



**UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS**

KALINE CATIELY CAMPOS SILVA

Caracterização da Acetilcolinesterase cerebral de tucunaré, *Cichla ocellaris* (BLOCH & SCHNEIDER, 1801): efeito de íons e pesticidas organofosforados e carbamatos sobre sua atividade

ORIENTADOR: RANILSON DE SOUZA BEZERRA

CO-ORIENTADOR: CAIO RODRIGO DIAS DE ASSIS

RECIFE

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Dissertação apresentada ao **Programa de Pós-Graduação em Ciências Biologia** para o cumprimento parcial das exigências para obtenção do título de **Mestre em Ciências Biológicas** pela **Universidade Federal de Pernambuco**

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Silva, Kaline Catiely Campos

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78 folhas : il., fig., tab.

Orientador: Ranilson de Souza Bezerra

Coorientador: Caio Rodrigo Dias de Assis

Dissertação (mestrado) – Universidade Federal de Pernambuco, Centro de Ciências Biológicas. Ciências Biológicas, 2012.

Inclui bibliografia

- 1. Enzimas 2. Pesticidas 3. Tucunaré (peixe) I. Bezerra, Ranilson de II. Assis, Caio Rodrigo Dias de III. Título.**

572.7

CDD (22.ed.)

UFPE/CCB-2012-064

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KALINE CATIELY CAMPOS SILVA

Esta dissertação foi julgada para a obtenção do título de **Mestre em Ciências Biológicas** e aprovada em 27/02/2012 pelo Programa de Pós-Graduação em Ciências Biológicas, em sua forma final. A comissão examinadora, composta pelos professores abaixo, sob a presidência do primeiro, considera a candidata **KALINE CATIELY CAMPOS SILVA** como **APROVADA**.

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DEDICATÓRIA

Ao meu querido Mecier, pelo amor, fé e exemplo de vida.

AGRADECIMENTOS

Agradeço infinitamente a Deus! Não deve ser fácil cuidar de mim, mais muito obrigada Senhor por me proteger sempre e provar a Tua maravilhosa presença em minha vida! É graças a Ti essa conquista.

Ao meu amor Mecier Soares que, principalmente nesses dois anos, com paciência, confiança e muito amor, me ensinou com exemplo, a importância de sermos um! Pela infinita paciência, atenção, compreensão e apoio em todas as minhas decisões, sem importar a distância! Pelo seu esforço e dedicação em me fazer feliz e incentivo para me fazer vencer!

Aos meus pais, Pedro e Dalvina pela confiança, noites em claro e orações que me dedicaram.

As minhas irmãs, Keyla e Kilma e sobrinhos, Emilly, Douglas, Nicolly, Murilinho e Nycolas pela alegria, carinho e fé nas minhas lutas.

Ao meu orientador, Ranilson de Souza Bezerra, pela porta que me abriu, pela simpatia, tolerância e confiança de que eu poderia conseguir!

Ao meu co-orientador, Caio Rodrigo Dias de Assis, pela paciência, dedicação, amizade, companheirismo, generosidade em transmitir seus valiosos conhecimentos.

Ao prefeito de Petrolândia, Lourival Simões, que me apoiou e me liberou para que essa conquista fosse possível. Muito obrigada!!!

As amigas Mileninha, Jussara e Juscly que me acolheram e se fizeram família enquanto a minha estava distante. Pela atenção, carinho e apoio de vocês ao dividirem comigo um lar, eu me fortaleci e consegui permanecer!

A Niedja Batista e Bruno Rocha, pela contribuição nos meus primeiros passos para realização desse trabalho.

A Seu Bezinho, Bruno, Neto, Sílvio e Rogério Viana, pela colaboração e esforço em pescar comigo e conseguir meus peixes!!

Aos meus companheiros de trabalho do Hospital Dr. Francisco Simões de Lima, Alda, Alessandra, Jores, Mônica, Patrícia, Raquel, Silvanice e especialmente Lucilene que várias vezes assumiu minha ausência.

Agradecimentos a CAPES, pelo apoio financeiro!

