbofuran cannot exceed, respectively, 0.005, 0.002, 0.001 and 0.002 mg/kg bw/day. It means that a person weighing 60 kg, for example, needs to drink 3 L of water which inhibited by 20% brain AChE of *C. ocellaris* to achieve the most demanding ADI for this compound.

AChE from the analyzed species still presented high sensitivity to the organophosphate TEPP (IC_{20} = 0.32 µM ~ 94.84 µg/L and IC_{50} = 0.37 µM ~ 107.37 µg/L). The only report about IC_{50} *in vitro* for TEPP using fish is from Assis *et al*. (2010) in which *Colossoma macropomum* brain AChE was exposed to the same concentrations of this pesticide and presented a value 10-fold higher than with *C. ocellaris*.

The fact that this enzyme had been less inhibited by ions such as copper, zinc, mercury and cadmium than other species in the literature and the lower costs of working with non-purified enzyme may become comparative advantages of using *C. ocellaris* AChE as a biomarker of anticholinesterase pesticides, particularly carbofuran.

10.5. Conclusions

Assay conditions were provided for the use of AChE from *C. ocellaris* through the determination of several physicochemical and kinetics features of this enzyme.

The enzyme had its activity influenced by five ions. However, the inhibitory concentration of such ions is a too high concentration (excepting Hg^{2+} and As^{3+}) to be found in natural samples not associated with mining or industrial effluents (Payne *et al*., 1996). In addition, the EDTA-resistant activity of the enzyme enables this chelate to be used in protection against some cations. On the other hand, analyzing the inhibition produced by these substances along with other methods, it is possible to use the enzyme also as a biomarker for the presence of mercury ion, according to the probable waste composition from a given area.

In this study, some of the highly toxic pesticides were analyzed in relation to *C. ocellaris* AChE sensitivity. Relevant levels of enzymatic inhibition were achieved in concentrations below or next to the Maximum Concentration Levels (MCLs) or Acceptable Daily Intakes (ADIs) for these pesticides contemplated in national and international legislation in force. According to such results, *C. ocellaris* brain AChE is a promising tool for use in environmental monitoring programs for the carbamate carbofuran.

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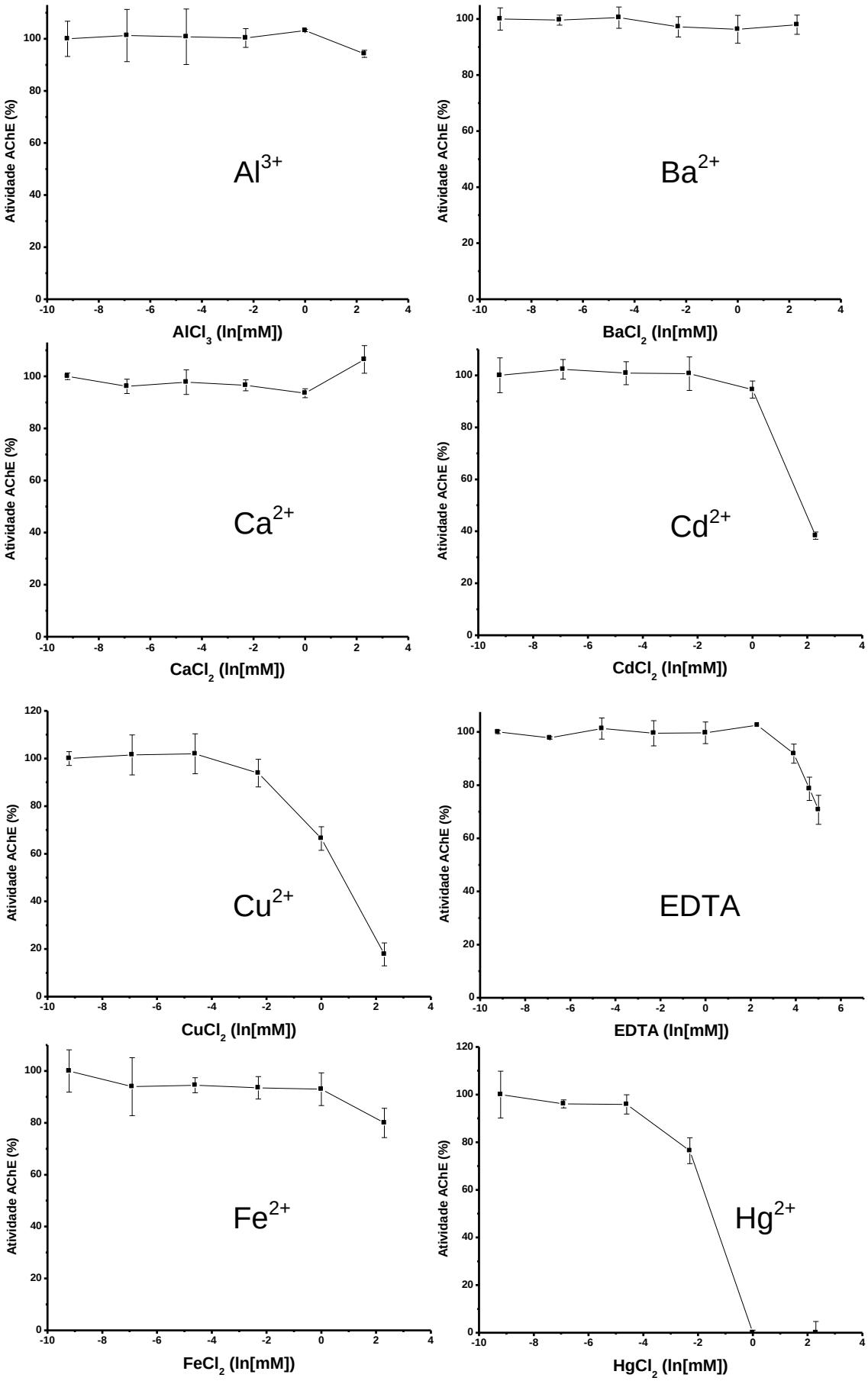
Supplementary data



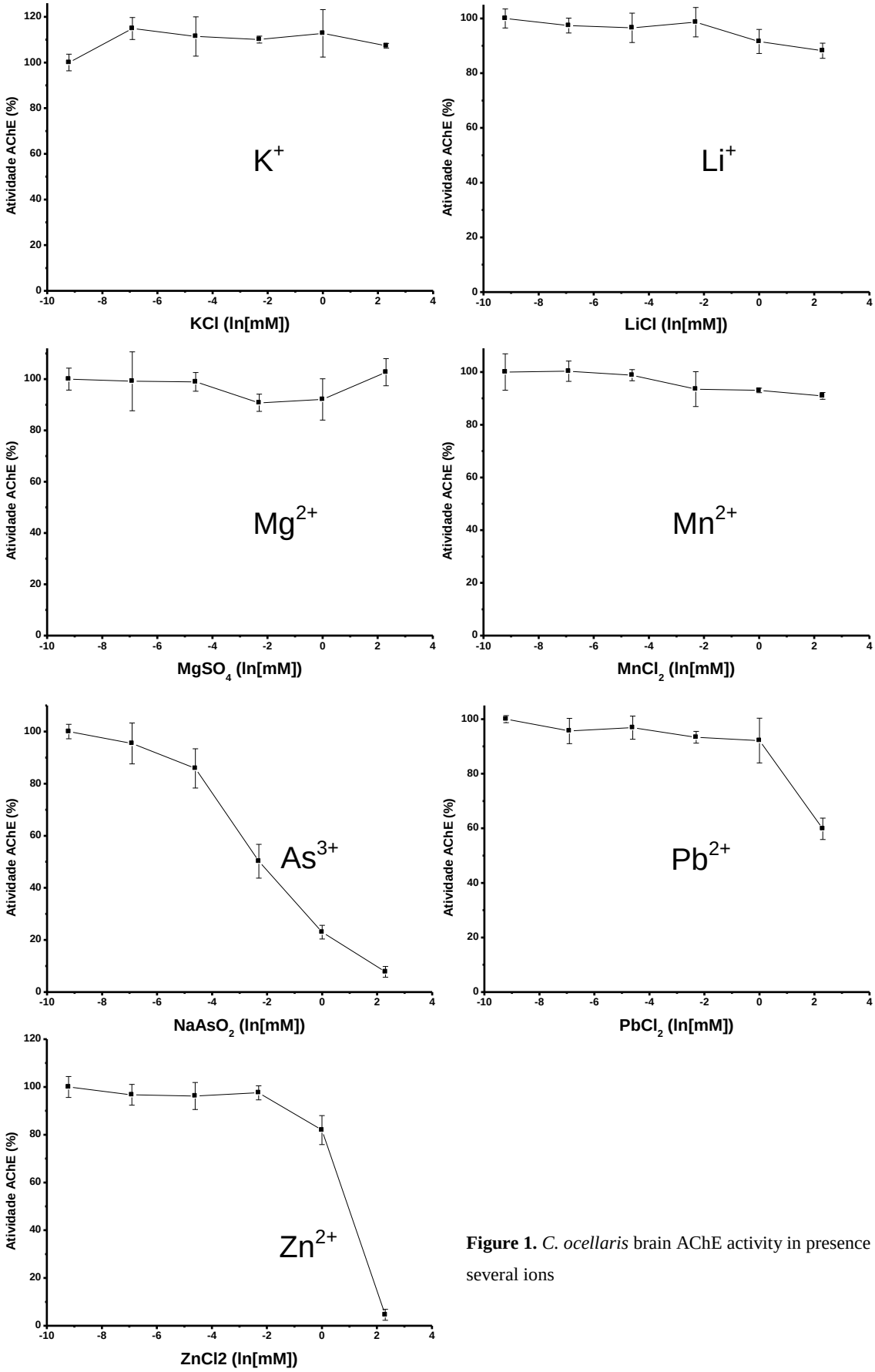


Figure 1. *C. ocellaris* brain AChE activity in presence of several ions

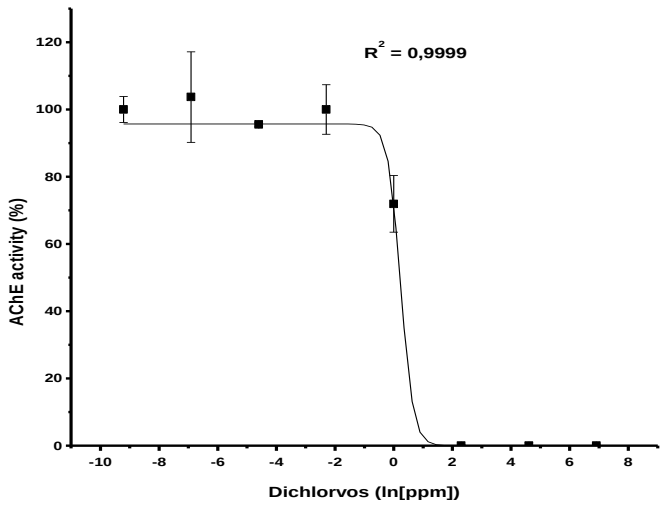


Figure 2. Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1,000 µg/mL) of the organophosphorus dichlorvos.

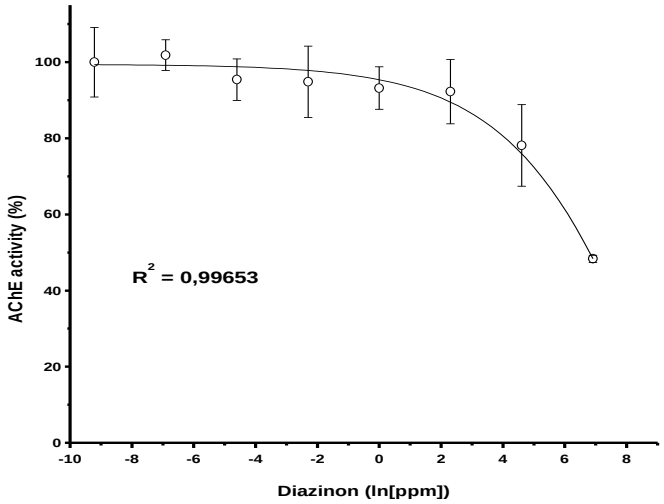


Figure 3. Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1,000 µg/mL) of the organophosphorus diazinon.

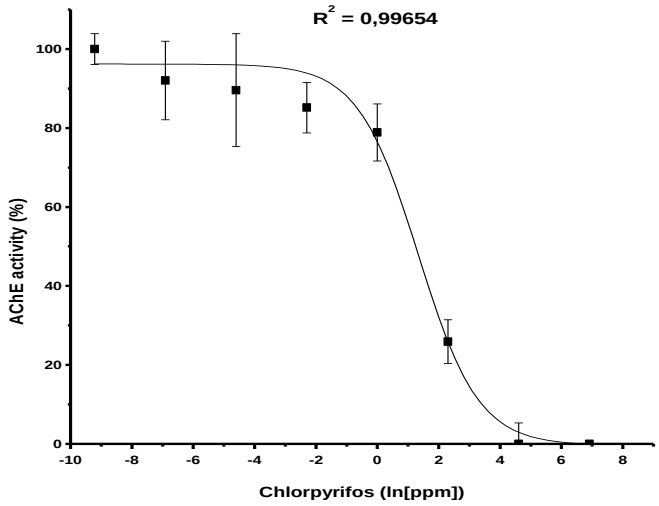


Figure 4. Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1,000 µg/mL) of the organophosphorus chlorpyrifos.

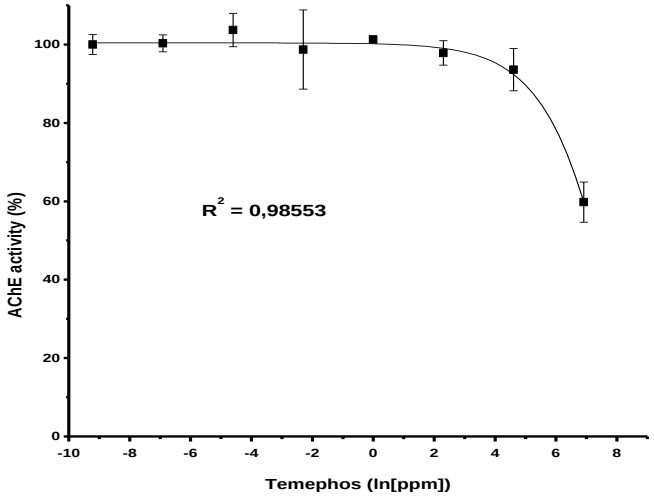


Figure 5. Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1,000 µg/mL) of the organophosphorus temephos.

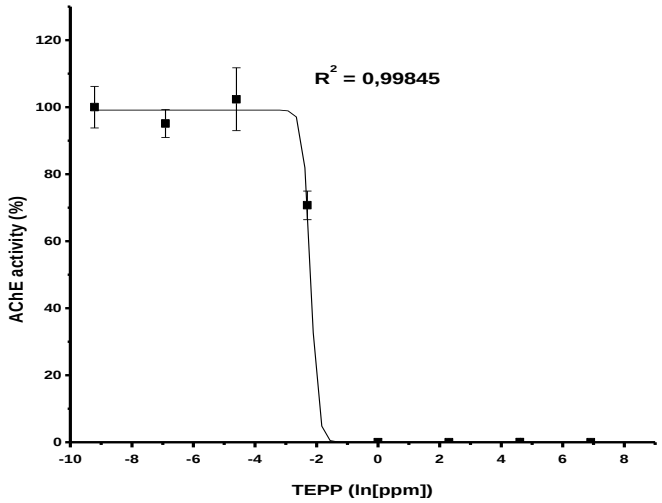


Figure 6. Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1,000 µg/mL) of the organophosphorus tetraethyl pyrophosphate (TEPP).

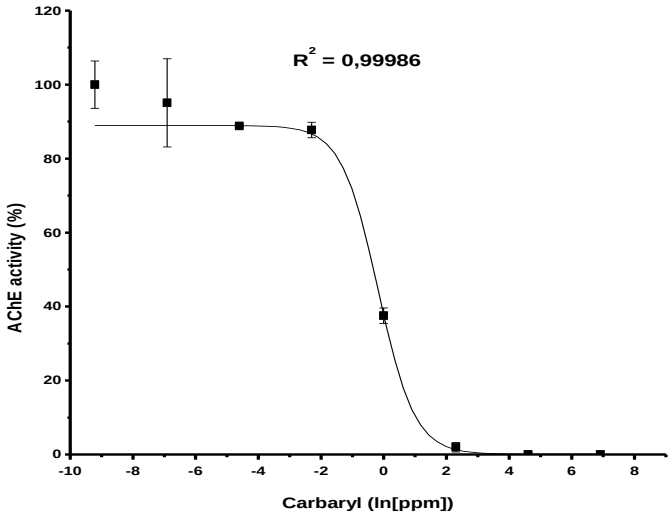


Figure 7. Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1,000 µg/mL) of the carbamate carbaryl.

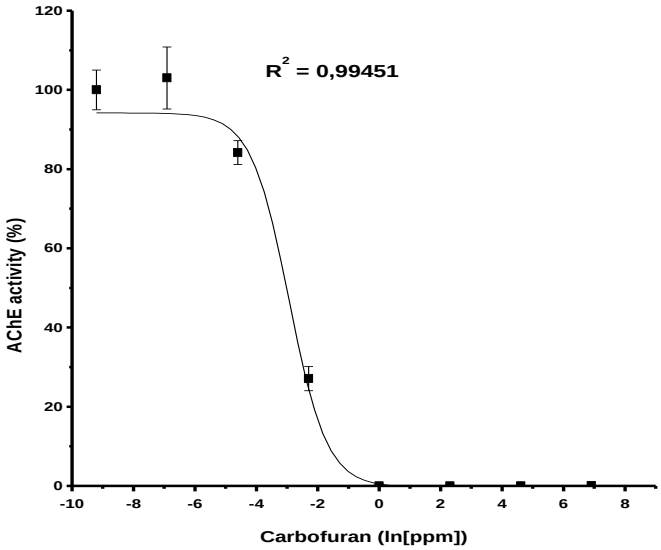


Figure 8. Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1,000 µg/mL) of the carbamate carbofuran.

11 – CAPÍTULO VII - EFFECT OF ORGANOPHOSPHATE AND CARBAMATE PESTICIDES ON HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE

ARTIGO ENVIADO À REVISTA HUMAN & EXPERIMENTAL TOXICOLOGY

Effect of organophosphate and carbamate pesticides on human erythrocyte acetylcholinesterase

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Abstract

A method to extract membranes from red blood cells (RBC) is described that were used to assay acetylcholinesterase (AChE) activity. The evidence for the enzyme activity was established by selective inhibition using BW284c51, Iso-OMPA and neostigmine. Blood samples were exposed to three organophosphorus (dichlorvos, chlorpyrifos, diazinon) and two carbamate (carbaryl and carbofuran) pesticides. Afterwards their membranes RBC AChE activities were determined. The IC_{50} for the pesticides were 10.66 µM (dichlorvos), 21.42 µM (chlorpyrifos), 109.98 µM (carbaryl) and 5.44 µM (carbofuran). The results related to 20% enzyme inhibition (level used in estimation of threshold limits for anticholinesterasic compounds) were below those Acceptable Daily Intakes (ADIs) values enacted by relevant national and international regulations. These results suggest that the proposed AChE extraction from RBC and assay could be a suitable method for monitoring occupational exposure to pesticides.

Key words: Acetylcholinesterase; Organophosphate; Carbamate; Biomarker, Erythrocyte.