RESUMO

A acetilcolinesterase (AChE) é uma enzima que vem sendo testada como biomarcador da presença de pesticidas. Trata-se de uma hidrolase, do grupo das colinesterases, que atua nos processos de transmissão de impulsos nervosos em vertebrados e invertebrados. A AChE hidrolisa rapidamente o neurotransmissor acetilcolina, na fenda sináptica, encerrando sua ação e garantindo a intermitência dos impulsos nervosos. A inibição desse mecanismo resulta no acúmulo de acetilcolina nas fendas sinápticas levando a uma hiperestimulação colinérgica. Tal inibição é o modo de ação de organofosforados e carbamatos, os inseticidas mais utilizados mundialmente. Diferentes compartimentos do ecossistema estão expostos aos agrotóxicos e uma vez presentes no ambiente aquático, eles podem ser absorvidos pelos organismos onde sofrerão bioacumulação, podendo ser utilizados como bioindicadores no monitoramento de pesticidas. O objetivo deste trabalho foi caracterizar físico-química e cineticamente a enzima acetilcolinesterase cerebral do tucunaré (*Cichla ocellaris*), bem como avaliar o efeito de pesticidas organofosforados e carbamatos, e íons sobre sua atividade com a finalidade de fornecer subsídios para o uso da referida enzima como ferramenta bioindicadora de contaminação ambiental. As amostras analisadas foram preparadas a partir de extratos de cérebro de tucunaré. Foram determinados parâmetros cinéticos como K_m e V_{max} . A atividade enzimática foi analisada após exposição aos inibidores selectivos BW284c51, Iso-OMPA, neostigmina e eserina que confirmaram AChE como responsável pela atividade analisada. A AChE foi submetida também a 05 pesticidas organofosforados (diclorvós, clorpirifós, diazinon, temefós e TEPP) e 02 carbamatos (carbofuran e carbaril), em diferentes concentrações. A atividade colinesterásica foi observada na presença de 14 íons catiônicos: Mn^{2+} ; Cu^{2+} ; Zn^{2+} ; Al^{3+} ; Ca^{2+} ; Pb^{2+} ; Cd^{2+} ; Hg^{2+} ; Fe^{2+} ; Ba^{2+} ; Mg^{2+} ; K^+ ; As^{3+} ; Li^+ e 1 íon complexo aniônico quelante: $EDTA^{2-}$. Os resultados demonstraram que o pesticida diclorvós provocou forte inibição na atividade da enzima estudada. O organofosforado tetraetil-pirofosfato (TEPP) também a inibiu fortemente e os dois carbamatos utilizados, sobretudo o carbofuran, apresentaram valores baixos de CI_{50} . Os íons que causaram maior inibição foram Cu^{2+} , Zn^{2+} , Hg^{2+} , Cd^{2+} , As^{3+} e Pb^{2+} , enquanto que o íon complexo $EDTA^{2-}$ só inibiu a enzima estudada a partir de 10 mM. Desta forma, a inibição in vitro da acetilcolinesterase de tucunaré demonstra ser uma ferramenta promissora para o monitoramento ambiental de recursos hídricos. A facilidade de obtenção e a sensibilidade da enzima aos inseticidas utilizados, apontam para a possibilidade de um monitoramento rotineiro e eficiente.

Palavras-Chave: Organofosforados, carbamatos, bioindicador, acetilcolinesterase, *Cichla*.

ABSTRACT

Acetylcholinesterase (AChE) is an enzyme that has been tested as a biomarker for the presence of pesticides. It is a hydrolase, of the group of cholinesterase, which acts in the process of transmission of nerve impulses in vertebrates and invertebrates. AChE rapidly hydrolyzes the neurotransmitter acetylcholine in the synaptic cleft, terminating its action and ensuring the blink of nerve impulses. Inhibition of this mechanism results in the accumulation of acetylcholine in the synaptic clefts and is therefore released in large quantities for their receptors leading to cholinergic overstimulation. Such inhibition is the mode of action of organophosphate and carbamate insecticides most commonly used worldwide. Different compartments of the ecosystem are exposed to pesticides and once in the aquatic environment, they can be absorbed by organisms which undergo bioaccumulation, which can be used as bioindicators for monitoring pesticides. The objective of this study was to characterize the physicochemical and kinetics of the brain acetylcholinesterase of peacock bass (*Cichla ocellaris*), and to evaluate the effect of organophosphate and carbamate pesticides, and ions on its activity in order to provide support for the use of this enzyme as a tool bioindicator of environmental contamination. The samples were prepared from brain extracts of peacock bass. Kinetic parameters as K_m and V_{max} were determined. The enzyme activity was analyzed after exposure to selective inhibitors BW284c51, Iso-OMPA, neostigmine and eserine which confirmed AChE activity as responsible for analyzed. AChE was also submitted to 05 organophosphate pesticides (dichlorvos, chlorpyrifos, diazinon, temephos and TEPP) and 02 carbamates (carbofuran and carbaryl) at different concentrations. The cholinesterase activity was observed in the presence of 14 cationic ions: Mn^{2+} , Cu^{2+} , Zn^{2+} , Al^{3+} , Ca^{2+} , Pb^{2+} , Cd^{2+} , Hg^{2+} , Fe^{2+} , Ba^{2+} , Mg^{2+} , K^+ , As^{3+} , Li^+ and an complex anionic chelating ion: $EDTA^{2-}$. The results showed that the pesticide dichlorvos caused a strong inhibition the AChE activity. The organophosphate tetraethyl pyrophosphate (TEPP) also strongly inhibited and the two carbamates used, especially carbofuran, showed low values of IC_{50} . The ions which are caused more inhibition Cu^{2+} , Zn^{2+} , Hg^{2+} , Cd^{2+} , Pb^{2+} and As^{3+} , while the ion complex $EDTA^{2-}$ inhibit the enzyme-only studied from 10 mM. Thus, *in vitro* inhibition of acetylcholinesterase of peacock bass proves to be a promising tool for environmental monitoring of water resources and the ease of obtaining the enzyme sensitivity to the insecticides used, indicate the possibility of a routine monitoring and efficient.