11.1. Introduction

Although the pesticides have provided an increase in agricultural productivity enabling high quality food at lower costs, the improper use of these chemicals can bring harm to human health and environmental negative impact (Domingues *et al*., 2004). It is estimated that only 0.1% of the applied pesticides in fact reach the target animals, while the rest spreads throughout the environment. The financial costs saved by pest control are partially wasted through the environmental and public health problems caused by pesticides (Hart and Pimentel, 2002). Agricultural workers are exposed to high risks of poisoning due to intense contact with pesticides. Such compounds can cause adverse effects on central and peripheral nervous system. In addition, they can present immunosuppressive action or be carcinogenic, among other injuries. The most used classes of insecticides and source of occupational poisoning are the organophosphates (OPs) and carbamates (CBs). In 2007, organophosphates accounted for 35% of all insecticides used in the United States (USEPA, 2011).

OPs and CBs are typical inhibitors of the cholinesterases (ChEs) and there are two accepted types of these enzymes. Firstly, acetylcholinesterase (AChE; EC 3.1.1.7) that occurs in brain, ganglia of the autonomic nervous system and motor endplates, is produced by the neurons where it plays primary function in the nerve impulse modulation at the synaptic clefts. Another form

of AChE is observed in the plasma membrane of red blood cells (RBC). This one is synthesized during the process of bone marrow haematopoiesis and has a half-life of approximately 120 days, the same as that of RBC. Secondly, butyrylcholinesterase (BChE; EC 3.1.1.8) is synthesized in liver and predominates in plasma, glial cells, pancreas and the walls of digestive tract and presents an *in vivo* half-life of 7 days (Chatonnet and Lockridge, 1989; Çokugras, 2003; Taylor, 1991).

The mechanism of action of OPs and CBs occurs through binding to the enzymes esteratic site with phosphorylation and carbamoylation for organophosphates and carbamates, respectively (Fukuto, 1990; Quinn, 1987). Inhibition by the organophosphorus compounds tends to irreversibility if untreated (WHO, 1986a). The inhibition by carbamates is reversible and the recovery of the enzyme may take from several minutes to hours (WHO, 1986b).

OPs and CBs are widely used in developing countries, predominantly in agricultural economies, to pest control and public health campaigns to eradicate disease vectors (WHO, 1986a, 1986b; ATSDR, 1997a). In developing world, the negative consequences of pesticide usage are conditioned by factors closely related, such as the mishandling of these substances, the high toxicity of some products, the lack of protective equipment and the poor surveillance. This situation is aggravated by low cultural and socioeconomic status for most of these workers. Markedly in these countries, the monitoring of occupational exposure to such compounds presents problems in rural locations distant of testing laboratories where there is no appropriate infrastructure for the analysis (Magnotti *et al*., 1987; Oliveira-Silva *et al*., 2000; Oliveira-Silva *et al*., 2001). Moreover, the monitoring of pesticides based on blood ChE inhibition recommended by World Health Organization (1967 and 1984) requires fresh blood samples of total ChE in the methods of Edson (1950) and Ellman at al. (1961 adapted by WHO 1984) or RBC AChE by George and Abernethy (1983). The disadvantage of these methods are the non-discriminating use of both blood enzyme activities in case of using total ChE samples and the less accuracy by hemoglobin interference in the case of those which use RBC AChE samples without complete red cell lysis and centrifugation. The method proposed by Oliveira-Silva and co-workers (2000) not only uses RBC AChE but also allows the freezing of samples for further analysis in an appropriate place without colorimetric interactions with hemoglobin.

Several works chose RBC AChE instead of BChE for many reasons, as follows: 1) determination of inter and intraindividual variation in both ChE activities was considered a critical knowledge for a blood esterase monitoring program. The class of AChEs is more homogeneous in terms of their primary structure than the class of BChEs (Rosenberry, 1975) and RBC AChE activity was pointed to be less variable than BChE (Nigg and Knaak, 2000; Lefkowitz, 2007); 2) the first one is more closely correlated with the AChE activity from nervous system (Tinoco-Ojanguren and Halperin, 1998); 3) more stability in frozen blood samples: 7days against 3 days for BChE (Oliveira-Silva, 2000); 4) BChE spontaneous recovery of inhibited forms are faster than AChE (Nigg and Knaak, 2000). In order to perform a successful monitoring program, it should be taken into account that some conditions other than pesticide exposure can change blood ChEs activities, hindering the evaluation and interpretation of results from studies. BChE activity can be decreased by liver diseases, malnutrition, alcoholism, nephritic syndrome, pregnancy, contraceptive pills and metoclopramide. Whereas RBC AChE activity is altered by pregnancy, anemia, hemorrhagic events and reticulocytosis. Other factors that may result in misinterpretation of ChE levels are collection, improper processing and transportation of samples and laboratory errors (Boiko *et al*., 2004; Del Prado-Lu, 2007). In addition, should be taken measures to minimize intra-individual variation as considering each person as his own control by collecting samples in periods of growing seasons before and after pesticide applications. Here, were evaluated the effect of three organophosphate and two carbamate pesticides on human RBC AChE comparing the results of inhibition with relevant national and international regulations in force.

11.2. Materials and Methods

11.2.1. Materials

Acetylthiocholine iodide, tetraisopropyl pyrophosphoramide (Iso-OMPA), 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51), neostigmine bromide, bovine serum

albumin, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), tris (hydroxymethyl) aminomethane and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Disodium ethylenediamine tetraacetic acid (EDTA) was obtained from Merck (Darmstadt, Germany). Analytical grade dichlorvos (98.8%), diazinon (99.0%), chlorpyrifos (99.5%), carbofuran (99.9%) and carbaryl (99.8%) were obtained from Riedel-de-Haën, Pestanal® (Seelze, Germany). A Bio-Rad xMark (Hercules Ca, USA) microplate spectrophotometer and an IKA RW - 20 digital (Staufen, Germany) tissue disrupter were used.

11.2.2. Methods

11.2.2.1. Blood samples

The blood was collected (9 mL) by venipuncture and gentle hand shaking homogenized with 10% EDTA (60 µL) from health students (n = 22) not exposed to pesticides.

These individuals were previously interviewed on the occurrence of diseases that could interfere with the activity of the enzyme: pregnancy, anemia, hemorrhagic events and reticulocytosis.

11.2.2.2. Sample processing and enzyme extraction

Aliquots (1.8 mL) of whole blood were incubated with 200 µL solutions of each pesticide prepared with 0.01 M Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl (TBS buffer). The used pesticides were the organophosphates dichlorvos, diazinon, chlorpyrifos and the carbamate pesticides carbaryl and carbofuran. The pesticides were firstly dissolved in DMSO and diluted with distilled water to attain thirteen solutions at final concentrations ranging from 0.01 to 100 µg/mL in 2% v/v DMSO.

The whole blood samples were also incubated with 200 µL of TBS only and TBS + 2% DMSO instead the pesticides as controls. The samples were then centrifuged at 2,000 x *g* for 4 min to obtain plasma and erythrocyte fractions. They were separated and aliquots of 500 µL of RBC were resuspended in 4.5 ml of lysis buffer (0.01m Tris-HCl pH 7.6 without salt). The samples were kept at -20°C for 24 hours for RBC lysis. After lysis, RBC samples were centrifuged three times at 4,000 x *g* for 15 min, discarding the supernatant after each centrifugation. Afterwards the pellet (RBC membranes or "ghost" suspension) was resuspended in 500 µL of enzymatic assay buffer (0.5 M Tris-HCl pH 7.4). Aliquots of the RBC membranes controls were also used to characterize RBC AChE selective inhibition.

11.2.2.3. Enzyme activity for selective inhibition

Samples of the RBC membranes from controls were exposed to selective inhibitors: BW284c51 for AChE, Iso-OMPA for BChE and neostigmine bromide as total cholinesterases inhibitor. The membranes samples (10 µL) were exposed inside of microplate wells to five concentrations of each inhibitor (10 µL) ranging from 0.001 to 10 mM during 1 h. After incubation, enzyme activity was determined using a modification of the Ellman method (1961) by Assis et al. (2010). Briefly, 0.25 mM DTNB (200 µL) prepared in 0.5 M Tris-HCl buffer, pH 7.4 was added to the incubated mixture and the reaction started by the addition of 62 mM acetylthiocholine iodide (20 µL). Enzyme activity was determined by reading the absorbance increase at 405 nm during 180 s. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 µM of substrate per minute. A blank was prepared with the assay buffer instead of ghost suspension sample. All these assays were carried out in quadruplicates.

11.2.2.4. Enzyme activity in blood samples exposed to pesticides.

The activity of membrane RBC AChE extracted from whole blood samples incubated with pesticides and controls were determined by mixing 200 µL of DTNB with 20 µL of ghost samples and finally adding 20 µL of the substrate acetylthiocholine iodide. The reaction was carried out and followed spectrophotometrically similar as above described (2.2.3.). All these assays were carried out in quadruplicates.

11.2.2.5. Protein determination

Protein content in the RBC membranes preparations was estimated according to Sedmak and Grossberg (1978), using bovine serum albumin as a standard. All tests were performed at room temperature (25ºC) in triplicates.

11.2.2.6. Estimation of IC_{20}, IC_{50} and Ki

The enzymatic activity values obtained from 11.2.2.3 and 11.2.2.4 were plotted *versus* selective

inhibitor or insecticide concentration. From the curves generated by non-linear regression fitting (using MicroCal™ Origin® Version 8.0) were estimated IC_{50} and IC_{20} (concentrations capable to inhibit the enzyme activity by 50% and 20%, respectively) for each selective inhibitor or pesticide. Their respective inhibition constants (Ki) were calculated using the Cheng and Prusoff equation (1973).

11.2.2.7. Comparative study of enzyme inhibition in accordance with current regulations

The IC_{20} found for the pesticides were converted from µg/mL to mg/Kg body weight/day (Acceptable Daily Intake – ADI) for comparison with the results reported by specialized agencies.

11.3. Results

AChE present in the RBC membrane preparation was strongly inhibited (about 50%) by its specific inhibitor BW284c51 at 0.001 mM (Fig. 1A) whereas under Iso-OMPA exposure (BChE specific inhibitor) its activity was only statistically reduced at 1 mM (Fig. 1B). On the other hand, neostigmine, a very potent inhibitor of total ChEs, abolished the enzyme activity even at 0.001 mM (Fig. 1C). These results show the ability of the enzyme extraction method in getting just the fraction of AChE from RBC. The IC_{50} and Ki values for BW284c51 and neostigmine were 0.92 µM; 0.0054 µM; 0.30 µM and 0.0018 µM, respectively. It was not possible to estimate these parameters for Iso-OMPA.

The effects of dichlorvos, diazinon, chlorpyrifos (organophosphorus) and carbaryl and carbofuran (carbamates) on RBC membrane AChE are displayed in Fig 2 and Fig. 3, respectively. Diazinon showed to be the less effective inhibitor among the five investigated pesticides while dichlorvos and carbofuran were the most powerful inhibitor. These findings are corroborated by their IC_{20}, IC_{50} and Ki (Table 1) estimated from the curves. These parameters are also confronted with the values of Acceptable Daily Intake found in national and international specific legislation in the same Table.

11.4. Discussion

The proposed extraction procedure to obtain AChE from RBC membrane was based on that

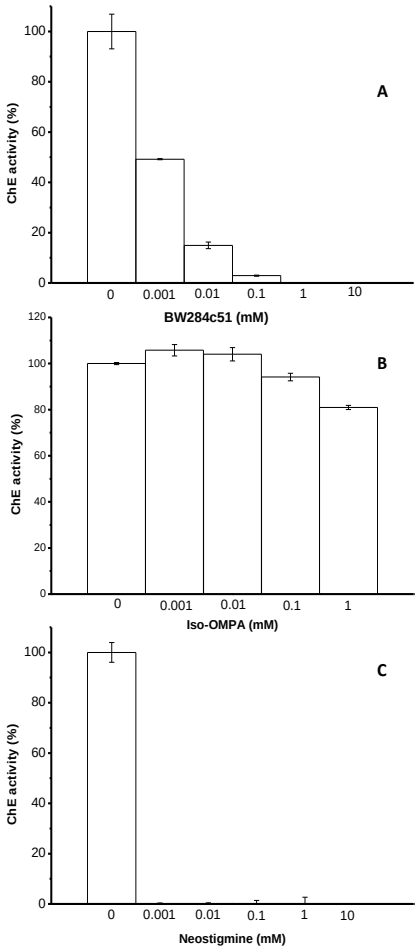

Figure 1 – Effect of (A) 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51), (B) tetraisopropyl pyrophosphoramide (Iso-OMPA) and (C) neostigmine bromide from 0.001 to 10 mM on frozen blood samples of human RBC AChE activity.

reported by Oliveira-Silva et al. (2000). Thus, the efficiency of the proposed method to extract the membrane RBC AChE was shown by selective inhibition by BW284c51 whereas Iso-OMPA (BChE specific inhibitor) did not impair. Also, regarding the inhibition effect of BW284c51 and neostigmine on membrane RBC AChE is worthwhile to register that the dissociation rate (Ki) of neostigmine was 3-fold slower than that of BW284c51. One of the more remarkable differences between AChE and BchE is the smaller cavity of AChE active site, lined by six aromatic amino acid residues that prevent the entrance of the selective BchE inhibitors and substrates (Harel *et al*., 1992).

Once established that membrane RBC AChE was properly extracted the whole blood was exposed to

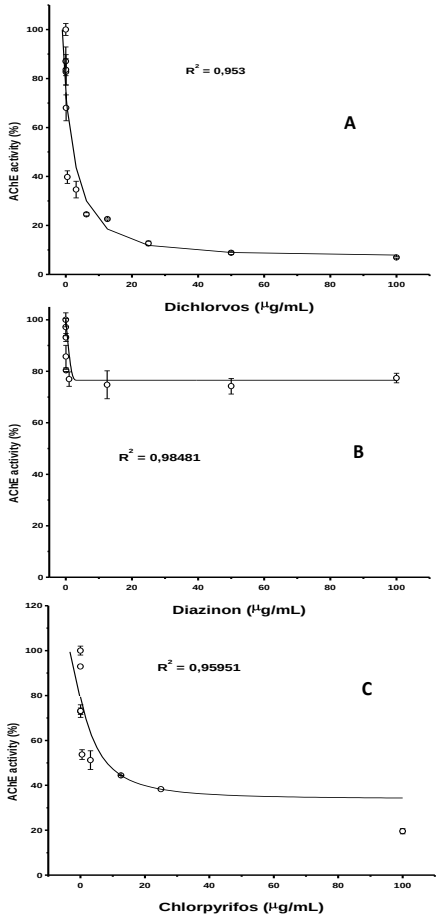

Figure 2 – Effect of (A) dichlorvos, (B) diazinon and (C) chlorpyrifos from 0.01 to 100 µg/mL on frozen blood samples of human RBC AChE activity.

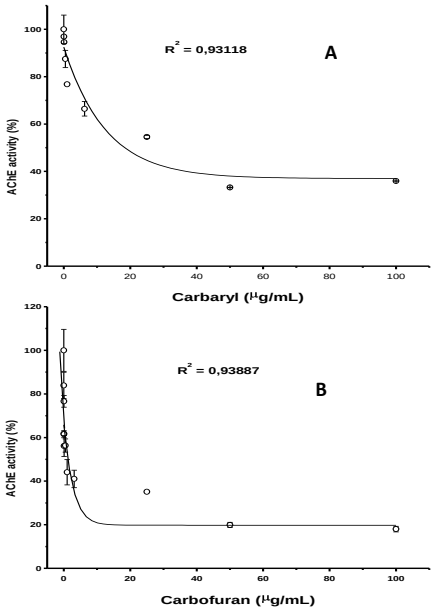

Figure 3 - Effect of (A) carbaryl and (B) carbofuran from 0.01 to 100 µg/mL on frozen blood samples of human RBC AChE activity.

pesticides simulating intoxication by them in order to evaluate the enzyme activity reduction as measure of this contact. The results showed the highest inhibitory action on the enzyme by dichlorvos compared to diazinon and chlorpyrifos. Dichlorvos is already bioactive as an oxon while the thion form (diazinon and chlorpyrifos form) needs biotransformation to enhance its toxic action (Vale, 1998; WHO, 1986a). However, another feature that interferes in the toxicokinetic of OP pesticides and that was decisive in the results is their specific lipophilicity. The most lipophilic compounds are rapidly absorbed and accumulated in fat and this contributes to the reduction of the primary effects of these pesticides (ChEs inhibition) while can also increase secondary effects in other biomolecules. Phosphorothioates (diazinon and chlorpyrifos) are more lipophilic than phosphates (dichlorvos) (Vale, 1998). In addition, serum oxonases seems to be more effective in the hydrolysis of diazinon and chlorpyrifos (Li et al., 1993; 1995; Manthripragada et al., 2010). Moreover, according to Rosenberry (1975) AChE is more sensitive to small acyl size condition fulfilled by dichlorvos compared to diazinon and chlorpyrifos.

Between the investigated carbamates carbofuran showed to be more toxic to membrane RBC AChE than carbaryl. This difference may be attributed to the 2,2-dimethylbenzofuranyl ring (carbofuran) that is more reactive than the naphthyl ring (carbaryl) when interacting with residues in the AChE active center. The controls with and without DMSO did not present significant difference.

The IC_{20} is the threshold limit to consider the presence of an anticholinesterasic compound while the IC_{50} represents the point at which clinical signs and symptoms appear and death occurs after 90% inhibition (FAO, 2007). Here, the IC_{20} values were converted to Acceptable Daily Intake (ADI) unit that stands for the highest concentration causing no effect (no-observed-adverse-effect-level, NOAEL) on the most susceptible species of mammal in long-term studies (chronic exposure).

To verify the possibility of using membrane RBC AChE as a biomarker for pesticides exposure the

Table 1 - ADI[1], IC_{20}[2] and IC_{50}[3] for human RBC AChE referring to pesticides under study

Pesticide	ADI (ref.) (mg/kg bw/day)	IC_{20} (mg/kg bw/day)	IC_{20} (µM)	IC_{50} (µM)	Ki (µM)
dichlorvos	0.004 (ANVISA, 2009a) 0.004 (LU, 1995) 0.003 (ATSDR, 1997a) 0.0005 (USEPA, 1994) 0.0008 (EFSA, 2005a)	0.0019	0.000135	10.66	0.131
diazinon	0.002 (ANVISA, 2009b) 0.002 (FAO/WHO, 2004) 0.0007 (ATSDR, 2001) 0.0002 (EFSA, 2005b)	0.0076	0.000394	- [4]	-
chlorpyrifos	0.01 (ANVISA, 2009c) 0.01 (WHO/FAO, 1999) 0.001 (ATSDR, 1997b) 0.01 (FAO, 2006a)	0.002	0.000085	21.42	0.262
carbaryl	0.003 (ANVISA, 2009d) 0.008 (FAO, 2006b) 0.008 (FAO/WHO, 2004) 0.0075 (EFSA, 2005c)	0.0033	0.00496	109.98	1.35
Carbofuran[5]	0.002 (ANVISA, 2009e) 0.002 (FAO/WHO, 2004) 0.005 (USEPA, 1984) 0.001 (EFSA, 2004)	0.0019	0.000135	5.44	0.066

1 – Acceptable Daily Intake; 2 - Concentration capable of inhibit AChE activity in 20%; 3 - Idem, in 50%; 4 – the maximum concentration used in the assays did not inhibit beyond 50%; bw – body weight; 5 - in process of cancellation of all licenses for the use in food production in the U.S.

IC_{20} values of each one (Table 1) should be below their respective ADIs. Thus, the values of IC_{20} for all studied pesticides were lower than those recommended by WHO, FAO, ATSDR (Agency for Toxic Substances and Disease Registry), EFSA (European Food Safety Agency) and ANVISA (Brazilian Sanitary Surveillance Agency) excepting diazinon. It is noteworthy to mention that carbofuran presented low IC_{20} value and has been outlawed nowadays in the U.S. for use in food farming (USEPA, 2009).

11.5. Conclusions

The results of the extraction method can be ascribed to RBC AChE according to selective inhibition. The used enzymatic assay allowed relevant levels of inhibition to be achieved and they were at pesticide concentrations below the majority of Acceptable Daily Intakes (ADIs) adopted for the analyzed organophosphates and carbamate insecticides in foods by national and international regulations. Regarding this, the method showed good accuracy to be used in human monitoring programs for occupational exposure of such pesticides and can be useful for sample collections in locations far from the laboratories.

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12 – CONCLUSÕES GERAIS

No presente trabalho, as espécies estudadas apresentaram apenas acetilcolinesterase em seus cérebros. Além disso, foi possível estimar parâmetros de eficiência catalítica (com valores dentro da faixa presente na literatura) a partir dos extrato brutos, algo que antes era feito apenas com enzimas purificadas.

Alguns dos pesticidas se mostraram altamente tóxicos em relação à acetilcolinesterase cerebral das cinco espécies de peixes. Níveis relevantes de inibição da atividade enzimática foram alcançados em concentrações abaixo dos limites máximos de resíduos (LMRs) para esses pesticidas previstos na legislação nacional e internacional em vigor.

Alguns íons estudados alteraram positiva ou negativamente a atividade das enzimas de peixes, o que poderia constituir uma desvantagem na análise de amostras complexas. Todavia, os mesmos causaram interferência em concentrações em torno de 1 mM, que é considerada uma concentração muito alta para ambientes naturais não associados a empreendimentos industriais e de mineração.

O método utilizado para extração da acetilcolinesterase eritrocitária humana foi satisfatório. A determinação da sua atividade a partir de amostras congeladas não compromete sua sensibilidade aos pesticidas estudados e proporciona o armazenamento das amostras para análise em laboratórios distantes dos locais de coleta. Além disso, a enzima foi sensível à concentrações abaixo das Ingestões Diárias Aceitáveis (IDAs) para os pesticidas presentes em regulamentos internacionais.

De acordo com esses resultados, a acetilcolinesterase das fontes propostas se constitui em ferramenta promissora para utilização em programas de monitoramento ambiental e ocupacional de pesticidas organofosforados e carbamatos.

Como perspectiva para o futuro está a utilização dessas enzimas em estudos de detecção de pesticida de acordo com a sensibilidade de cada espécie, tanto em ensaios com o extrato bruto, como purificadas e imobilizadas em biossensores de diversos princípios de detecção.

13 - ANEXOS

13.1. Indicadores de produção 2009-2011

13.1.1. Participação em artigos

Effect of *in vivo* and *in vitro* aluminum exposure on brain, muscle, and digestive tract hydrolases of juvenile Nile tilapia (*Oreochromis niloticus*).

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Abstract

Heavy metals are one of the most dangerous substances that can accumulate in aquatic biota. Aluminium is a ubiquitous metal with no biological function set and focus for further studies due to constant evictions in aquatic environments through anthropogenic sources, industrial and domestic. In this study, the aluminum *in vivo* and *in vitro* effects over the activity of seven enzymes of Nile tilapia (*Oreochromis niloticus*): brain AChE, muscle AChE, muscle BChE, pepsin, trypsin, chymotrypsin and intestinal amylase were evaluated. The *in vivo* test was carried out of uninterrupted cultivation period of 14 days, with daily measurement of the physical-chemical parameters of water quality, photoperiod 12:12 and *ad libitum* feeding, dynamic system of exchange of water (80%) in all experimental groups (TGC – without exposure; TG1 – with 1 mg / L of $Al_2(SO_4)_3$ and TG3 - with 3 mg / L of $Al_2(SO_4)_3$). Samples of brain, muscles, stomach and intestine were collected for determination of enzyme activity. The *in vitro* induction by incubation corresponded directly with the enzymatic extract metal from the group not exposed to *in vivo* test (TGC) for a period of 1 h. No changes were observed in gills and liver of exposed fish. The three cholinesterase enzyme activity increased in the control group (100%). The highest mean muscle BChE activity were obtained by, 168.28 ± 5.49% (TG1) and 196.17 ± 4.08% (TG3), *in vitro* assay. In contrast to cholinesterase activity, the digestive enzymes showed decreased activity in both *in vivo* and *in vitro*. Pepsin *in vivo* activity were 65.01 ± 1.04% and 61.06 ± 1.90%, whereas intestinal amylase activity were 87.21 ± 5.76% and 60.20 ± 3.95% to TG1 and TG3 treatments, respectively. These results suggest that digestive enzymes cholinesterase and evaluated fry *O. Niloticus*, in combination, can be used as an alternative to biomonitoring in routine screening of aluminum in the aquatic environment.

Key words: Aluminum, Biomarker, Cholinesterase, Digestive Enzymes, Hydrolase.

5.1 Introduction

Aluminum (Al) is the most abundant metallic element and constitutes about 8% of the Earth's crust, and the third most abundant element after oxygen and silicon (Atwood and Yearwood, 2000) occurring only in combined form (oxides, silicates). Despite its abundance, this metal has no defined biological role and is considered non-essential (Tuzen and Soylak, 2007; Exley, 2009). Aluminum and its salts are used not only in water treatment as flocculating agent (Silva et al., 2007; Camargo et al., 2009), as a food additive in the manufacture of cans, shingles, aluminum foil, in the

pharmaceutical industry, among others (García-Medina et al., 2011). As a coagulant, aluminum can reduce organic matter and microorganism levels. Besides, aluminum sulfate ($Al_2(SO_4)_3$) is applied in order to reduce the development of phytoplankton and thus improve water transparency (Wauer et al., 2004). Due of its environmental distribution is considered ubiquitous (Nayak, 2002; Gourier-Fréry, 2004). When present in aquatic environments, can accumulate in the sediment and in fish (Walton et al., 2010). The accumulation can occur in mitochondria (Kumara et al., 2009), in lysosome and and/or in the cell nucleus chromatin (Nayak, 2002). The physiological changes commonly observed in different species of fish exposed are related mainly to cardiovascular (Laitinen and Valtonen, 1995), hematologic (Barcarolli and Martinez, 2004), metabolic (Brodeur et al., 2001), respiratory (Póleo, 1995), nervous systems (Meyer-Baron et al., 2007) and osmoregulatory (Camargo et al., 2009). Al^{3+} binds to nuclear chromatin and acts on the transcription of genetic information in susceptible neurons, possibly increasing the stability of linker histone-DNA adducts (Kiss et al., 1996). In addition, the $Al_2(SO_4)_3$ can also cause deleterious and cytotoxic changes in the DNA of exposed organisms (Garcia-Medina et al., 2011).

The rates of occurrence of acute toxicity caused by aluminum reported in the literature are low. Due to its better bioavailability, soluble forms of aluminum (aluminum chloride - $AlCl_3$, aluminum fluoride-AlF_3, aluminum sulfate - $Al_2(SO_4)_3$ and citrate aluminum - $AlC_5H_5O_7$) are toxically more important than the insoluble forms such as aluminum hydroxide ($Al(OH)_3$), for example (Gourier-Fréry, 2004). In water, the metal may occur in different forms and is influenced by pH, temperature and presence of fluorides, sulfates, organic matter and other ligands. The aluminum dissolved in rivers that have acidified pH when in contact with the gills, can cause a fish death by suffocation (Teien et al., 2006).

The Al concentration in natural waters can vary significantly, depending on several physical-chemical and mineralogical features of the water bodies. Aluminum concentrations dissolved in water with neutral pH values usually range from 0.001 to almost 0.05 mg/L, while 0.5-1 mg/L or more is found in acidic waters rich in organic matter. An aluminum level in drinking water varies with the levels found in water supply and charges of this metal used as coagulant during water treatment. In experimental animals, its absorption via the gastrointestinal tract is usually less than 1% (WHO, 2003), while in humans is 0.1-0.3% (DeVoto and Yokel, 1994). Complexing organic compounds, notably citrate, increase absorption. The uptake of aluminum may interact with transport systems for calcium and iron. Once absorbed, Al is distributed in most organs within the body, with accumulation occurring mainly in bone at high dose levels. To some extent, but as yet undetermined, Al passes the blood brain barrier and can be distributed to the fetus. He is effectively eliminated in the urine (WHO, 2003).

Through the viscera (liver, gills, brain, intestine, kidney, stomach) such as: Atlantic salmon *Salmo salar* (Monette et al., 2010), freshwater fish *Mogurnda mogurnda* (Camilleri et al., 2003), roach *Rutilus rutilus* (Camilleri et al., 2003; Keinänen et al., 2004), neotropical freshwater fish *Prochilodus lineatus* (Camargo et al., 2009), European perch *Perca fluviatilis*, Freshwater bream *Abramis brama*, Silver carp *Hypophthalmichthys molitrix* (Wauer and Teien, 2010), torrentfish *Cheimarrichthys fosteri* (Greig et al., 2010), demersal fish *Triglia lucerna* (Yilmaz et al., 2010) and Nile tilapia *Oreochromis niloticus* (Birungi et al., 2007) were the main aquatic organisms used as environmental bioindicators, using their chemical, biochemical, physiological or morphological parameters.

Laboratories tests are being used in the field of aquatic toxicology, where specific experimental conditions are controlled and target organisms are exposed to different concentrations from the xenobiotic for observation and quantification of its toxic effects. The aim of this study was to evaluate the influence of aluminum (Al) on the activity of brain and muscle acetylcholinesterase (AChE, *EC*, 3.1.1.7), muscle butyrylcholinesterase (BChE, *EC*, 3.1.1.8), pepsin (*EC*, 3.4.23.1), chymotrypsin (*EC*, 3.4 .21.1), trypsin (*EC*, 3.4.21.4) and intestinal amylase (*EC*, 3.2.1.1) from Nile tilapia *Oreochromis niloticus*, besides evaluating the effects of metal on the morphology of gills and liver of fish treated and propose the species as a bioindicator and

its enzymatic parameters used as biomarkers for this metal.

5.2 Materials and methods

5.2.1 Chemicals and equipment

Aluminum sulfate ($Al_2SO_4)_3$ was acquired from Vetec fine chemical (Rio de Janeiro, RJ, Brazil). Acetylthiocholine iodide, azocasein, bovine serum albumin (BSA), BApNA (Nα-benzoyl-DL-arginine-p-nitroanilide), DTNB (5,5'-dithiobis(2-nitrobenzoic) acid), Suc-Phe-p-Nan (Succinyl-DL-phenylalanine-p-nitroanilide), Tris (hydroxymethyl) aminomethane and DMSO (dimethyl sulfoxide) were purchased from Sigma. Glycine was acquired from Amersham Biosciences. HCl were obtained from Merck. The spectrophotometer used was Bio-Rad Smartspec™ 3000. Microplate spectrophotometer used was Bio-Rad xMark™. The centrifuges were BioAgency Bio-Spin and Software MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

5.2.2 Experimental design

Fingerlings, males and females, of *Oreochromis niloticus* (n = 90) were obtained from the aquaculture station of Universidade Federal Rural de Pernambuco - UFRPE (Recife/PE, Brazil) and brought to the Laboratório de Enzimologia of Universidade Federal de Pernambuco - UFPE (Recife/PE, Brazil). Before starting the experiments, fish were evaluated biometrically (weight and length) and were acclimatized in 10 days in glass aquaria (90 L each, 45 cm x 54 cm x 45 cm) and photoperiod of 12h, fed *ad libitum* (32% protein) and water exchange (80%). After this period, fish were divided in three treatments (n = 10 per aquarium) in triplicate, comprising the following groups: TGC - control, without exposure to aluminum sulfate - $Al_2(SO_4)_3$; TG1 - exposed to 1 mg / L of aluminum sulfate - $Al_2(SO_4)_3$, and TG3 - exposed to a concentration of 3 mg / L of aluminum sulfate - $Al_2(SO_4)_3$. The physical and chemical parameters of water quality (temperature, pH, dissolved oxygen) were continuously monitored. After the experimental period, animals were sacrificed by immersion in ice, following removal of the brain, muscle and digestive organs for further processing. Water samples and muscle were collected and analyzed for the presence of aluminum, using atomic absorption spectrophotometer. Organs such as liver and gills were selected for histopathology evaluation.

5.2.2.1 *In vivo* assay

The *in vivo* assay corresponded to the period of continuous exposure of 14 days, by which the animals were subjected to different concentrations of the metal. All parameters were maintained in culture for the period of acclimatization. On the 14th day of exposure, the animals were killed by immersion in ice, their biometric parameters measured, to be subsequently withdrawn their viscera for further analysis, both of control (TGC) and the exposed (TG1 and TG3).

5.2.2.2 *In vitro* assay

The *in vitro* analysis was performed using the animals treatment control (TGC) of the *in vivo* treatment. Samples (crude extract) of 30 fish in the control group (TGC) were subjected to an incubation period of 1 hour in aluminum concentrations of 1 and 3 mg / L. Then analyses were accomplished by conventional standard methodology.

5.2.3 Cholinesterase enzyme extraction

Brains and muscle were immediately removed, pooled per treatment and homogenized in 0.5 mol/L Tris-HCl buffer, pH 8.0 keeping the ratio of 20 mg per ml of buffer using a Potter-Elvehjem tissue disrupter. These homogenates were centrifuged for 10 min at 10,000 x *g* (4ºC) and the supernatants (crude extracts) were frozen at -20ºC and then used for further assays (Assis et al., 2010).

5.2.4 Digestive enzyme extraction

Intestines and stomach were immediately collected and homogenized (40 mg/mL) in 0.01 mol/L Tris-HCl pH 8.0 and 0.01 mol/L Glycine-HCl pH 2.0, respectively, both containing 0.9% NaCl (w/v), using a tissue homogenizer. The resulting preparations were centrifuged at 10,000 x g for 10 min at 4^{o}C to remove cell debris and nuclei. The supernatants (crude extracts) were frozen at -20 ^{o}C and used in further assays (Díaz-López et al., 1998; Bezerra et al., 2005).

5.2.5 Protein concentration determination

The concentration of brain and digestive proteins were determined according to Sedmak and Grossberg (1977) using bovine serum albumin (BSA) as standard.

5.2.6 Non-specific digestive enzymes assay

Determined according to Bezerra et al. (2005) where a microcentrifuge tube (triplicates), 1% azocasein (50 µL) prepared in 0.1M Tris-HCl buffer pH 8.0 was incubated with intestine crude extract (30 µL) for 60 min at 25°C. Then, 240 µL of 10% trichloroacetic acid (TCA) were added to stop the reaction. After 15 min, centrifugation was carried out at 8,000 x g for 10 min. The supernatant (70 µL) was added to 1 mol/L sodium hydroxide (NaOH, 130 µL) in a 96-well microtiter plate and the absorbance of this mixture was measured at 450. One unit (U) of enzymatic activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001 change in absorbance per minute per milligram of protein.

5.2.7 Enzyme Activity Assay

5.2.7.1 Acetylcholinesterase and butyrylcholinesterase

Acetylcholinesterase and butyrylcholinesterase activity was determined using extract (20 µL) and chromogenic reagent DTNB 0.25 mM (200 µL). The reaction was monitored on a microplate spectrophotometer at 405 nm for 3 minutes after adding of 62 mM acetylthiocholine and S-butyrylthiocholine iodide (20 µL), respectively for AChE and BChE. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 µmol of substrate per minute (Assis et al., 2010).

5.2.7.2 Chymotrypsin and Trypsin

Trypsin and chymotrypsin activity was determined using 8mM Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA) and 8mM Succinyl-DL-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan) in dimethyl sulfoxide (DMSO). Crude extract intestine (30 µL) was incubated with 0.1M Tris-HCl buffer pH 8.0 (140 µL) and respective substrates (30 µL). The absorbance was measured at 405 nm. Enzymatic activity was performed in triplicates. Trypsin and chymotrypsin units of activity were expressed as change in absorbance per minute per milligram of protein (Bezerra et al., 2005).

5.2.7.3 Pepsin

Proteolytic activity of enzyme was determined using hemoglobin as substrate according to the method of Nalinanon et al. (2010) with some modifications. To initiate the reaction, 200 µL of enzyme solution were added into the assay mixture containing hemoglobin 2% (200 µL), distilled water (200 µL) and reaction buffer 0.1 M glycine–HCl (625 µL). The reaction was conducted at pH 2.0 and 37°C for 20 min. To terminate enzymatic reaction, trichloroacetic acid (TCA) 50% (w/v) (200 µL) were added. Unhydrolysed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifugation at 10,000*g* for 10 min. The oligopeptide content in the supernatant was measured at 280 nm. One unit of activity was defined as the amount which causes an increase of 1.0 in absorbance at 280 nm per min. A blank was run in the same manner, except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

5.2.7.4 Intestinal amylase

Amylase activity was evaluated according to Bernfeld (1955) using starch 2% as substrate: 60 µL intestine crude extract were incubated with 375 µL starch solution and 375 µL 10mM phosphate buffer pH 8.0 containing 15mM NaCl at 25°C. After 20 min 3.5-dinitro salicylic acid (DNSA) was added and the solution was submitted to 100º C for 10 min. After temperature equilibration the absorbance was measured at 570 nm against a blank similarly prepared except that 10mM phosphate buffer replaced the crude extract sample. Enzymatic activity was perceived in triplicates. One unit of enzymatic activity was defined as the amount of enzyme required to hydrolyze 1mg of maltose per milligram of protein per min.

5.2.8 Polyacrylamide gel electrophoresis (PAGE) and zymogram

Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970), using a 6% (w/v) stacking gel and a 12.5% (w/v) separating gel. The gels were stained for protein overnight in 0.01% (w/v) Coomassie Brilliant Blue. The background of the gel was destained by washing in 10% (v/v) acetic acid. The molecular weight of the Nile tilapia protease band was estimated using the protein standards (Sigma) bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29 kDa), trypsinogen (24.0 kDa) and a-lactalbumin (14.2 kDa). Zimogram were also carried out according to Garcia-Carreño et al. (1993).

5.2.9 Statistical analysis

Data are expressed as mean±SEM. Mean values for the different treatments were compared using one-way analysis of variance (ANOVA) followed by the Tukey's test. The significance level adopted was 95% ($\rho < 0.05$), modeling using the software MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

5.3 Results

5.3.1 Biometric parameters and physico-chemical quality of water

During the cultivation period, from the three proposed treatments (TGC, TG1 and TG3), the physico-chemical parameters of water quality were measured on the inherent temperature (27.29 ± 0.34°C, 27.30 ± 0.0°C and 27.38 ± 0.07°C), dissolved oxygen (6.31 ± 0.42 $mgO_2.L^{-1}$, 6.09 ± 0.29 $mgO_2.L^{-1}$ and 6.01 ± 0.09 $mgO_2.L^{-1}$) and pH (6.50 ± 0.13, 6.26 ± 0.33 and 6.13 ± 0.10). After this period, the animals were biometrically measured, obtaining the average weight of 21.87 ± 0.98, 21.93 ± 0.11 e 22.33 ± 0.61 g and long the of 10.33 ± 0.11, 10.13 ± 0.17 e de 10.48 ± 0.44 cm) in different treatments. The presence of aluminum in water was quantified with a mass spectrometer at the Technological Institute of Pernambuco (ITEP) was found in each treatment 0.22 $\mu g.L^{-1}$, 0.44 $\mu g.L^{-1}$ and 0.73 $\mu g.L^{-1}$, respectively, groups for TGC, TG1 and TG3.