Keywords: Organophosphates, carbamates, bioindicator, acetylcholinesterase, *Cichla*.

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LISTA DE ABREVIações

Ach	Acetilcolina
AChE	Acetilcolinesterase
ANVISA	Agência Nacional de Vigilância Sanitária
ATSDR	Agency for Toxic Substances and Disease Registry
BChE	Butirilcolinesterase
CB	Carbamato
CI ₅₀	Concentração capaz de inibir a enzima em 50% de sua atividade
DDE	Dicloro-Difenil-Etano
DDT	Dicloro-Difenil-Tricloroetano
DMSO	Dimetilsulfóxido
DTNB	ácido 5,5' Ditiobis (2-nitrobenzóico)
EDTA	ácido etileno diamino tetracético
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
Iso-OMPA	Tetraisopropil pirofosforamida
IC ₂₀	Concentração que inibe a atividade enzimática em 20%
IC ₅₀	Median inhibition concentration
K _{cat}	Turnover number, número de renovação da enzima
K _{cat} /K _m	Eficiência catalítica
K _i	Constante de inibição
K _m	Constante de Michaelis-Menten
OMPA	Octametil-pirofosforamida
OMS	Organização Mundial da Saúde
OP	Organofosforado
PC	peso corporal
TEPP	Tetraetil-pirofosfato
Tris	Tris-hidróximetil-aminometano
V _{max}	Velocidade máxima de catálise atingida por uma enzima
WHO	World Health Organization

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1. INTRODUÇÃO

As colinesterases são enzimas do grupo das hidrolases que catalizam a hidrólise dos ésteres de colina. Duas enzimas têm sido designadas como colinesterases: a acetilcolina hidrolase ou acetilcolinesterase (AChE EC 3.1.1.7) que predomina nos eritrócitos, neurônios, gânglios do sistema nervoso autônomo e placas motoras terminais e a acilcolina hidrolase, também conhecida como butirilcolinesterase, (BChE EC 3.1.1.8) que predomina no plasma, fígado, neuróglia, pâncreas e paredes do tubo digestivo.

A neurotransmissão mediada pela acetilcolina é fundamental para o correto funcionamento do sistema nervoso central, e representa o sistema neurotransmissor mais antigo do ponto de vista filogenético (GOTTI e CLEMENTI, 2004). Os neurônios colinérgicos inervam a musculatura voluntária do sistema somático e também são encontrados no sistema nervoso central (SOREQ e SEIDMAN, 2001).

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 (TÔUGU, 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). Seu mecanismo de ação se dá através da ligação com o sítio esterásico da acetilcolinesterase, com fosforilação para organofosforados 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, porém 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, 1986). 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).

Em geral, quanto maior a concentração de pesticidas e mais longo o tempo de exposição, maiores as chances dos impactos negativos atingirem níveis superiores de organização biológica,

como comunidades e ecossistemas. Se um estresse dura tempo suficiente para levar à morte uma população de organismos, afetando as taxas de crescimento, de reprodução e impedindo o recrutamento de novas espécies, ele é então capaz de alterar a estrutura da comunidade (CAIRNS e PRATT, 1993). Geralmente, os efeitos em tais níveis podem ser diretamente ligados à exposição aos agentes contaminantes.