5.3.2 AChE and BChE activity in the brain and muscle

The cholinesterase activity determined brain AChE enzyme, muscle AChE and muscle BChE are described in Table 1. For brain AChE was not detected significant differences between treatments *in vivo* assay (ANOVA, Tukey test, $P < 0.05$), while *in vitro* differences were recorded (ANOVA, Tukey test, $P > 0.05$) between groups TGC and TG1, and between TG1 and TG3. In the determination of cholinesterase activity of muscle AChE and BChE was no significant difference ($P > 0.05$) among all treatments, both *in vivo* and *in vitro*.

5.3.3 Peptidases and amylase activity in the digestive tract

Quantified the activities of digestive enzymes are described in Table 1. At the *in vivo* assay, pepsin activity was significantly different ($P > 0.05$) between treatments TGC and TG3. The enzyme in both *in vivo* and *in vitro* treatment, showed statistical difference ($P > 0.05$). In the analysis between the means of chymotrypsin assay *in vivo* were no statistical differences among all treatments ($P > 0.05$), while the same was not observed *in vitro* assay ($P < 0.05$). Data on intestinal amylase showed statistical differences in both *in vivo* and *in vitro* assay ($P > 0.05$).

5.4 Discussion

Tropical species such as tilapia usually have thermal comfort at temperatures between 27 and 32°C, external factor that can influence the digestive enzyme activity of fish water (Kubitza et al., 1998). They are very tolerant of low dissolved oxygen (Popma and Masser, 1999), live with a pretty broad range of acidity (Camargo et al., 2009) and alkalinity in the water, grow and even breed in brackish water and salty (Póleo and Hytterod, 2003). Changes in these parameters are detrimental to metabolism and development of fish and can result in increased mucous secretion causing clogging gill (Rosseland et al., 1990), irritation and swelling in the gills, resulting in the destruction of gill tissue, affecting the dynamics of fish (Kubitza, 2000). Even in alkaline water (pH 7.5 to 9.5), accumulation of the metal tends to alter the physicochemical properties of water (Camilleri et al. 2003; Póleo and Hytterod, 2003), which in turn influence the polymerization of Al in order to alter the physiology of the species, especially in the ion exchange guelrras (Póleo, 1995; Wauer et al. 2004; Alstad et al. 2005; Tria et al. 2007; Roberts and Palmeiro, 2008; Camargo et al., 2009; Monette et al., 2010).

Aluminum substantially increased the activity of the enzyme cholinesterase, brain AChE and muscle AChE, and pseudo cholinesterase muscle BChE, fish induced exposure in both trials, *in vivo* and *in vitro*, as described in **Table 1**, indicating the influence of metal on the activity both brain and muscle cholinergic, acting as a potent activator. These results are consistent to that reported by Zatta et al. (2002), demonstrating that the toxic potential of this metal as the determining factor is the length of exposure to which animals are subjected. The activation or inhibition of this class of enzymes by heavy metals still need to have their metabolism interaction clearly elucidated, given the contradictions between modulation studies (Zatta et al. 2002). It is

Table 1 - Enzyme activity (mU/mg) measured as biomarkers of exposure to aluminum: *in vivo* and *in vitro* assay.

Enzyme	*In vivo* assay			*In vitro* assay		
	TGC*	TG1**	TG3***	TGC*	TG1**	TG3***
Brain AChE	100.0 ± 7.07% (a)	119.64 ± 6.13% (a)	126.93 ± 13.20%(a)	100.0 ± 2.56% (a)	117.76 ± 13.11%(b)	160.13 ± 10.92%(b)
Muscle AChE	100.0 ± 2.32%(a)	104.49 ± 1.97%(b)	163.81 ± 0.30%(c)	100.0 ± 3.26%(a)	109.75 ± 1.76%(b)	138.77 ± 4.07%(c)
Muscle BChE	100.0 ± 2.05%(a)	105.15 ± 2.64%(b)	163.60 ± 4.15%(c)	100.0 ± 4.05%(a)	168.28 ± 5.49%(b)	196.17 ± 4.08%(c)
Pepsin	100.0 ± 1.22%(a)	65.01 ± 1.04%(b)	61.06 ± 1.90%(b)	100.0 ± 2.03%(a)	88.12 ± 2.63%(b)	82.52 ± 2.58%(b)
Trypsin	100.0 ± 0.09%(a)	95.0 ± 0.54%(b)	91.0 ± 0.59%(c)	100.0 ± 2.10%(a)	89.0 ± 2.89%(b)	83.0 ± 0.52%(b)
Chymotrypsin	100.0 ± 0.60%(a)	92.0 ± 0.33%(b)	73.0 ± 1.8%(c)	100.0 ± 3.21%(a)	97.0 ± 2.07%(a)	91.0 ± 3.68%(a)
Intestinal amylase	100.0 ± 0.32%(a)	87.21 ± 5.76%(b)	60.20 ± 3.95%(b)	100.0 ± 0.30%(a)	99.45 ± 4.36%(a)	91.62 ± 6.05%(a)

* TGC - Treatment group control; ** TG1: Treatment Group 1 mg / L.; *** Treatment Group 3 mg / L.
**** Data comparison at 0,05 level of significance (a, b, c).

known, so pervasive that in animals, Al tends to cause neurobehavioral changes (decreased activity and motor coordination) (Rosseland et al., 1990), histopathological changes in the brain (vacuolation and/or neuronal degeneration nuclear) (Banks et al., 1996, Platt et al., 2001), and disruption of biochemical processes (second messenger cascade and oxidative damage, changes in cholinergic activity) (Gourier-Fréry e Fréry, 2004), including the degradation of peptides amyloid and cause changes in the metabolism of acetylcholine and thus act as a possible co-pathogenic factors (Banks et al. 1996; Zatta et al. 2002).

Thus, the enzyme cholinesterase, with special attention to the brain AChE has been widely used as biomarkers of exposure (Sturm et al., 1999; Whitehead et al. 2005; Assis et al., 2010), to detect changes in the biological system caused by metals such as aluminum (Zatta et al., 2002), copper (Romani et al., 2003), cadmium, zinc, mercury (Olson and Christensen, 1980), and other substances, as is the case of inhibitors of both AChE and BChE as BW284c51, iso-OmpA (Sturm et al. 1999; Adresi, 2003; Rodríguez-Fuentes et al., 2004; Rendón-Von Osten et al., 2005) organophosphate and carbamato (Olson and Christensen, 1980; Whitehead et al., 2005; Rodríguez-Fuentes et al., 2008), the latter causing disturbances and peripheral nervous systems, and can result in death of fish (Assis et al., 2010). Muscle is one of the places of bioaccumulation of heavy metals, organophosphates and other xenobiotics, promoting a change in the enzyme dynamics (Rodríguez-Fuentes et al., 2008). The accumulation of metals in this organ has been subject of study. Tuzen and Soylak (2007) detected concentrations of aluminum variants between 0.45 to 1.50 µg/g in five different species of fish. The variation of aluminum content found in three fish species for Türkmen et al. (2005) was 0.02 to 5.41 $mg.kg^{-1}$ dry weight, while Ranau et al. (2001) found values between 0.032 to 5.346 µg/g dry weight. As illustrated in **Figure 1**, a comparison *in vitro* of aluminum chloride with sulphate, is shown a regulating enzyme cholinesterase in order to increase the excitement of the enzyme.

Unlike the results of cholinesterase, the digestive enzymes showed reduced in direct proportion the amount of metal exposure induced both *in vivo* assays as *in vitro* assays. The most important aspect of the gastrointestinal tract in relation to the capture of Al is his change of pH,

2-3 in the stomach to 3-8 in the intestine (DeVoto and Yokel, 1994). The ion Al (III) is easily connected to many substances, and determining its metabolism by affinity with each of its ligands (Ganrot, 1986). The materials with which Al is complexed in the intestinal lumen affect the extent of absorption. Eg, transferrin, which when complexed with the metal, tends to accelerate absorption. An important factor in the absorption of this metal are the intestinal pH interfere by directly facilitating the solubility of the metal (Drüeke, 2002).

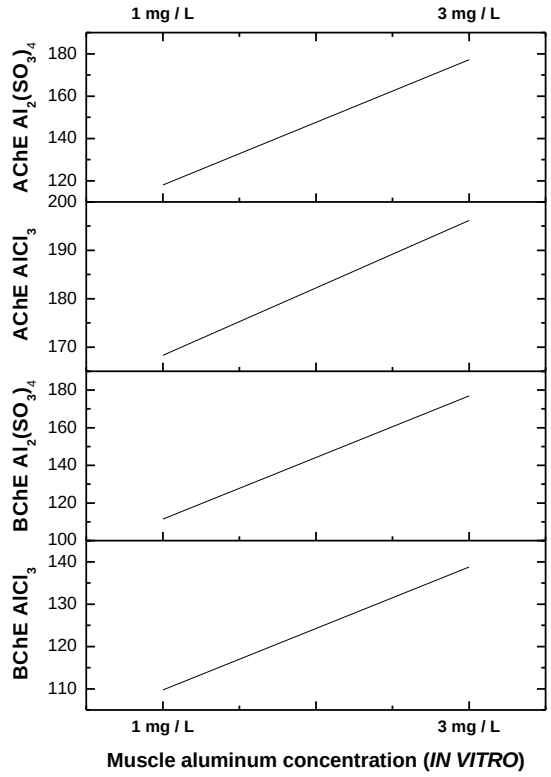

Figure 1- Cholinesterase activity (mU/mg) in vitro: effects of sulfate and chloride.

The pepsin, the main protease enzyme of the stomach metabolism (Nalinanon et al., 2010), demonstrated the greatest reduction at in activity *in vivo*, 65.01 ± 1.04% (TG1) and 61.06 ± 1.90% (TG3), and *in vitro*, 88.12 ± 2.63% (TG1) and 82.52 ± 2.58% (TG3), compared with other analyzed enzymes, trypsin, chymotrypsin and intestinal amylase, as described in **Table 1**. The results with pepsin may be explained by the ability of gut absorption of Al, as given by DeVoto and Yokel, (1994), since the low pH of the stomach allows the complete dissolution, for example, $Al(OH)_3$ to free forms of Al (III), free form of this metal. Despite differences in testing conditions *in vitro* to *in vivo* inhibitory potential of 35% of this metal in pepsin can be explained by the strong interactions that Al undergoes inside the body (Ganrot, 1986), conditions not provided *in vitro* tests when the enzyme is directly exposed to the metal after successive runs. Thus, it is noteworthy that changes in the activity of pepsin may serve as a biomarker of exposure to this metal, even if the exposure occurred in alkaline conditions and cause disturbances in the process of degradation of food, undermining in this way, the metabolism of the digestive fish, influenced, for example, in its growth. Data about the pepsin inhibited by metallic elements are scarce, making it promising sources of new attacks.

There were also reductions in the activity of intestinal proteases studied, trypsin and chymotrypsin in both assays. The highest percentage of inhibition of trypsin was *in vitro* assay (17%, TG3), while chymotrypsin was the *in vivo* (27%, TG3), an effect proportional to increasing amount of aluminum added to the exhibition. Inhibition of fish trypsins by ions of Al (III) *in vitro* have been reported by Bezerra et al. (2005), Souza et al. (2007), Lu et al. (2008) and Wang et al. (2010). The results with chymotrypsin corroborate the assertion Zatta et al. (1993) and Lupidi et al. (2002) that Al (III) has strong influence on the binding properties of this enzyme, affecting enzyme recognition process through a modulation of the active site, between the substrate and the enzyme, favoring the metal. Kinetically, the mechanisms of aluminum *in vivo* may involve passive transfer (simple diffusion) or specialized transport (active transport, facilitated diffusion or pinocytosis). Physiologically, the intestinal absorption can be intracellular or extracellular. The main pathway is the paracellular extracellular through the "leaky" junctions between the cells (Berthon, 2002).

The use of substrate SDS-PAGE (**Figure 2A**) revealed interesting results. In fact, according Garcia-Carreño et al. (1993), this technique is a biochemical tool several times more sensitive than others methods for detecting proteinase composition of crude extracts of tissues, which also allows the observation of enzyme activity zones cause by proteinase inhibitors. Through

analyses of zymogram (**Figure 2B**) it was possible to identify: There was no decrease in enzyme expression in the group exposed to 1 mg/L compared to control, which was visualized as the decreased expression of the enzyme exposed to a concentration of 3 mg/L of the metal. By PMSF, inhibitor of serine proteases, we observed that most bands in the group expressed serine, among which trypsin and chymotrypsin.

The trypsin of *Daphnia magna* was used as a biomarker for Cd, Cr and Hg (De Coen e Janssen, 1997; De Coen et al., 1998). Alayse-Danet et al. (1979)

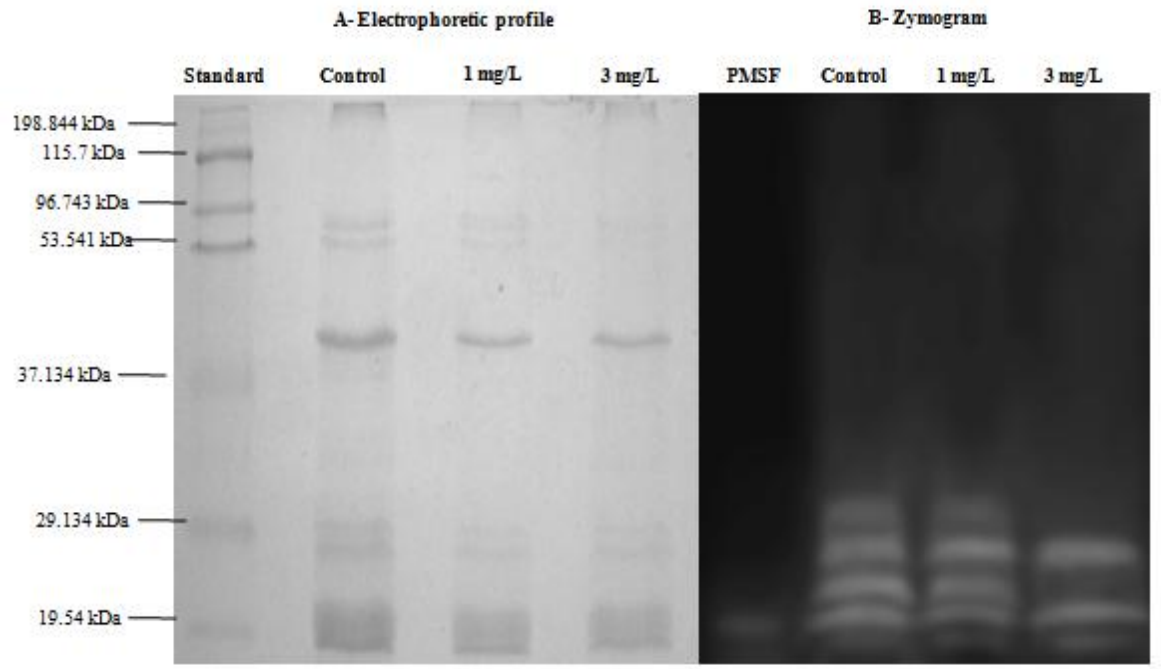

Figure 2 – Protein profile of alkaline. Electrophoretic profile of proteins (SDS-PAGE) and zymogram. 3A - Alkaline electrophoretic profile of proteins (SDS-PAGE). Samples of control (TGC), 1 mg / L (TG1) and 3 mg / L (TG3). 3B – Zymogram consisting Control group (TGC), exposed to 1 mg / L (TG1) group exposed to 3 mg / L (TG3), an inhibitor of serine proteases (PMSF).

Table 2: Inhibitory effect of aluminum on the activity (mU/mg) of digestive proteases of Nile tilapia*

Enzyme	Conc.	*In vitro* $Al_2(SO_4)_3$	*In vitro* $AlCl_3$
Pepsin	*1* mg / L	34.98 ± 0.56%	22.98 ± 0.83%
	3 mg / L	38.93 ± 1.21%	34.31 ± 1.25%
Trypsin	*1* mg / L	11.42 ± 0.72%	4.49 ± 3.20%
	3 mg / L	17.26 ± 0.61%	10.90 ± 1.79%
Chymotrypsin	*1* mg / L	3.40 ± 0.48%	6.59 ± 0.38%
	3 mg / L	8.68 ± 0.45%	12.09 ± 0.35%

*****comparison of the degree of inhibition afforded by sulfate and aluminum chloride by means of *in vitro* (1 h incubation **).**

detected a reduction in growth which coincided with a clear decrease of enzymatic activity of trypsin and amylase in brine shrimp exposed for 72 hours at sub-lethal concentrations of copper (2 mg/L) and zinc (5 mg/L). Sastry and Gupta (1979) evaluated the effect of concentration of 6,8 mg/L of $CdCl_2$ in the digestive system of teleost fish *Heteropneustes fossilis* and found a decrease in activity of trypsin and an increased activity of pepsin. In our research, as illustrated in **Table 2**, aluminum proved a reduction of activity in both the chemical form of aluminum chloride and, indicating the action of the metal.

The amylase, an enzyme carbohydrases activity was reduced in the groups exposed to the metal. The group TG3 showed the biggest drop in the enzymatic activity of all analyzed enzymes, including pepsin, with an inhibition percentage of about 40%. There are rare works that make use of this intestinal enzyme biomarker a tool

for environmental stress, being a promising source of further attacks, since it is related to digestive metabolism of omnivores and herbivores such as tilapia (Moreau et al. 2001), due to metals such as aluminum, silver, mercury, copper, manganese, zinc, iron, cobalt, cadmium and lead are potential inhibitors of this enzyme (Gupta et al., 2003).

Data from this experiment suggest the use of cholinesterase enzyme (brain AChE, muscle AChE, muscle BChE) and digestive (pepsin, trypsin, chymotrypsin and intestinal amylase) through a combination of results - the activation of cholinesterase's studied and monitored the reduction activity of digestive enzymes mentioned above, suggests the presence of aluminum in aquatic systems - as useful tools in the evaluation of biomarkers of exposure to the presence of this metal, even though the induction occurred under alkaline conditions, as demonstrated in our work. We also emphasize the need for further studies to elucidate the interaction between the metal element in the rise of industrial point of view and domestic and digestive enzymes, metabolic by its relevance to aquatic organisms, thus contributing to the monitoring and environmental management of areas affected by this xenobiotic.

Acknowledgement

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13.1.2. Revisão de artigos

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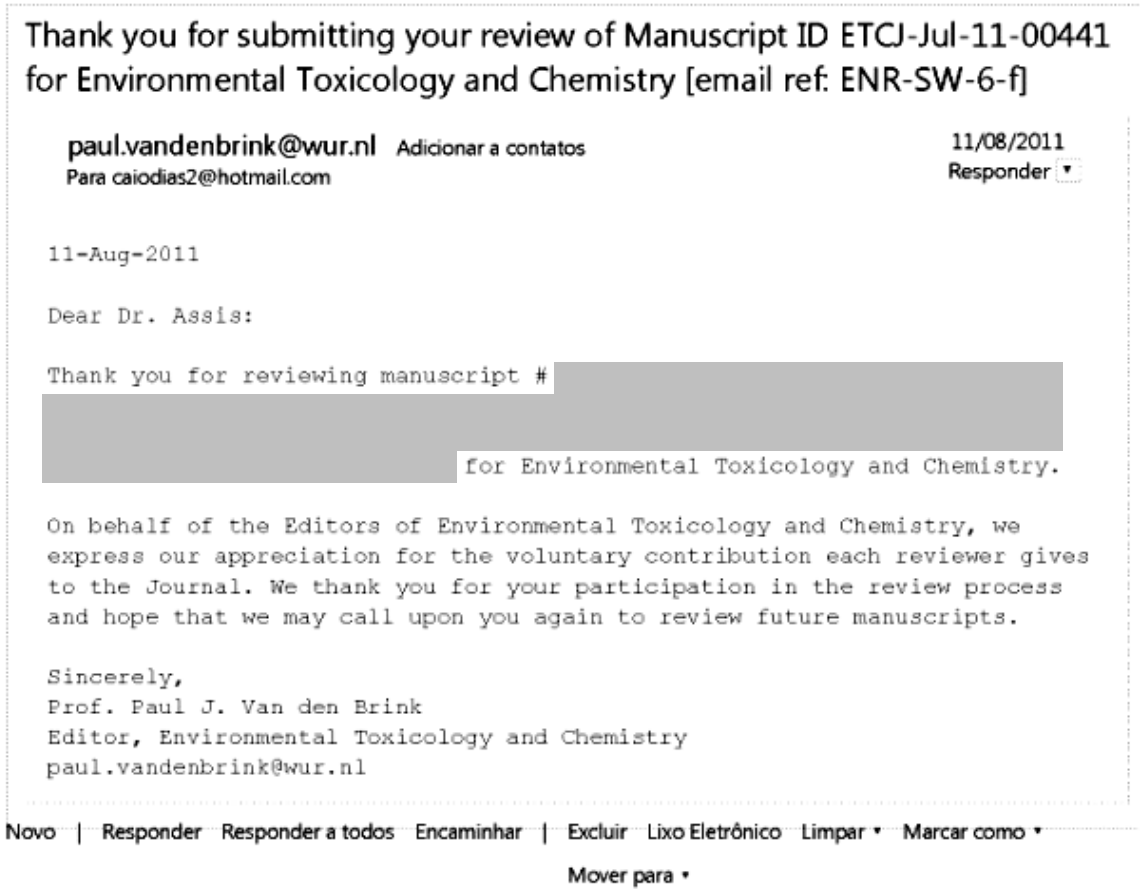

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13.1.3. Resumos em congressos

XXXIX Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq
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Resumos no SBBq 2010

R - 2871 Effect of organophosphorus and carbamate pesticides on human erythrocyte acetylcholinesterase and its potential use as biomarker of occupational exposure

Linhares, A.G, Assis, C.R.D, Carvalho Jr, L.B., Bezerra, R.S.

Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica, and Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco

Pesticides play an important role in the productive process and concomitantly became the source of most cases of human poisoning, occupational or not. Organophosphorus and carbamate are the major classes of pesticides in use around the world. Both classes are inhibitors of acetylcholinesterase (AChE; EC 3.1.1.7). This work aimed to investigate the inhibitory effect of five pesticides on the activity of human erythrocyte acetylcholinesterase. The enzyme activity was assayed by the Ellman colorimetric method. The samples were prepared from the human blood. The enzyme was exposed to the organophosphate pesticides dichlorvos, chlorpyrifos, diazinon and the carbamate pesticides carbaryl and carbofuran. The results showed inhibition about 50% of enzyme activity (IC_{50}) using 10.66 µg/mL of the pesticide dichlorvos. The same inhibition of enzyme activity was obtained with concentrations of 21.42 µg/mL (chlorpyrifos), 109.98 µg/mL (carbaryl) and 5.44 µg/mL (carbofuran). The findings related to 20% enzyme inhibition by dichlorvos, chlorpyrifos, carbaryl and carbofuran were lower than the values of Acceptable Daily Intakes (ADI's) determined by relevant national and international laws. These results suggest that human erythrocyte acetylcholinesterase might be an appropriate bioindicator in the prevention and diagnosis of occupational exposure to some of these pesticides.

Key words: organophosphate and carbamate pesticides, occupational exposure, biomarker, acetylcholinesterase, erythrocyte.

Apoio financeiro: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL e EMBRAPA.

R-8079 Effect of heavy metals, ions and pesticides on the acetylcholinesterase from four fish: an in vitro comparison

Assis, C.R.D.[1], Linhares, A.G.[1], Carvalho, E.V.M.M.[1], Castro, P.F. [2], Amaral, I.P.G.[1], Carvalho Jr, L.B.[1], Bezerra, R.S.[1]

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Acetylcholinesterase (AChE; EC 3.1.1.7) has been used as a biomarker of environmental contamination by pesticides. Aquatic species are commonly chosen for it, since their environments are being contaminated with those compounds. This study aimed to evaluate the behavior of AChE from four species, *Arapaima gigas* , *Colossoma macropomum*, *Rachycentron canadum* and *Electrophorus electricus* in the presence of fifteen heavy metals and ions (Al^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, $EDTA^{2-}$, Fe^{2+}, Hg^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, As^{3+}, Pb^{2+} and Zn^{2+}) and seven pesticides (dichlorvos, diazinon, chlorpyrifos, temephos, tetraethyl pyrophosphate-TEPP, carbaryl and carbofuran). The exposure to heavy metals and ions was performed at concentrations ranging from 0.001 to 10 mM. All species presented inhibition by Hg^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+} and As^{3+}. Pesticide exposure concentrations ranged from 0.001 to 100 µg/mL and the IC_{50} values estimated for them were lower than the Maximum Residue Levels (MRL's) established by national and international regulations, mainly for TEPP, dichlorvos and carbofuran. These results provide assay conditions and data about AChE from these species in relation to its use as the biocomponent of biosensors in heavy metals and pesticide routine screening in the environment. Financial support: FACEPE.

Key words: Organophosphorus, carbamate, Acetylcholinesterase, Biomarkers, Fish.

Apoio financeiro: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL e EMBRAPA.

R-8184 Effect *in vivo* and *in vitro* of Aluminum on Acetylcholinesterase from brain of *Oreochromis niloticus*

Oliveira, V.M.[1], Assis, C.R.D.[1], Costa, H.M.S.[1], Freitas Jr., A.C.V.[1], Silva, J.F.[1], Andrade, D.H.H.[1], Santos, F.M.S.[1], Silva, J.F.X.[1], Carvalho Junior, L.B.[1], Bezerra, R.S.[1]

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Acetylcholinesterase (AChE, EC 3.1.1.7) has been used as a biomarker. *O. niloticus* is the freshwater fish most cultivated in Brazil making it an abundant source of this enzyme. This study evaluated the effect *in vivo* and *in vitro* of Al3+ on AChE activity from brain of *O. niloticus*. Exposure to Al3+ was performed at 0.5ppm and 1.0ppm using $Al_2(SO_4)_3$ for *in vivo* assay and $Al_2(SO_4)_3$ and $AlCl_3$ for *in vitro* analysis. At *in vivo* assays (96h), 45 juveniles specimens were cultured in tanks with fed *ad libitum*. For the *in vitro* assays were incubated (1h) $Al_2(SO_4)_3$ and $AlCl_3$ in extracts samples from brains of 15 specimens from control treatment of *in vivo* assays, using the same concentrations above. The enzymatic activity was determined by the method of Ellman et al. (1961) using 20µL of extract, 200µL of reagent DTNB 0.25mM. The *in vitro* results showed no difference in the use of $Al_2(SO_4)_3$ and $AlCl_3$. $AlCl_3$ increased AChE activity to (0.5 and 1.0ppm, respectively) 91.4% and 160.1%, such as $Al_2(SO_4)_3$ 93,3% and 156,3%. The *in vivo* AChE activity was 117.7% and 213.2%. The results provide conditions of *O. niloticus* AChE to be used as bioindicator of the presence of Al^{3+} in the environment.

Keywords: Acetylcholinesterase, Aluminum, *Oreochromis niloticus*.

Apoio financeiro: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL e EMBRAPA.

V Reunião Regional da Federação de Sociedades de Biologia Experimental - FeSBE
27 a 29 de maio de 2010 – Hotel Parque dos Coqueiros, Aracaju, Sergipe.

Título: EFEITO DE ÍONS E METAIS PESADOS SOBRE A ATIVIDADE DA ACETICOLINESTERASE CEREBRAL DE TILÁPIA-DO-NILO, *Oreochromis niloticus*

1Assis, C.R.D*., 1Oliveira, V.M., 1Costa, H.M.S., 1Linhares, A.G., 1Carvalho Junior, L.B., 1Bezerra, R.S. 1Laboratório de Enzimologia, Departamento de Bioquímica e Fisiologia, Universidade Federal de Pernambuco, Recife/PE.

Diversos estudos apontam para a influência dos íons e metais pesados sobre a atividade da enzima acetilcolinesterase (AChE; EC 3.1.1.7) (Mol. Pharmacol. 18:33, 1980; Env. Res. 21:327-335, 1980). Biossensores para detecção de agentes anticolinesterásicos não levam em conta essa influência. Fato que pode levar a falsos positivos ou negativos e interpretações errôneas na análise dos resultados. Este trabalho objetivou investigar o efeito de diferentes íons e metais pesados (As3+, Ba2+, Ca2+, Cd2+, Cu2+, EDTA2-, Hg2+, K+, Li+, Fe2+, Mg2+, Mn2+, Pb2+ e Zn2+) passíveis de influenciar a atividade da AChE cerebral de tilápia-do-Nilo, *O. niloticus*, tendo em vista seu potencial uso como biomarcador da presença de agentes anticolinesterásicos.

Peixes, de ambos os sexos, foram cultivados por um período de 216 horas, em aquários com 60 L, 26,76 ºC, pH 5,48, 86,25% de oxigênio dissolvido, com limpeza diária e troca dinâmica da água, fotoperíodo de 12:12 h e alimentação *ad libitum*. Após esse período, os animais foram sacrificados por resfriamento. Os cérebros foram colhidos, pesados e homogeneizados em solução tampão Tris-HCl pH 8,0 a 0,5 M até a concentração de 20 mg de tecido por mL de tampão. Em seguida, foi realizada a incubação, por 40 min, dos diferentes íons e metais pesados: As3+, Ba2+, Ca2+, Cd2+, Cu2+, EDTA2-, Hg2+, K+, Li+, Mg2+, Mn2+, Pb2+, Fe2+ e Zn2+ em concentrações de 0,001; 0,01; 0,1; 1 e 10 mM, nos extratos provenientes de cérebros de 15 espécimes juvenis de *O. niloticus*. A atividade enzimática foi determinada através de uma modificação do método colorimétrico de Ellman et al. (Biochem. Pharmacol. 7:88, 1961), utilizando 20 µL de extrato, 200 µL do reagente cromogênico DTNB a 0,25 mM. A reação foi acompanhada em espectrofotômetro de microplacas a 405 nm, durante 3 minutos, após a adição do substrato. Os resultados da análise de íons e metais pesados em 1 mM demonstraram não haver efeito significativo para o Ba2+, Ca2+, EDTA2-, Fe2+, K+, Li+, Mg2+, Mn2+, enquanto que para o As3+, Cd2+, Cu2+, Hg2+, Pb2+ e Zn2+ houve decréscimo da atividade de 61%, 35%, 18%, 100%, 18% e 29%, respectivamente.

A análise dos resultados indica que a maioria dos íons utilizados não influenciou na atividade da acetilcolinesterase cerebral de *O. niloticus*, não sendo, portanto, interferentes importantes durante o uso da referida enzima como biomarcador de agentes anticolinesterásicos. Os íons com potencial para influenciar a atividade colinesterásica, só o fizeram em torno da concentração de 1 mM, a qual é uma concentração bastante elevada para os mesmos em amostras ambientais que não estejam associadas a minerações ou empreendimentos industriais. Além disso, a acetilcolinesterase de *O. niloticus* apresentou um alto potencial para atuar como biomarcador da presença do íon mercúrio (Hg^{2+}).

Apoio financeiro: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL e EMBRAPA.

Título: ACETILCOLINESTERASE CEREBRAL DA TILÁPIA (*Oreochromis niloticus*) COMO BIOINDICADOR DA PRESENÇA DE ALUMÍNIO (Al3+): ABORDAGENS *IN VIVO* E *IN VITRO*

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Objetivos: O alumínio vem se tornando um poluente importante em corpos d'água de todo o mundo. Sua presença está associada a doenças como aluminose, osteodistrofia e anemia microcítica hipocrômica. Estudos indicam neurotoxicidade causada por esse íon (Brain Res. Bull. 59:41, 2002) e um dos seus alvos é a enzima acetilcolinesterase (AChE; EC 3.1.1.7). O presente trabalho teve como objetivo avaliar o efeito *in vivo* e *in vitro* do alumínio (Al3+) sobre a atividade da AChE proveniente do cérebro de Tilápia-do-Nilo, *O. niloticus*, e verificar a possibilidade de uso da referida enzima como biomarcador da presença deste íon.
Métodos e resultados: Os peixes passaram por 120 h de adaptação ao ambiente. O ensaio *in vivo* foi conduzido durante de 96 horas em aquários com 60 L, utilizando 45 espécimes juvenis de *O. niloticus*. Os animais foram divididos em grupo controle (não submetidos ao metal: 26,76ºC; pH 5,48; 86,25 OD) e dois grupos experimentais expostos ao sulfato de alumínio ($Al_2(SO_4)_3$) em concentrações de 0,5 ppm (26,75ºC; pH 4,73; 78,8% OD) e de 1,0 ppm (26,79ºC; pH 5,13; 96,92% OD). Todos com alimentação *ad libitum*, limpeza diária com troca dinâmica da água e fotoperíodo de 12:12 h. Após esse período, os animais foram sacrificados por resfriamento. Os cérebros foram extraídos, pesados e homogeneizados em tampão Tris-HCl pH 8,0, 0,5 M até a concentração de 20 mg de tecido por mL de tampão e em seguida foram centrifugados. Para o ensaio *in vitro* foi realizada a incubação, por 1 h, do sulfato do alumínio nos extratos provenientes de cérebros de 15 espécimes juvenis de *O. niloticus* do tratamento controle, com exposição às concentrações de 0,5 ppm e 1,0 ppm. A atividade enzimática foi determinada através de uma modificação do método colorimétrico de Ellman et al. (Biochem. Pharmacol. 7:88, 1961), utilizando 20 µL de extrato, 200 µL do reagente DTNB 0,25 mM. A reação foi acompanhada em espectrofotômetro de microplacas a 405 nm, durante 3 minutos, após a adição de do substrato. Para o ensaio *in vivo* o $Al_2(SO_4)_3$ apresentou uma atividade colinesterásica de 117,7 ± 13,1% (0,5 ppm) e 213,2 ± 22,8% (1,0 ppm), em relação ao grupo controle. A análise *in vitro* apresentou atividade de 93,3 ± 7,0% (0,5 ppm) e 156,3 ± 10,9% (1,0 ppm), em relação ao respectivo controle. Cloreto de alumínio ($AlCl_3$) foi também utilizado, *in vitro*, com resultados similares (91,45 ± 7% para 0,5 ppm e 160,14 ± 10,9% para 1,0 ppm, em relação ao controle).
Conclusão: Houve um aumento da atividade enzimática da acetilcolinesterase com o aumento da concentração de exposição ao sulfato de alumínio. Estes dados confirmam outros estudos sobre o funcionamento da acetilcolinesterase, *in vivo* e *in vitro*, na presença de alumínio. Além disso, auxiliam em estudos para uma possível utilização como bioindicador da presença do íon em amostras ambientais, dada sua natureza fortemente ativadora, não observada na presença de outros íons em estudos anteriores.

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PHYSICOCHEMICAL AND KINETIC CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM FIVE FISH SPECIES

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Objectives: Acetylcholinesterase (AChE; EC 3.1.1.7) is the enzyme which hydrolyzes the neurotransmitter acetylcholine and its rapid action is responsible for modulating neuronal communication. AChE is the primary target for the action of several pesticides and medicines. The pesticides have become an environmental and public health problem. Monitor and control the presence of these compounds in the environment is of vital importance. To identify changes in enzyme behavior caused by these or other compounds, first is necessary to investigate its normal activity. This study aimed to characterize physicochemical and kinetic parameters of the AChE from five fish species in order to be used in biosensor devices.
Methods and results: The enzymes used were from *E. electricus* (commercial from Sigma) and juvenile specimens of *C. macropomum* (30.0 ± 4.2 cm; 512.5 ± 123.7 g), *A. gigas* (76.8 ± 8.7 cm; 4,118 ± 207.9 g), *O. niloticus* (12.0 ± 3.0 cm; 7.9 ± 1.2 g) and *R. canadum* (51.67 ± 1.5 cm; 1,575 ± 329.6 g). The animals were sacrificed by an ice bath and the brains were removed and weighed. The homogenates were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer. The preparation were centrifuged at 1000 x *g* during 10 min. The optimal pH and temperature were determined, respectively, assaying the activity of the extracts in a pH range from 2.5 to 9.0 and temperatures from 0 to 80ºC. The thermal stability was determined submitting the extracts to the same temperatures during 30 min and, after equilibration, assaying the remaining activity. Kinetic parameters k_m and V_{max} were calculated after determinate the activity under increasing concentrations (0.8 to 20.8 mM final concentration) of the substrate acetylthiocholine iodide. The extracts were assayed in presence of specific cholinesterases inhibitors (concentrations from 0.001 to 10 mM) BW284c51 for AChE, Iso-OMPA for BChE and neostigmine bromide for total ChEs. The enzymatic activity was determined by a colorimetric method (Biochem. Pharmacol. 7:88-95, 1961). Optimum pH was found to be next to 8.0 and optimum temperature was from 35 to 45ºC for all enzymes. *A. gigas*, *C. macropomum* and *O. niloticus* retained between 66 and 81% of activity after incubation at 50ºC for 30 min. The kinetic parameter k_m for all species ranged from 0.12 to 0.48 mM. The extract from *C. macropomum* was less inhibited by BW284c51 at 0.01 mM and was the most inhibited by Iso-OMPA.
Conclusion: Several physicochemical and kinetic features of acetylcholinesterase from five species were observed and these characteristics can be useful as assay conditions for these biomolecules in biotechnological applications such as biosensors.
Financial support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL and EMBRAPA.

EFFECT OF PESTICIDES AND IONS ON BRAIN ACETYLCHOLINESTERASE FROM BEIJUPIRÁ (*Rachycentron canadum* Linnaeus, 1766)

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Objectives: Organophosphorus and carbamate pesticides are classic inhibitors of acetylcholinesterase (AChE; EC 3.1.1.7). These compounds have become an environmental and public health problem. Monitor and control the presence of these compounds in the environment is of vital importance. *R. canadum* is one of the most important marine species cultured. The use of its AChE as biomarker for these compounds can constitute a valuable tool for environmental and food monitoring, in addition of being a useful destination for discarded tissues (brains) produced in the cultivation. This work aims to investigate the *in vitro* effect of organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos and TEPP), carbamates (carbaryl and carbofuran) and interfering ions on the AChE activity in order to analyze its possible use as a biocomponent of sensor devices.
Methods and results: Three juvenile specimens (51.67 ± 1.5 cm; 1,575 ± 329.6 g) were cultured in cages 11 km away from the coast of Pernambuco. The animals were sacrificed by an ice bath and the brains were removed and weighed. The homogenates were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer. The preparation were centrifuged at 1000 x *g* during 10 min. Then, the extracts were incubated with organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos, TEPP) and carbamates (carbaryl and carbofuran) which concentrations were from 0.001 to 1000 µg/mL. In other assays AChE was exposed, during to 15 ions and heavy metals (As3+, Ba2+, Ca2+, Cd2+, Cu2+, EDTA2-, Hg2+, K+, Li+, Mg2+, Mn2+, Pb2+, Fe2+ and Zn2+) and the concentrations ranged from 0,001 to 10 mM. The enzymatic activity was determined by modification of a colorimetric method (Biochem. Pharmacol. 7:88-95, 1961). The values of the pesticides median inhibitory concentration (IC_{50}) were under 9.0 µM, excepting chlorpyrifos, diazinon and temephos. At 1 mM, Ca2+, Mg2+ and Mn2+ induced an increase in the AChE activity while the other ions (excepting K+ and Al3+) inhibited enzyme activity.
Conclusion: AChE from *R. canadum* was extremely sensitive to the pesticides, presenting significative decrease at concentrations lower than the values set by the most of international regulations related to maximum residue levels. Most of the ions and heavy metals with potential to influence the cholinesterase activity, have done it only from 1 mM, which is a concentration not commonly found in environmental samples for most of them.