No prosseguimento da cadeia alimentar, os pesticidas chegam até os alimentos e demais produtos de origem agroindustrial utilizados pelos homens. A ingestão diária e durante longo prazo de alimentos contaminados com tais agentes, mesmo que em pequenas doses, pode levar a quadros de intoxicação de diversos graus (UFF, 2000), 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).

A exposição a esses produtos tóxicos pode vir a causar diversos males à saúde humana e ambiental. Como afirma Waichman (2008), os animais silvestres e domésticos podem se contaminar a partir da água, do solo, e de outros recursos ambientais contaminados nos quais vivem ou dos quais se alimentam. Estas rotas de exposição e a quantificação da exposição são avaliadas a partir da determinação dos níveis de agrotóxicos nos diferentes compartimentos do ecossistema e nos organismos.

Uma vez presente no ambiente aquático, eles podem se associar ao material em suspensão, aos sedimentos no leito do corpo d'água ou serem absorvidos pelos organismos onde sofrerão bioacumulação ou detoxificação (NIMMO, 1985).

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, 2007).

As substâncias conhecidas como bioindicadores são compostos de origem animal ou vegetal 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, 2003). 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).

No monitoramento ambiental de pesticidas e outros contaminantes em recursos hídricos, existem diversas técnicas que utilizam organismos aquáticos como bioindicadores, seja pela estimativa da densidade populacional e alterações comportamentais, seja por uma característica fisiológica desses organismos que os torna sensíveis a determinados poluentes. A escolha desses organismos se dá através de características como habitat, ecologia, hábitos alimentares, abundância da espécie e facilidade de captura.

Já no monitoramento da qualidade alimentar, busca-se determinar, de forma acurada, resíduos de pesticidas e outros contaminantes presentes em partes comerciais de vegetais, animais e seus derivados, visando à observância dos prazos de inativação das substâncias utilizadas, previstos na legislação (WHO, 1990).

A espécie escolhida neste trabalho foi o tucunaré. Estas características, aliadas à excelência da qualidade da sua carne, tornam o tucunaré uma espécie potencialmente utilizável em piscicultura intensiva.

Os peixes são importantes no biomonitoramento uma vez que várias espécies estão no topo de cadeias alimentares em seus habitats e os processos de bioacumulação a que estão submetidos permitem que os efeitos dos compostos sejam detectados dias ou semanas após os resíduos dos agrotóxicos terem desaparecido da água (STURM et al. 1999).

No presente estudo, a espécie escolhida como fonte de acetilcolinesterase foi o tucunaré (*Cichla ocellaris*), que segundo (FONTENELE, 1948; SILVA et al., 1980) é um peixe pertencente ao gênero *Cichla* sp. (Teleostei, Actinopterygii, Cichlidae), incluído entre as espécies nativas de grande importância para a pesca esportiva. Originário da bacia amazônica, o tucunaré é uma espécie de hábito alimentar carnívoro e tem demonstrado considerável eficiência no controle de peixes invasores em represas. Sua utilização prende-se não apenas à importância econômica e facilidade de aquisição, mas também por ser uma espécie sedentária e não migratória, característica capaz de evidenciar diferenças entre os locais de amostragem.

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 ambiental utilizando peixes.

2 REVISÃO DE LITERATURA

2.1 Recursos hídricos

A crescente degradação ambiental, principalmente em relação aos recursos hídricos, vem afetando diretamente a qualidade da água em rios e reservatórios, bem como sua distribuição nas cidades brasileiras, fato que compromete cada vez mais as gerações futuras.

Além do impacto sobre ecossistemas naturais, existe a ação nociva sobre grandes reservatórios de água, construídos principalmente nas décadas de 1960 e 1970, os quais são utilizados, não só para a produção de energia hidrelétrica, mas também para irrigação, navegação e abastecimento público. (MELO et al., 2006)

Como sistemas complexos, os reservatórios de água apresentam um padrão dinâmico, com rápidas mudanças em mecanismos de funcionamento e gradientes horizontais e verticais. De um modo geral, interferem nos rios em que são construídos alterando o fluxo e os sistemas terrestres e aquáticos de uma forma drástica e efetiva (TUNDISI, 2003). Isto decorre do fato da construção de uma barragem implicar na imediata conversão de um ambiente lótico em lântico, promovendo um considerável aumento do tempo de residência na água. Essa transformação inicial é a principal responsável por uma série de modificações nas características limnológicas observadas, provocando a substituição de espécies que predominam em rios por aquelas características de ambientes lânticos (LANSAC-TÔHA et al. 1999), onde espécies mais bem adaptadas às novas condições do ambiente podem excluir as espécies originais e serem mais tarde, por sua, vez excluídas.