Financial support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL and EMBRAPA.

ORGANOPHOSPHORUS AND CARBAMATES EFFECTS ON BRAIN ACETYLCHOLINESTERASE FROM PIRARUCU (*Arapaima gigas* Schinz, 1822)

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Objectives: The enzyme acetylcholinesterase (AChE; EC 3.1.1.7) is inhibited by organophosphate and carbamate pesticides. These compounds have been distributed around the world and have become an environmental and public health problem. It is necessary to monitor and control the presence of these compounds in the environment. AChE has potential to be a valuable biomarker for this task. Pirarucu (*Arapaima gigas*) is a species of high farming potential and the use of discarded tissues like the brain constitutes a good environmental destination for part of the waste generated in the cultivation. The objective of this study is to investigate the *in vitro* effect of organophosphorus and carbamates (here represented by dichlorvos, diazinon, chlorpyrifos, temephos, TEPP, carbaryl and carbofuran) on the activity of AChE from *A. gigas*, evaluating its possibility to be used as a component of sensor devices.
Methods and results: Three juvenile specimens (76.8 ± 8.7 cm; 4,118 ± 207.9 g) were cultured in 200 x 50 m pond. The animals were sacrificed by an ice bath and the brains were excised and weighed. The crude extracts were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer. The extracts were centrifuged at 1000 x *g* during 10 min. Organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos, TEPP) and carbamate pesticides (carbaryl and carbofuran) were incubated in the concentrations from 0.001 to 1000 µg/mL, for 60 min, in the crude extracts from the brain of eight *A. gigas* juvenile especimens. The enzymatic activity was determined by modification of a colorimetric method (Biochem. Pharmacol. 7:88-95, 1961). In the determination of the pesticides median inhibitory concentration (IC_{50}), the compounds strongly inhibited the enzyme, especially TEPP (< 0.01 µM). Diazinon and temephos did not show significant inhibition, capable for estimating IC_{50} for this species.
Conclusion: Excepting temephos and diazinon, the other pesticides showed high inhibitory effect on AChE activity, presenting significative decrease at concentrations lower than the values set by the most of international regulations related to maximum residue levels of these pesticides in natural water bodies. The pesticide of greater potential for biomonitoring by *A. gigas* acetylcholinesterase was TEPP, for which the enzyme were extremely sensitive.

Financial support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL e EMBRAPA.

ORGANOPHOSPHORUS AND CARBAMATES ACTION AND EFFECT OF IONS ON ACETYLCHOLINESTERASE FROM PORAQUÊ (*Electrophorus electricus* Linnaeus, 1766)

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Objectives: Organophosphorus and carbamate are the major class of insecticides and fungicides used worldwide. Together, they share about 50% of the world market of pesticides. They are classic inhibitors of the enzyme acetylcholinesterase (AChE; EC 3.1.1.7). These compounds have become an environmental and public health problem. It is necessary to monitor and control the presence of these compounds in the environment. AChE from *E. electricus* has potential to be a useful tool for environmental and food monitoring. The objective of this contribution is to investigate the *in vitro* effect of organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos and TEPP), carbamates (carbaryl and carbofuran) and interfering ions on *E. electricus* AChE activity.
Methods and results: The commercial enzyme from the electric organ of *E. electricus* was diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 0.1 µg/mL. Then, the organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos, TEPP) and carbamates (carbaryl and carbofuran) were incubated in the concentrations from 0.001 to 1000 µg/mL, during 60 min, with the enzyme preparation. In other assays AChE was exposed to each of the 15 ions and heavy metals (As3+, Ba2+, Ca2+, Cd2+, Cu2+, EDTA2-, Hg2+, K+, Li+, Mg2+, Mn2+, Pb2+, Fe2+ and Zn2+), during 40 min and the concentrations were from 0,001 to 10 mM. The enzymatic activity was determined by a modification of a colorimetric method (Biochem. Pharmacol. 7:88-95, 1961). The median inhibitory concentrations (IC_{50}) for all pesticides were less than 8 µM. At 1 mM, Al3+, Ca2+, Mg2+ and Mn2+ induced an increase in the enzyme activity while the other ions (excepting K+) inhibited enzyme activity, especially Hg2+ which inhibited the activity in 100% at that concentration.
Conclusion: All the pesticides used showed high inhibitory effect on AChE activity, presenting significative decrease at concentrations lower than the values set by the most of international regulations related to maximum residue levels of these pesticides in natural water bodies. Most of the ions and heavy metals with potential to influence the cholinesterase activity, have done it only from 1 mM, which is a high concentration for environmental samples that are not associated with mining or industrial enterprises. In addition, AChE from *E. electricus* showed a great potential to serve as a biomarker for the mercury ion (Hg^{2+}).

Financial support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL e EMBRAPA.

EFFECT OF FIVE ORGANOPHOSPHATE AND TWO CARBAMATE PESTICIDES ON BRAIN ACETYLCHOLINESTERASE FROM NILE TILAPIA (*Oreochromis niloticus* Linnaeus, 1758)

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Objectives: Organophosphates and carbamates are classic inhibitors of acetylcholinesterase (AChE; EC 3.1.1.7). Indiscriminate use of such pesticides has become a public health problem in many countries. It is necessary to monitor and control the presence of these compounds in the environment. Nile tilapia (*Oreochromis niloticus*) is the most farmed fish in Brazil, making it an abundant source of AChE. This study aims to investigate the *in vitro* effect of five organophosphates (dichlorvos, diazinon, chlorpyrifos, temephos and TEPP) and two carbamates (carbaryl and carbofuran) on the brain AChE from *O. niloticus* as a way to evaluate the possibility of its use as a biomarker of these pesticides.
Methods and results: Fifteen specimens of both sexes, were cultured for a period of 10 days, in the following conditions: 60 L aquaria, temperature 26.76 ºC, pH 5.48, 86.25% dissolved oxygene, on a dynamic exchange of water, photoperiod of 12:12 h and feeding *ad libitum*. After the adaptation period, the animals were sacrificed by an ice bath, the brains were excised and weighed. The crude extracts were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until 20 mg of tissue per mL of buffer. The extracts were centrifuged at 1000 x *g* during 10 min. Then, the organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos, TEPP) and carbamate (carbaryl and carbofuran) were incubated in the concentrations from 0.001 to 1000 µg/mL, during 60 min, in the crude extracts from the brain of 15 *O. niloticus* juvenile especimens. The enzymatic activity was determined by modificating of a colorimetric method (Biochem. Pharmacol. 7:88-95, 1961). In the determination of the pesticides median inhibitory concentration (IC_{50}), were found values under 6 µM for carbofuran and dichlorvos. Diazinon and temephos did not induce significant inhibition on AChE from this species.
Conclusion: All the pesticides used, excepting temephos and diazinon, showed high inhibitory effect on AChE activity, presenting significative decrease at concentrations lower than the values set by the most of international regulations related to maximum residue levels of these pesticides in natural water bodies. The pesticides of greater potential for biomonitoring by *O. niloticus* acetylcholinesterase were carbofuran and dichlorvos, which caused 50% of inhibition at lower concentrations.
Financial support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL e EMBRAPA.

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Caracterização físico-química, cinética e conteúdo cerebral de acetilcolinesterase em cinco espécies de peixes

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A acetilcolinesterase (AChE; EC 3.1.1.7) é a enzima responsável por hidrolisar o neurotransmissor acetilcolina e sua rápida ação modula a comunicação neuronal. A AChE é o alvo primário da ação de vários pesticidas e medicamentos. Devido à sua crescente utilização, os pesticidas tornaram-se problema de saúde pública e ambiental, portanto, o monitoramento e o controle da presença de tais compostos no meio ambiente é de vital importância. Para identificar mudanças no comportamento enzimático causadas por essas e outras substâncias, é necessário investigar sua atividade em condições normais. Este estudo teve como objetivo caracterizar parâmetros físico-químicos e cinéticos da AChE de cinco espécies, bem como seu conteúdo cerebral em quatro dessas espécies. As enzimas utilizadas foram provenientes de poraquê (*E. electricus*, enzima comercial do órgão elétrico) e espécimes juvenis de tambaqui (*C. macropomum*, 30,0 ± 4,2 cm; 512,5 ± 123,7 g), pirarucu (*A. gigas*, 76,8 ± 8,7 cm; 4118 ± 207,9 g), tilápia do Nilo (*O. niloticus*, 12,0 ± 3,0 cm; 7,9 ± 1,2 g) e beijupirá (*R. canadum*, 51,67 ± 1,5 cm; 1575 ± 329,6 g). Os animais foram sacrificados em gelo e os cérebros foram removidos e pesados. Os extratos brutos foram preparados em homogeneizador de tecidos e diluídos em tampão Tris-HCl 0,5 M pH 8,0 até a concentração 20 mg de tecido por mL de tampão. Os homogenatos foram centrifugados a 1000 x *g* durante 10 min. O pH e temperatura ótimos foram determinados, respectivamente, ensaiando a atividade dos extratos em uma faixa de pH de 2,5 a 9,0 e de temperatura de 0 a 80ºC. A estabilidade térmica foi determinada submetendo os extratos à mesma faixa de temperatura durante 30 min e, após equilibração com a temperatura ambiente, observando a atividade remanescente. Os parâmetros cinéticos km e Vmax foram calculados a partir da atividade enzimática na presença de concentrações crescentes (0,8 a 20,8 mM de concentração final) do substrato iodeto de acetiltiocolina. A atividade dos extratos foi analisada em presença dos inibidores seletivos de colinesterases (concentrações de 0,001 a 10 mM) BW284c51 para AChE, Iso-OMPA para butirilcolinesterase (BChE) e brometo de neostigmina para colinesterases totais. A atividade enzimática foi determinada através de um método colorimétrico utilizando 20 µL de extrato enzimático, 200 µL do reagente cromogênico DTNB. A reação foi acompanhada durante 3 min a 405 nm em espectrofotômetro de microplacas imediatamente após a adição do substrato (20 µL). O pH ótimo encontrado para todas as espécies foi em torno de 8,0 e a temperatura ótima variou de 35 a 45ºC. *A. gigas*, *C. macropomum* e *O. niloticus* retiveram entre 66 e 81% da atividade após exposição dos extratos a 50ºC por 30 min. O parâmetro cinético km, para as espécies, variou de 0,12 a 0,48 mM. O extrato de *C. macropomum* foi o menos inibido pelo

BW284c51 a 0.01 mM e foi o mais inibido pelo Iso-OMPA. Várias características físico-químicas e cinéticas de acetilcolinesterases das cinco espécies foram observadas e tais peculiaridades podem ser úteis como condições experimentais dessas moléculas em aplicações biotecnológicas tais como biossensores.
Palavras-chave: acetilcolinesterase, butirilcolinesterase, caracterização, inibidor específico, peixe

Apoio: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL e EMBRAPA.

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EFFECT OF IONS AND HEAVY METALS ON BRAIN ACETYLCHOLINESTERASE FROM TUCUNARÉ (*Cichla ocellaris* SCHNEIDER, 1801)

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Several studies point to the influence of ions and heavy metals on the activity of acetylcholinesterase (AChE, EC 3.1.1.7). Biosensors for detection of anticholinesterase agents do not take into account this influence. This fact can lead to false positive or negative and erroneous interpretations in the analysis. This study aimed to investigate the effect of fourteen different ions and heavy metals that might influence the activity of brain AChE from *C. ocellaris*, because of its potential use as a biomarker for the presence of anticholinesterase agents. Juvenile specimens were captured from São Francisco River. the animals were killed by cooling. The brains were collected and homogenized in Tris-HCl buffer 0.5 M, pH 8.0, until reach 20 mg of tissue per mL buffer. Then, incubation for 40 min with ions and heavy metals were performed: As3+, Ba2+, Ca2+, Cd2+, Cu2+, EDTA2-, Hg2+, K+, Li+, Fe2+, Mg2+, Mn2+, Pb2+ and Zn2+ at five concentrations from 0.001 to 10 mM in extracts from brains of *C. ocellaris*. The enzyme activity was determined using 20 µL of extract and 200 µL chromogenic reagent DTNB 0.25 mM. The reactions were monitored at 405 nm for 3 minutes after substrate addiction. The results of the analysis of ions and heavy metals at 1 mM showed no significant effect on Ba2+, Ca2+, Cd2+, EDTA2-, Fe2+, K+, Li+, Mg2+, Mn2+, Pb2+, while for As3+, Cu2+, Hg2+, and Zn2+ the activity decreased 77%, 35%, 100% and 18% respectively. The analysis indicates that most of the ions used had no effect on brain acetylcholinesterase activity of *C. ocellaris*, which are not important interferents during the use of this enzyme as a biomarker of anticholinesterase agent. Ions with potential to influence the cholinesterase activity, done it around 1 mM, which is a very high concentration for them in environmental samples not associated with mining or industrial enterprises. In addition, acetylcholinesterase from *C. ocellaris* showed a high potential to serve as a biomarker for the presence of mercury ion (Hg^{2+}).
Keywords: heavy metals, ions, acetylcholinesterase.

Financial support: CNPq, FINEP/RECARCINE, FACEPE, PETROBRAS, EMBRAPA.

ORGANOPHOSPHORUS AND CARBAMATES EFFECTS ON BRAIN ACETYLCHOLINESTERASE FROM TUCUNARÉ (*Cichla ocellaris* SCHNEIDER, 1801)

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The enzyme acetylcholinesterase (AChE; EC 3.1.1.7) is inhibited by organophosphate and carbamate pesticides. These compounds have been distributed around the world and have become an environmental and public health problem. It is necessary to monitor and control the presence of these compounds in the environment. AChE from fish has potential to be a valuable biomarker for this task. The objective of this study is to investigate the *in vitro* effect of five organophosphorus and two carbamates on the activity of AChE from *C. ocellaris*, evaluating its possibility to be used as a component of sensor devices. Four juvenile specimens were captured from São Francisco River. The animals were sacrificed by an ice bath and the brains were excised and weighed. The crude extracts were prepared in tissue disrupter, diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer and then centrifuged. Organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos, TEPP) and carbamate pesticides (carbaryl and carbofuran) were incubated in seven concentrations from 0.001 to 1000 µg/mL, for 60 min, in the crude extracts from the brain of *C. ocellaris* juvenile specimens. The enzymatic activity was determined using 20 µL of crude extract and 200 µL of DTNB 0.25 mM. The reaction was followed at 405 nm, during 180 s, after substrate addition. In the determination of pesticides median inhibitory concentration (IC_{50}), were found the following values: 0.21 µM (carbofuran), 5.52 µM (dichlorvos), 4.41 µM (carbaryl), 0.37 µM (TEPP) and 10.13 µM relating to chlorpyrifos. Diazinon and temephos did not show significant inhibition. Excepting temephos and diazinon, the other pesticides showed high inhibitory effect on AChE activity, presenting significative decrease at concentrations lower than the values set by the most of international regulations related to maximum residue levels of these pesticides in natural water bodies. This fact shows the accuracy of the present method. The pesticides of greater potential for biomonitoring by *C. ocellaris* acetylcholinesterase were carbofuran and TEPP, for which the enzyme were extremely sensitive.
Keywords: organophosphorus, carbamates, acetylcholinesterase.

Financial support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRÁS AMBIENTAL and EMBRAPA.

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R8842 - Effect of heavy metals on the Acetylcholinesterase from fish (Nile tilapia and tucunaré)

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The literature reports the influence of some heavy metals on the acetylcholinesterase (AChE, EC 3.1.1.7) activity. AChE-based biosensors for detection of anticholinesterase agents do not take into account this influence. This fact can lead to erroneous interpretations in the analysis. This study aimed to investigate the effect of five heavy metals (Arsenic, Copper, Mercury, Lead and Zinc) that might influence the activity of brain AChE from two cichids fish: Nile tilapia (Oreochromis niloticus) and tucunaré (Cichla ocellaris). The brains were collected and homogenized in Tris-HCl buffer 0.5 M, pH 8.0 (20 mg of tissue per mL buffer). The metals at five concentrations (from 0.001 to 10 mM) were incubated with the brain extracts that were also exposed to EDTA2- (from 0,001 to 150 mM) due to its ability to protect AChE from other sources against divalent cations. The enzyme activity was determined using 20 µL of extract + metals and 200 µL chromogenic reagent DTNB 0.25 mM. The reactions were monitored at 405 nm for 3 minutes after substrate addiction. The results of the analysis of heavy metals showed that AChE activity from both species was significantly inhibited by all metals under study and EDTA2- only inhibits the enzymes at high concentration (50 mM). The highest inhibitory effect was observed when the enzymes were exposed to arsenic (61 and 75%, inhibition at 1 mM for O. niloticus and C. ocellaris, respectively) and mercury (100% for both species at 1 mM).

Key words: heavy metals, acetylcholinesterase, fish, cichlids

Supported by: CNPq, FINEP/RECARCINE, FACEPE, PETROBRAS, EMBRAPA

R8687 - Effect of Larvicidal Extract from Moringa oleifera Flowers on Trypsin and Acethylcholinesterase Activities of Aedes aegypti Larvae

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The control of Aedes aegypti population is fundamental to reduce dengue fever spreading. This work reports the investigation of constituents from Moringa oleifera flower extract and its effect on fourth (L4) larval instar of A. aegypti. The extract contains proteinaceous trypsin inhibitor (MoFTI, 25.9 kDa, Ki= 2.5 nM), triterpene (β-amyrin), sterol (β-sitosterol) as well as flavonoids (kaempferol and quercetin) and did not contain lectin. Larvicidal activity was detected on L4 (LC50 of 0.925% w/v corresponding to 1.851 mg/ml of protein). The flower extract inhibited L4 gut trypsin (Ki= 3.8 nM) and did not affect acetylcholinesterase (AChE) activity from total L4. In vivo assay showed that gut trypsin activity from L4 treated with M. oleifera flower extract decreased along the time (0 to 1440 min) and was strongly inhibited (98.6 %) after 310 min incubation; AChE activity from total L4 extract was not affected in this period. The study points out M. oleifera flower extract as a biodegradable tool for A. aegypti larvae control and suggest that larvicidal mechanism involves inhibition of L4 gut trypsin.

Word Keys: Moringa oleifera; Aedes aegypti; gut trypsin; acethylcholinesterase.

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06 a 10 de Junho de 2011 – Centro de Convenções de Natal, Rio Grande do Norte.

ACETYL AND BUTYRYLCHOLINESTERASE FROM BRAIN OF FIVE TROPICAL FISH

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Currently, there are two accepted types of cholinesterases: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). AChE classical function is modulating the nerve impulses by the deactivation of neurotransmitter acetylcholine in cholinergic synapses and neuromuscular junctions. BChE physiological function is not well defined although studies point to the possibility of acting in hydrolysis of xenobiotics like cocaine, heroin and as scavenger of pesticides and other anticholinesterasic agents. They have been extensively studied for being the primary targets of compounds used in agriculture, medicine, public health campaigns and chemical weapons. Cholinesterase inhibition is a widely known biomarker of exposure to organophosphorus and carbamate pesticides in aquatic environments due to its sensibility to such compounds. These enzymes have been assayed in several aquatic organisms because of its ability to assess the environmental impact even when these compounds are not present in the water. The aims of the present study were to characterize the cholinesterase relative content in brain of five tropical fish.

Brain ChE content from five fish, pirarucu (*Arapaima gigas*), tambaqui (*Colossoma macropomum*), cobia (*Rachycentron canadum*), electric eel (*Electrophorus electricus* commercial enzyme) and Nile tilapia (*Oreochromis niloticus*) were characterized by the Ellman method using the substrates acetylthiocholine iodide and S-butyrylthiocholine iodide. Selective inhibitors were used: BW284c51 as AChE inhibitor, Iso-OMPA as BChE inhibitor and neostigmine bromide as total ChE inhibitor. By the exposure to these compounds was possible to estimate the IC_{50} and dissociation constant (Ki) for the enzymes.

The content of BChE observed in the brain of *C. macropomum* shows the higher residual activity (retaining 10% activity) even at 10 mM of BW284c51, while the other species presented negligible activity at 1 mM. This species was the only that presents a sharp drop in its ChE activity at 1 mM of Iso-OMPA. The IC_{50} and Ki related to each inhibitor is presented in Table 1. The molecular weight of AChE and BChE were between 202 to 300 KDa. The results suggested that *C. macropomum* brain has a higher relative content of BChE compared to other species studied here or its AChE shows structural changes that represent an intermediary size of active center between it and BChE, implying in resistance to AChE selective inhibitor.

Table 1 IC_{50} and Ki of ChE selective inhibitors in relation to five species

Species	IC50 (µM)	Ki (µM)
BW284c51		
Colossoma macropomum	320	2.19
Arapaima gigas	7.52	0.05
Rachycentron canadum	3.96	0.007
Oreochromis niloticus	4.12	0.02
Electrophorus electricus	0.85	0.006
Iso-OMPA		
Colossoma macropomum	318	2.19
Arapaima gigas	-	-
Rachycentron canadum	-	-
Oreochromis niloticus	-	-
Electrophorus electricus	-	-
Neostigmine		
Colossoma macropomum	0.59	0.004
Arapaima gigas	0.151	0.001
Rachycentron canadum	0.17	0.0003
Oreochromis niloticus	0.16	0.0009
Electrophorus electricus	0.17	0.001

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SEASONAL CHANGES IN TOTAL PROTEIN AND LECTINS, RELEVANT IMMUNE PROTEINS IN FISH TAMBAQUI *Colossoma macropomum* (Cuvier, 1818) SERUM

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The tambaqui is a freshwater fish native to the Northern Region of Brazil. This fish has great economic importance due to the high growth pattern and excellent quality of fillet. The cultivation of tambaqui is very difficult in cold regions due to high mortality rates. The optimal temperature is around 25 to 30 ^{0}C therefore ideal for its cultivation in the Northern, Northeast and Midwest Regions of Brazil. Lectins are proteins with the ability to bind, selectively, carbohydrates. The discovery of these proteins in fish has added a new dimension in the immunology of these animals. Lectins of serum recognize foreign cells as "non-self" through the carbohydrates expressed on the surface acting as opsonins and encouraging its destruction by complement and/or phagocytic cells. Our group has recently purified lectins with antimicrobial activity in tambaqui serum and the aim of our current work is to analyze the activity of these lectins against seasonality.

The blood from an adult tambaqui was collected, through its caudal vein, throughout the year, covering the major stations in the North and Northeast Regions of Brazil (summer, autumn and winter). After blood coagulation, the serum was removed. Lectin activity (hemagglutinating activity-HA), was carried out according to Correia e Coelho [1]. The serum was serially diluted in 0.15 M NaCl before adding rabbit erythrocytes. Titer was expressed as the highest dilution exhibiting hemagglutination. The protein concentration was estimated according to Bradford [2]. Specifc HA (SHA) was defined as the ratio between the titer of HA and protein concentration (mg/mL)

There were significant differences to HA and SHA between summer and winter (p <0.05, Tukey test), seasons where we have sudden changes in temperature in the Region (Table 1).

Table 1: HA and SHA of lectins in the serum of tambaqui. Data collected for 4 years.

Seasons of the Year	HA (titre^{-1})	SHA
Spring (Sept-Nov)	512	25.6
Summer (Dec-Feb)	1024	51.2
Autumn ((Mar-May)	512	25.6
Winter (June-Aug)	64	3.2

The lectins present in the serum of tambaqui possess antimicrobial activity against pathogenic bacteria for freshwater fish. The seasonality of HA corroborate the fact that tambaqui, during winter, becomes more susceptible to mortality from diseases caused by bacteria and fungi [3, 4]. With this information new tools may be offered to a better understanding of the role of these proteins in the immune system of the tambaqui, in order to improve the management of this fish by pisciculturists of the Region.

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BUTYRYLCHOLINESTERASE ACTIVITY IN MUSCLE OF *Oreochromis niloticus* EXPOSED IN VIVO AND IN VITRO TO ALUMINUM

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Aim: Aluminum has become a major pollutant in water bodies around the world. Its presence is associated with diseases such aluminosis, osteoarthritis, Alzheimer's, Parkinson's disease and microcytic hypochromic anemia. Butyrylcholinesterase (BChE; EC 3.1.1.8) is one of the main detoxification enzymes which hydrolyze or scavenge a broad range of xenobiotics and its rapid action is partly responsible for homeostatic conditions by protecting acetylcholinesterase (AChE; EC 3.1.1.7) against aggressive agents, such as heavy metals. BChE has been used as a biomarker in aquatic organisms. Nile Tilapia, *Oreochromis niloticus*, is the freshwater fish most cultivated in Brazil, making it an abundant source of this enzyme. This study evaluated the *in vivo* and *in vitro* effect of aluminum (Al^{3+}) on BChE activity in the muscle from *O. niloticus*. Exposure to Al^{3+} was performed at the Department of Biochemistry, Universidade Federal de Pernambuco.

Methods and results: The trial lasted 14 days after a period of 5 days of adaptation, using 90 juvenile specimens, grown in 90 L aquaria comprising daily cleaning with dynamic exchange of water, photoperiod of 12:12 h and feeding *ad libitum*. The animals were divided into three groups, control (not subjected to metal: 27.29±0.34° C; pH 6.50±0.13; 6.31±0.32 $mg.L^{-1}$ DO), exposed to 1 ppm (27.30±0.05°C, pH 6.26±0.33, 6.09±0.29 $mg.L^{-1}$ DO) and exposed to 3 ppm (27.38±0.07°C, pH 6.13±0.10, 6.01±0.09 $mg.L^{-1}$ DO), performed in triplicate. The animals were sacrificed by an ice bath and the muscle were excised and weighed. The crude extracts were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer. The extracts were centrifuged at 1,000 x *g* during 10 min. The *in vitro* assay was performed incubating the Al^{3+} in extracts from muscle of 30 juvenile specimens of the control treatment for a period of 1h, exposed to concentrations from 1 and 3 ppm. The enzyme activity was determined using 20 µL of extract, 200 µL of the chromogenic reagent DTNB 0.25 mM. The reaction was monitored on a microplate spectrophotometer at 405 nm for 3 minutes after adding 20 µL of 62 mM butyrylthiocholine. The activity of butyrylcholinesterase *in vivo*, in concentrations of 1 and 3 ppm, in relation to the controls, were 105.15 ± 2.64% and 163.60 ± 4.15%, while *in vitro* were 168.28 ± 5.49% and 196.17 ± 4.08%, respectively.

Conclusion: There was an increase in enzymatic activity of BChE, both *in vivo* and *in vitro* concomitantly with increasing concentration of exposure to aluminum sulfate. The results provide conditions for the use of muscle BChE from *Oreochromis niloticus* as a biomarker for the presence of aluminum in environments impacted by such xenobiotics.

Sources of research support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRAS AMBIENTAL and EMBRAPA.

PHYSICOCHEMICAL AND KINETIC CHARACTERIZATION OF CHOLINESTERASES FROM BRAIN OF TUCUNARÉ (*Cichla ocellaris*)

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Objectives: Acetylcholinesterase (AChE; EC 3.1.1.7) is the enzyme which hydrolyzes the neurotransmitter acetylcholine and its rapid action is responsible for modulating neuronal communication. Butyrylcholinesterase (BChE; EC 3.1.1.8) is one of the main detoxification enzymes which hydrolyze or scavenge a broad range of xenobiotics and its rapid action is partly responsible for homeostatic conditions by protecting against anticholinesterasic pesticides such as organophosphorus and carbamates. This study aimed to characterize physicochemical and kinetic parameters of the AChE and BChE-like activity from brain of *C. ocellaris* in order to be used in biosensor devices.
Methods and results: The enzymes used were from juvenile specimens of *C. ocellaris* (31.17 ± 2.47 cm; 322 ± 14.28 g). The animals were sacrificed by an ice bath and the brains were removed, pooled and weighed. The homogenates were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer. The preparation were centrifuged at 1000 x *g* during 10 min. The optimal pH and temperature were determined, respectively, assaying the activity of the extracts in a pH range from 2.5 to 9.0 and temperatures from 0 to 80ºC. The thermal stability was determined submitting the extracts to the same temperatures during 30 min and, after equilibration, assaying the remaining activity. Kinetic parameters km and Vmax were calculated after determinate the activity under increasing concentrations (0.8 to 20.8 mM final concentration) of the substrate acetylthiocholine and S-butyrylthiocholine iodide. The extracts were assayed in presence of specific cholinesterases inhibitors (concentrations from 0.001 to 10 mM) BW284c51 for AChE, Iso-OMPA for BChE, neostigmine and eserine for total ChEs. The enzymatic activity was determined by a colorimetric method (Biochem. Pharmacol. 7:88-95, 1961). Optimum pH was found to be 8.0-8.5 and optimum temperature was around 35ºC. *C. ocellaris* retained 35% of its AChE activity after incubation at 50ºC for 30 min. The kinetic parameter km for AChE activity was 0.76 mM. BChE-like activity was negligible and only presented a peak at pH 8.5. The extract from *C. ocellaris* was weakly inhibited (13%) by Iso-OMPA at 10 mM and was highly inhibited (92%) by BW284c51 at 0.1 mM. Neostigmine and eserine strongly inhibited ChE activity at 0.001 and 0.01 mM, respectively.
Conclusion: Some physicochemical and kinetic features of cholinesterases from brain of *C. ocellaris* were observed and these characteristics can be useful as assay conditions for these biomolecules in biotechnological applications such as biosensors. Moreover, the brain of this species has negligible BChE content or the very little BChE-like activity can be ascribed to the ability of AChE to hydrolyze the substrate butyrylthiocholine.
Financial support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRAS AMBIENTAL and EMBRAPA.

CHARACTERIZATION OF BUTYRYLCHOLINESTERASE-LIKE ACTIVITY FROM BRAIN OF BEIJUPIRA (*Rachycentron canadum*)

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Aim: Butyrylcholinesterase (BChE; EC 3.1.1.8) is one of the main detoxification enzymes which hydrolyze or scavenge a broad range of xenobiotics and its rapid action is partly responsible for homeostatic conditions by protecting acetylcholinesterase (AChE; EC 3.1.1.7) against anticholinesterasic pesticides such as organophosphorus and carbamates. In some cases, these pesticides present an inhibitory action on BChE activity which is stronger than on AChE (Hum Ecol Risk Assess 8; 165, 2002). Monitor and control the presence of these compounds in the environment is of vital importance. To identify changes in enzyme behavior caused by these compounds, first is necessary to investigate its normal activity. This study aimed to characterize physicochemical and kinetic parameters of the BChE-like activity from brain of *R. canadum* in order to be an alternative component in biosensor devices.

Methods and results: The enzymes used were from juvenile specimens of *R. canadum* (51.67 ± 1.5 cm; 1.575 ± 0.329 kg). The animals were sacrificed by an ice bath and the brains were removed, pooled and weighed. The homogenates were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer. The preparation were centrifuged at 1000 x *g* during 10 min. The optimal pH and temperature were determined assaying the activity of the extracts in a pH range from 2.5 to 9.0 and temperatures from 0 to 80ºC, respectively. From temperature data was estimated the activation energy (Ea) of the enzymatic reaction by Arrhenius plot. Kinetic parameters km, Vmax, km ratio and Vmax ratio were calculated after determinate activity under increasing concentrations (0.8 to 20.8 mM final concentration) of the substrates S-butyrylthiocholine, acetylthiocholine and propionylthiocholine iodides. The extracts were assayed in presence of specific cholinesterases inhibitors (concentrations from 0.001 to 10 mM) BW284c51 for AChE, Iso-OMPA for BChE, neostigmine and eserine for total ChEs. The enzymatic activity was determined by a colorimetric method (Biochem. Pharmacol. 7:88-95, 1961). Optimum pH was found to be next to 8.5 and optimum temperature was 35 ºC. The km and Vmax of *R. canadum* BChE-like activity were 4.23 mM and 31.9 mU/mg protein, respectively. The activity was almost completely inhibited at 0.01 mM eserine and was inhibited by Iso-OMPA 1mM. The Ea estimated for the enzymatic reaction was 7.1701 Kcal/mol.

Conclusion: Part of cholinesterase activity in the brain of *R. canadum* can be ascribed to BChE-like activity. Several physicochemical and kinetic features of this activity were observed and these characteristics can be useful as assay conditions for these biomolecules in biotechnological applications such as biosensors.

Sources of research support: CNPq, SEAP\PR, FINEP/RECARCINE, FACEPE, PETROBRAS AMBIENTAL and EMBRAPA.

CHARACTERIZATION OF BRAIN ACETYLCHOLINESTERASE FROM COBIA (*Rachycentron canadum*) AND EFFECT OF ORGANOPHOSPHORUS AND CARBAMATE PESTICIDES

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ABSTRACT

Organophosphorus and carbamates are the major classes of pesticides in use around the world. However, their high toxicity to mammals and other non-target organisms is a threat for human and environmental health. Both classes are cholinesterase inhibitors and several methodologies have been developed in order to monitor their presence in natural samples. Aquatic species are commonly chosen for it, since their environments are being contaminated with those compounds. Here, acetylcholinesterase (AChE; EC 3.1.1.7) from brain of cobia (*Rachycentron canadum*) was partially characterized, and its activity was assayed in presence of five organophosphate and two carbamate insecticides: dichlorvos, diazinon, chlorpyrifos, temephos, tetraethyl pyrophosphate (TEPP), carbaryl and carbofuran, respectively. The km, Vmax, activation energy (AE), rate enhacement, optimum pH, temperature and thermal stability were determined. Three isoforms of AChE were found in brain of *R. canadum* and their molecular weights were estimated. The enzyme was sensitive to all pesticides and the lowest IC_{50} estimated was 0.08 µM referring to the carbamate carbofuran. These results suggest that AChE from *R. canadum* brain could be useful for routine carbofuran screening.

Keywords: acetylcholinesterase, characterization, biomarker.

INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) is the enzyme which hydrolyzes the neurotransmitter acetylcholine and its rapid action is responsible for modulating neuronal communication. AChE is the primary target for the action of several pesticides and medicines (SILMAN *et al.,* 2003; TÕUGO, 2001). Organophosphorus and carbamate pesticides are the major classes of insecticides in the world market and they (NAUEN *et al.,* 2002). These pesticides are classic inhibitors of AChE. They bind to catalytic site of the enzyme through phosphorylation or carbamoylation, causing inhibition, which tends to irreversibility, in the case of organophosphate exposure. The disruption of the nerve function occurs by the accumulation of the acetylcholine in the synaptic cleft. Loss of AChE activity may lead to a range of effects resulting from excessive nervous stimulation and culminating in respiratory failure and death. The pesticides have become an environmental and public health problem. Monitor and control the presence of such compounds in the environment is of vital importance. To identify changes in enzyme behavior caused by these or other compounds, first is necessary to investigate its normal activity (STURM *et al*., 1999). *R. canadum* is one of the most important marine species cultured. The use of its AChE as biomarker for these compounds can constitute a valuable tool for environmental and food monitoring, in addition of being a useful destination for discarded tissues (brains) produced in the cultivation. This work aims to characterize kinetic and physicochemical features of AChE from *R. canadum* brain in addition to investigate the *in vitro* effect of organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos and TEPP), carbamates (carbaryl and carbofuran) on its activity in order to analyze its possible use as a biocomponent of sensor devices.

MATERIALS AND METHODS

Juvenile specimens (51.67 ± 1.5 cm; 1,575 ± 329.6 g) were cultured in cages 11 km away from the coast of Pernambuco. The animals were sacrificed by an ice bath and the brains were removed and weighed. The homogenates were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer. The preparation were centrifuged at 1000 x *g* during 10 min. Enzymatic activity was determined by a modification of the colorimetric method of Ellman (ASSIS *et al*., 2010) using 20 µL of crude extract, 200 µL of 0,25 mM chromogenic agent DTNB and 20 µL of acetylthiocholine iodide 62 mM. The increase in absorbance was followed spectrophotometricaly during 180 s at 405 nm after substrate addition. Were determined the optimal pH and temperature. Activation energy (AE) and rate enhancement of the enzymatic reaction was estimated by Arrhenius plot. The extracts were incubated during 60 min with organophosphorus (dichlorvos, diazinon, chlorpyrifos, temephos, TEPP) and carbamates (carbaryl and carbofuran) which concentrations were 0.001; 0.01; 0.1; 1; 10; and 100 µg/mL. The enzyme parameters km and vmax as well as IC_{50} related to all pesticides under study were determined by non linear regression fitting using Microcal Origin® 8.0 software while the inhibition constant (ki) for each pesticide was estimated by the CHENG and PRUSOFF equation (1973). Molecular weight of brain AChE isoforms was estimated by staining of non-denaturing 6-10% polyacrylamide gel according to MOHAMED *et al.* (2007).

RESULTS AND DISCUSSION

Tab. 1 shows operating parameters of brain AChE from *R. canadum* where can be observed the high rate enhancement in relation to non-enzymatic reaction and the high affinity for the substrate acetylthiocholine expressed by the low value of km. In relation to the thermal stability, the enzyme retained 70% its activity after 30 min at 45ºC. Tab. 2 displays molecular weights estimated for the three isoforms found in brain of *R. canadum*. In the exposure to pesticides, the strongest inhibitory effect was achieved by the carbamate carbofuran (Tab. 3) which is known to be a potent anticholinesterasic agent (THAM *et al*., 2009; ASSIS *et al*., 2010). Diazinon and temephos did not present IC_{50} in the concentration range used in the present study.

Km [mM]	Vmax [mU/mg protein]	Optimum pH	Optimum Temperature [ºC]	Activation energy [Kcal/mol]	Rate enhancement	Source	Life stage
0.435	243.2	8.0	35	7.4937	10^7	Brain	juvenile

Table 1 – Kinetics and physicochemical parameters of AChE from *R. canadum*.