No Estado de Pernambuco, a maioria das águas usadas para abastecimento provém de reservatórios originários de rios que, por percorrerem várias cidades, encontram-se em processo de degradação concentrando grandes quantidades de poluentes que comprometem a qualidade da água usada para o consumo da população. (SOBRAL et al., 2006).

Desde que o reservatório de Itaparica entrou em operação, em 1988, com a inundação de 805 km², tem-se verificado uma série de problemas ambientais, decorrentes do uso descontrolado das margens do lago por atividades agrícolas e ocupações urbanas (SOBRAL et al., 2006).

Como observado por Melo (2006), em várias partes do reservatório não está sendo respeitada a faixa de 100 m de área de preservação permanente prevista na resolução nº 04/85 do CONAMA, sendo observados desmatamentos para agricultura e construções irregulares no entorno

do reservatório. Além disso, a utilização de agroquímicos em grande parte de seus perímetros irrigados, somada à falta de fiscalização adequada, possibilita o lançamento de efluentes sem tratamento diretamente no reservatório.

Um dos principais projetos em execução no Brasil é o *Projeto de Integração do Rio São Francisco com as Bacias Hidrográficas do Nordeste Setentrional*. Trata-se de um empreendimento do Governo Federal, sob a responsabilidade do Ministério da Integração Nacional, destinado a assegurar a oferta de água, em 2025, a cerca de 12 milhões de habitantes de pequenas, médias e grandes cidades da região semi-árida dos estados de Pernambuco, Ceará, Paraíba e Rio Grande do Norte. Neste projeto, o rio será integrado ao Semi-Árido Setentrional por meio de dois canais, que conduzirão água até os principais açudes da região, possibilitando seu uso com garantia de atendimento. Um dos canais terá seu ponto de captação no reservatório de Itaparica (BRASIL, 2004). Entretanto, de acordo com Melo (2006), isso pode aumentar os conflitos de uso múltiplo da água e do solo no entorno, devido à ampliação das atividades agrícolas e da aquicultura que vem sendo realizadas pelos moradores locais próximo às margens do lago, as quais contribuem para o acréscimo nos níveis de agrotóxicos e fertilizantes na água dos reservatórios.

A origem e a concepção dos projetos de irrigação existentes em Petrolândia – PE foram marcadas por fatos bastante significativos, no que diz respeito às questões ambientais, que contribuíram e contribuem para a formação de um quadro de grande desequilíbrio nos ecossistemas da região. Essa é uma situação que se repete em todos os perímetros de irrigação existentes no País (CONDEPE-FIDEM, 2001).

Com o passar dos anos esta situação foi piorando por conta de novos desmatamentos realizados pela própria população, que de forma desordenada retirava madeira e/ou fabricava carvão, principalmente nas margens dos riachos e de seus afluentes, em busca de alternativas econômicas para o sustento familiar, uma vez que o atraso na entrega dos lotes irrigados por parte do Governo Federal provocou uma grande ociosidade da mão-de-obra potencialmente disponível nas agrovilas. A grande maioria das manchas de solo do Projeto, onde foram implantadas as áreas irrigadas, são de areia quartzosa, com índice extremamente baixo de matéria orgânica, contribuindo para o desequilíbrio nutricional das culturas, e, conseqüentemente, tornando-as alvo fácil das infestações, principalmente de pragas e doenças. Além disso, a matéria orgânica proveniente de fontes externas é escassa na região sendo, por isso, muito cara para ser adquirida pelas famílias reassentadas, e os solos arenosos não retêm a umidade favorecendo o carreamento dos fertilizantes químicos solúveis para o lençol freático com mais rapidez (CONDEPE-FIDEM, 2001).