MW (KDa)		
AChE1	**AChE2**	**AChE3**
244.57	218.05	207.56

Table 2 – Molecular weights of brain cholinesterases from *R. canadum*.

Pesticide	**IC_{50} (µM)**	**Ki (µM)**
Dichlorvos	6.9	1.36×10^{-2}
Diazinon	-	-
Chlorpyrifos	30.24	5.94×10^{-2}
Temephos	-	-
TEPP	8.1	1.59×10^{-2}
Carbaryl	8.31	1.63×10^{-2}
Carbofuran	0.082	1.61×10^{-4}

Table 3 – Pesticide IC_{50} and Ki values for *in vitro* exposure.

International regulations (ANVISA, 2006; WHO/FAO, 2004; USEPA, 1984 and EFSA, 2004) advocate that acceptable daily intake (ADI) for carbofuran can not exceed, respectively, 0.002, 0.002, 0.005 and 0.001 mg/kg bw/day. It demonstrates that AChE from *R. canadum* can detect this compound at levels far below from those thresholds.

CONCLUSIONS

AChE from *R. canadum* was sensitive to the pesticides, mainly to carbofuran, presenting significative decrease at concentrations lower than the values set by the most of international regulations related to maximum ingestion levels for this compound and can be useful as a biomarker for that compound.

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EFFECT OF IONS AND HEAVY METALS ON BRAIN ACETYLCHOLINESTERASE FROM COBIA (*Rachycentron canadum*)

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ABSTRACT

Several studies point to the influence of ions and heavy metals on the activity of acetylcholinesterase (AChE, EC 3.1.1.7). Biosensors for detection of anticholinesterase agents do not take into account this influence. This fact can lead to false positive or negative and erroneous interpretations in the analysis. This study aimed to investigate the effect of fourteen different ions and heavy metals that might influence the activity of brain AChE from *R. canadum*, because of its potential use as a biomarker for the presence of anticholinesterase agents. The enzyme was assayed in presence of As^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, $EDTA^{2-}$, Hg^{2+}, K^{+}, Li^{+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+} at five concentrations from 0.001 to 10 mM in extracts from brain of *R. canadum*. The results of the analysis of ions and heavy metals at 1 mM showed no significant effect of Al^{3+}, Fe^{2+} and K^{+} while Ca^{2+}, Mg^{2+} and Mn^{2+} activated the enzyme. In the other hand, As^{3+}, Ba^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Li^{+}, Pb^{2+}, Zn^{2+} caused inhibition. The enzyme presented EDTA-resistant activity until 1 mM. Kinetic analysis suggests that mercury ion presents a competitive inhibition behavior on brain AChE of *R. canadum*, while Cu^{2+}, Ba^{2+} and Li^{+} presented a mixed-type inhibition behavior. Pb^{2+} seems to be an uncompetitive inhibitor.

Keywords: acetylcholinesterase, heavy metals, biomarker.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is a crucial enzyme for the development and functioning of the nervous system. Its classical function is to modulate the nerve impulse through the hydrolysis of the neurotransmitter acetylcholine in the synaptic cleft (QUINN, 1987). AChE inhibition is the mechanism of action of organophosphorus and carbamate pesticides. AChE have been also used for monitoring these pesticides and other compounds (RENDÓN-VON OSTEN *et al*., 2005). The investigation of AChE inhibitors and interfering substances are relevant to identify the usefulness of this enzyme as a tool in environmental monitoring. Several studies reported the influence of ions and heavy metals on the activity of AChE (REDDY *et al*., 2003). Therefore, high content of these ions in water samples from rivers, lakes and reservoirs can influence the detection of anticholinesterasic pesticides. These findings must be taken in account when biosensors based on AChE activity are proposed to analyze pesticide presence in some environment conditions. This fact can lead to false positives or negatives and misinterpretations in the analysis of results. *R. canadum* is one of the most important marine species cultured. The use of its AChE as biomarker for these compounds can constitute a valuable tool for environmental and food monitoring, in addition of being a useful destination for discarded tissues (brains) produced in the cultivation. This study aimed to investigate the effect of different ions and heavy metals (Al^{3+}, As^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, $EDTA^{2-}$, Hg^{2+}, K^{+}, Li^{+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}) that could influence the activity of brain AChE from R. canadum, providing information to support its potential use as a biomarker for the presence of anticholinesterase agents.

MATERIALS AND METHODS

Juvenile specimens (51.67 ± 1.5 cm; 1,575 ± 329.6 g) were cultured in cages 11 km away from the coast of Pernambuco. The animals were sacrificed by an ice bath and the brains were removed and weighed. The homogenates were prepared in tissue disrupter and diluted in Tris-HCl buffer 0.5 M pH 8.0 until reach 20 mg of tissue per mL of buffer. The preparation were centrifuged at 1000 x *g* during 10 min. Enzymatic activity was determined by a modification of the colorimetric method of Ellman (ASSIS *et al*., 2010) using 20 µL of crude extract, 200 µL of 0,25 mM chromogenic agent DTNB and 20 µL of acetylthiocholine iodide 62 mM. The increase in absorbance was followed spectrophotometricaly during 180 s at 405 nm after substrate addition. The extracts were incubated during 40 min with fourteen ions and heavy metals (Al^{3+}, As^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, K^{+}, Li^{+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}), including the complex chelating ion $EDTA^{2-}$, whose concentrations were 0.001; 0.01; 0.1; 1 and 10 µg/mL. Lineweaver-Burke kinetic study of ion inhibition mechanism was carried out incubating the same ion concentrations during 60 min and ranging substrate concentration from 0.83 to 20.83 mM. The parameter IC_{50} related to the inhibiting ions under study were determined by non linear regression fitting using Microcal Origin® 8.0 software while the inhibition constant (ki) for each pesticide was estimated by the CHENG and PRUSOFF equation (1973).

RESULTS AND DISCUSSION

Tab. 1 shows the effect of some ions and heavy metals on brain AChE from *R. canadum* where can be observed activation by Ca^{2+}, Mg^{2+} and mainly Mn^{2+}. The enzyme was extremely sensitive to Hg^{2+}, Cu^{2+} and As^{3+} at 1 mM. Tab. 2 displays the estimated values of IC_{50} and Ki referring to the inhibitory ions and can be noted that mercury ion can inhibit AChE activity in 50% at 0.001 mM. The behavior of inhibitory effect of some ions can be seen in Lineweaver-Burke plots: Fig. 1 shows a probable competitive inhibition while in Fig. 2, copper seems to be a mixed-type inhibitor as well as barium and lithium (Figs. 3 and 4) while lead presents a kinetic similar to uncompetitive inhibitors.

As^{3+}	Ba^{2+}	Ca^{2+}	Cd^{2+}	Cu^{2+}	Hg^{2+}	Li^{+}	Mg^{2+}	Mn^{2+}	Pb^{2+}	Zn^{2+}
63%	14%	10% (a)	33%	75%	100 %	10%	15% (a)	38% (a)	15%	23%

Table 1 – Inhibition or activation (a) of the AChE activity from *R. canadum* by ions and heavy metals at 1 mM ($p < 0.05$).

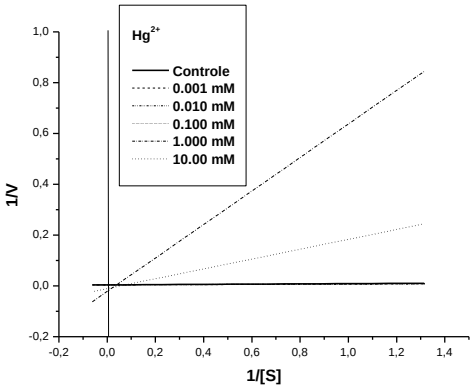

Fig. 1 – kinetic behavior of *R. canadum* AChE exposed to 5 concentrations of Hg^{2+}.

Ion	IC_{50} (mM)	Ki (mM)
As^{3+}	0.21	0.002
Cd^{2+}	1.10	0.012
Cu^{2+}	0.37	0.004
Hg^{2+}	0.12	0.001
Zn^{2+}	6.29	0.071

Table 2 – Ions IC_{50} and Ki values for in vitro exposure.

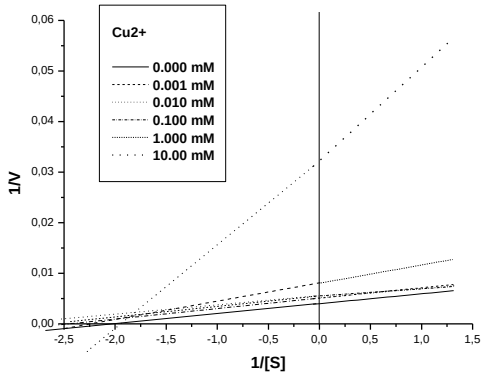

Fig. 2 – kinetic behavior of *R. canadum* AChE exposed to 5 concentrations of Cu^{2+}.

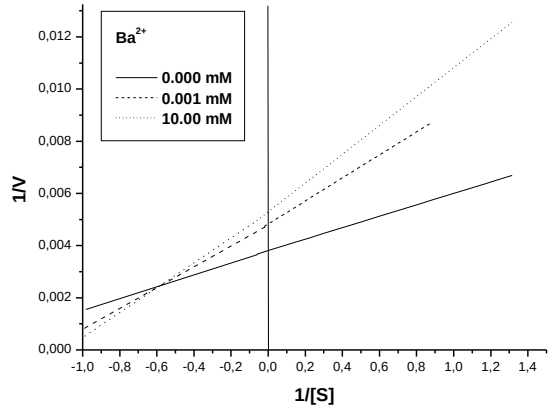

Fig. 3 – kinetic behavior of *R. canadum* AChE exposed to 2 concentrations of Ba^{2+}.

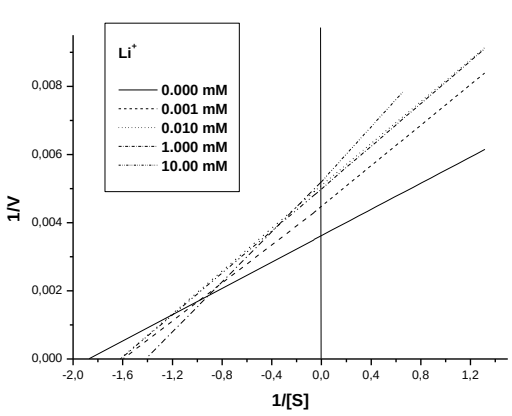

Fig. 4 – kinetic behavior of *R. canadum* AChE exposed to 4 concentrations of Li^{+}.

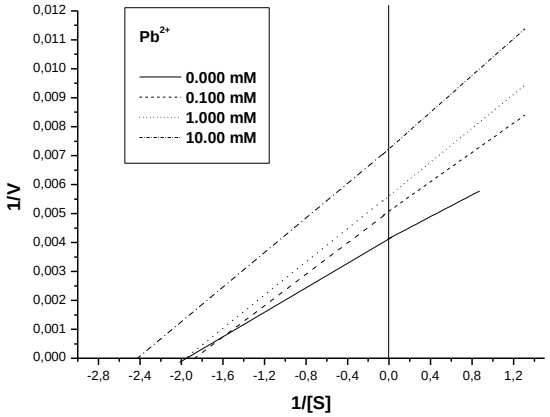

Fig. 5 – kinetic behavior of *R. canadum* AChE exposed to 3 concentrations of Pb^{2+}.

CONCLUSIONS

Several ions present potential to strongly influence the *R. canadum* AChE activity. However, the EDTA-resistant activity makes this chelate a useful tool to complex inhibitory and activating ions present in environmental samples. In addition, acetylcholinesterase from *R. canadum* showed a high potential to serve as a biomarker for the presence of mercury ion (Hg^{2+}).

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ENZYMATIC PROFILE OF FREE-LIVING NEMATODES IN TWO COASTAL ENVIRONMENTS

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ABSTRACT

The free-living nematodes are the most abundant animals of the marine and estuarine sediments. In its organic composition proteins represent the largest biochemical class. This study aimed to determine the partial enzymatic content of nematofauna in two coastal environments as well as digestive enzymes. Nematofauna from two coastal environment (Maracaípe beach and Pina estuary, Pernambuco, Brazil) were analyzed for their enzymatic composition. For each treatment were used 200 individuals per replica. Were analyzed three treatments: (S1) fixed with 4% neutral formalin, (S2) 4% formaldehyde buffered with borax and (S3) without formaldehyde. Total proteolytic activity was colorimetric assayed as well as trypsin, chymotrypsin, carboxipeptidase A2, dipeptidase, pepsin, acetylcholinesterase and butyrylcholinesterase activities. S3 presented total proteolytic activity 12.3-fold higher than the other treatments. Analysis of selective substrate and inhibitors showed the presence of trypsin, chymotrypsin, carboxipeptidase A2, acetylcholinesterase and butyrylcholinesterase. Chymotrypsin seems to be more important than trypsin in these organisms. The results presented considerable differences between the collection environments.

Keywords: enzymes, proteases, acetylcholinesterase, nematodes

INTRODUCTION

The free-living nematodes are the most abundant animals of the marine and estuarine sediments, considered the most representative components of the Metazoa (GIERE, 2009). In its organic composition proteins represent the largest biochemical class (DANOVARO *et al.*, 1999). Biochemical composition of marine nematofauna can vary with the degree of eutrophication and the pollution of the environment they live in. Such variation is important when comparing the biochemical parameters of groups from different areas. Organisms of meiofauna can be useful as biomonitors and/or suppliers of molecules of environmental and biomedical interest. Considering the variety of biotechnological applications of proteins and mainly enzymes, is very important to prospect the protein profile of these organisms. Proteases have being used in a range of industrial process such as the production of food, cloth, cosmetics and medicines. In addition, inhibition of proteases *in vitro* can be tested as biomarkers as well as detoxifying enzymes and cholinesterases. Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pepsin (EC 3.4.23.1), carboxypeptidase A2 (EC 3.4.17.15), dipeptidase (EC 3.4.13.18), acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) were the chosen enzymes in the present work. This study aimed to determine the partial profile of enzymes from the nematofauna in two coastal environments including digestive enzymes.

MATERIALS AND METHODS

Methods and results: The collection was performed in sediments from Maracaípe beach and Pina estuary, Pernambuco, Brazil. Nine replics were taken from random samples to constitute three treatments: (S1) fixed with 4% neutral formalin, (S2) 4% formaldehyde buffered with borax and (S3) without formaldehyde. The biosedimentological samples were elutriated (at least 10 times) and supernatant from this procedure was poured into 0.045 mm geological sieves for nematofauna extraction. For each treatment were used 200 individuals per replica which were sonicated and centrifuged to analyze protein content by BCA method. Total proteolytic activity was colorimetric assayed as follows: samples incubated with azocasein 1% during 60 min had interrupted their reaction

with TCA 10%. After waiting 15 min, samples were centrifuged at 8,000 x *g* for 5 min and the supernatant were collected, added to NaOH 1 M in microplates and read at 450 nm (BEZERRA *et al*., 2005). Activity of trypsin were estimated using Nα-benzoyl-DL-arginine-*p*-nitroanilide (BApNa) as substrate while for chymotrypsin were used succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SApNA) and N-succinyl-L-phenylalanine-*p*-nitroanilide (Suc-Phe-p-Nan) as substrate. The samples were added to tris-HCl 0.1 M pH 8.0 and after substrate was applied to start the reaction which occurred during 15 min (for trypsin) and 10 min (for chymotrypsin) and was followed at 405 nm. For these two enzymes were used selective inhibitors: N-*p*-tosyl-L-lysin chloromethyl ketone (TLCK) for trypsin and N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) for chymotrypsin (BUARQUE *et al*., 2009). Dipeptidase and carboxipeptidase were estimated by using N-Glycyl-L-leucine (Gly-Leu for dipeptidase) and Z-Glycyl-L-Phenylalanine (Z-Gly-Phe for carboxipeptidase) as substrate. The mixture of samples and L-amino oxidase reagent (LAOR) were incubated during 20 min at 37ºC and after the reaction was stopped with sulphuric acid 50%. The developed color was read at 530 nm and the absorbance was converted to activity by the equations obtained from the curves of L-leucine and L-phenylalanine previously prepared (NICHOLSON *et al*., 1974). For pepsin was used an adaptation of BERGKVIST (1956) in which the samples in pH 2.0 were added to water, buffer glycine-HCl pH 2.0 and denaturated hemoglobin 2%. The mixture was incubated for 20 min and the reaction was stopped with TCA 50% and precipitated during 60 min at 4ºC. After that was centrifuged at 10,000 x *g* for 10 min. The supernatant was collected and read at 280 nm. For AChE and BChE measurements, were used acetylthiocholine and S-butyrylthiocholine iodide, respectively, as substrate. In this assay, samples were mixed to the chromogenic agent DTNB and the substrate was added and the reaction was followed at 405 nm during 3 min (ASSIS *et al*., 2010).

RESULTS AND DISCUSSION

The protein content of extracts obtained from treatments without formaldehyde (S3) were approximately 75% (beach) and 30% (estuary) lower than the extracts of S1 and S2 treatments (approximately 115 µg/mL). However, the total proteolytic activity of S3 were about 12.3-fold higher than treatments using formaldehyde. Trypsin activity exposed to 8 mM TLCK was 31.7% of the control (45.3 mU/mg protein) while chymotrypsin activity exposed to 8 mM TPCK were 27.6% (SApNA as substrate) and 1.7% (Suc-Phe-p-NAN as substrate) of controls (103.7 mU/mg protein). Chymotrypsin specific activity was shown to be higher than the same parameter for trypsin in these organisms. Dipeptidase and BChE showed no detectable activities while carboxipeptidase showed 1,309 mU/mg protein (beach) and 5,733 mU/mg protein (estuary) (Tab. 1). Pepsin presented low activity and only in the estuarine environment while AChE presented no activity in this environment and very low activity in beach. BChE presented no activity in both environments (Tab. 1). The increase of carboxipeptidase A2 and pepsin activity in estuary seems to occur as a result of the higher degree of eutrophication in this environment and consecutive higher content of their substrates. On the other hand, the absence of trypsin, chymotrypsin and AChE activity in estuarine environment can be consequence of pollutants (including anticholinesterasic pollutants) in Pina estuary which is an urban estuary.

	Trypsin	Chymotrypsin	Carboxipeptidase A2	Dipeptidase	Pepsin	AChE	BChE
Beach	45.3	103.7	1,309	NA	NA	2.87	NA
Estuary	NA	NA	5,733	NA	2.36	NA	NA

Table 1 – Activity of controls (S3 treatment) of the enzymes from marine free-living nematodes in two coastal environments (mU/mg protein)

Inhibitor	Substrates				
	BApNA	SApNA[1]	Suc-Phe-p-NAN[1]	Acetylthiocholine[2]	Butyrylthiocholine[3]
TLCK	31.7	-	-	-	-
TPCK	-	27.6	1.7	-	-
BW284c51	-	-	-	0.0	0.0
Iso-OMPA	-	-	-	0.0	0.0

Table 2 – Residual activity (%) of trypsin, chymotrypsin[1], AChE[2] and BChE[3] from nematodes exposed to selective inhibitors (S3 treatment).

Exposure to selective inhibitors (concentration of 8 mM for TLCK and TPCK while 10 mM for BW284c51 and Iso-OMPA) and subsequent inhibition confirmed that the activities observed were from some of the enzymes under analysis (Tab. 2).

CONCLUSIONS

Although, there is a need for more studies about the protein composition of marine and estuarine nematodes, the initial results presented here, showed that there is considerable variation between environments. Trypsin and chymotrypsin in addition to cholinesterases seems to be capable to detect pollutants present in environment.

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