2.2 Pesticidas

De acordo com a Lei Federal nº 7.802 de 11/07/89 (BRASIL, 2000) os agrotóxicos, genericamente denominados de pesticidas, podem ser definidos como: os produtos e os componentes de processos físicos, químicos ou biológicos destinados ao uso nos setores de produção, armazenamento e beneficiamento de produtos agrícolas, nas pastagens, na proteção de florestas nativas ou implantadas e de outros ecossistemas e também em ambientes urbanos, hídricos e industriais, cuja finalidade seja alterar a composição da flora e da fauna, a fim de preservá-la da ação danosa de seres vivos considerados nocivos, bem como substâncias e produtos empregados como desfolhantes, dessecantes, estimuladores e inibidores do crescimento. O termo “Agrotóxico”, ao invés de “Defensivo Agrícola”, passou a ser utilizado, no Brasil, para denominar os venenos agrícolas, após grande mobilização da sociedade civil organizada. Mais do que uma simples mudança da terminologia, esse termo coloca em evidência a toxicidade desses produtos ao meio ambiente e à saúde humana (LARINI, 1979).

De acordo com a espécie que se pretende eliminar, esses compostos são classificados como inseticidas, fungicidas, herbicidas, rodenticidas, moluscicidas e outros (ANWAR, 1997). Baseado em seu alvo tóxico, os pesticidas podem ser ainda classificados como anticoagulantes, anticolinérgicos, etc. Já a classificação recomendada pela Organização Mundial da Saúde (WHO/UNEP/ILO/IPCS, 2006) considera o grau de toxicidade exibido por esses compostos e são baseados na toxicidade aguda oral e dérmica verificada em algumas espécies. De acordo com essa classificação os pesticidas podem ser descritos como extremamente tóxicos (classe I a), altamente tóxicos (classe I b), moderadamente tóxicos (classe II) e discretamente tóxicos (classe III). Finalmente, de acordo com a forma de classificação a classe química desses compostos, os mesmos agrupados em organoclorados, organofosforados, carbamatos, piretróides, etc (HE, 1993).

Amaraneni & Pillala (2001) encontraram resíduos de vários agrotóxicos nas duas espécies de peixes usados como bioindicadores, coletados no lago Kolleru, Índia. Os resultados demonstraram que os peixes continham resíduos de agrotóxicos em níveis superiores aos padrões estabelecidos pela Food and Agriculture Organization (FAO, 2007), organismo das Nações Unidas, se constituindo em mais uma fonte de exposição dos habitantes da região aos agrotóxicos. Os resultados refletiram também o nível de poluição por estes compostos naquele lago bem como o perigo ao qual os habitantes estavam expostos ao consumirem os peixes contaminados.

Younes e Galal-Gorchev (2000) ressaltam que a capacidade dos agrotóxicos persistirem e produzirem efeitos tóxicos sobre a saúde humana e sobre o meio ambiente é muito variada em função das inúmeras classes químicas existentes. Além disto, em função de seu amplo uso, os agrotóxicos podem estar presentes inclusive em água de abastecimento.

Estudos epidemiológicos de exposição ao DDT (Dicloro-Difenil-Tricloroetano) verificaram um aumento de câncer de mama em mulheres com altas taxas plasmáticas de DDE (Dicloro-Difenil-Etano), um metabólito do DDT. Essa ação está relacionada com a ligação deste componente a receptores de estrógeno, mimetizando a ação deste hormônio (JAGA et al., 2000). Outras ações causadas pelo efeito estrogênico de organoclorados incluem: diminuição da quantidade de sêmen e câncer de testículo nos homens; indução de anormalidades no ciclo menstrual e aborto espontâneo em mulheres; diminuição do peso ao nascer e alteração no amadurecimento sexual (CARLSEN et al. apud MEYER et al., 1999; TOFT et al., 2004). Diversos casos de contaminação ocupacional por organofosfatados têm sido relatados, sendo o envenenamento letal (SUNGUR e GUVEN , 2001) .

Além disso, foi verificada a morte de crianças prematuras, quando mulheres foram expostas em longo prazo aos inibidores da acetilcolinesterase (HEEREN et al. , 2003) . A análise toxicológica dos fungicidas mostrou um potencial mutagênico e teratogênico em células de mamíferos e linfócitos humanos (PEROCCO et al., 1997).

2.2.1 Organofosforados e Carbamatos

Os pesticidas anticolinesterásicos são representados por duas principais classes de pesticidas, os compostos organofosforados (OP) e carbamatos (CB). São as classes de pesticidas mais utilizadas em todo mundo, juntos respondem por mais de 50% do que é comercializado (Tabela 1). 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; ATSDR, 2005).

Esses pesticidas tiveram seu uso intensificado depois da proibição de utilização da maioria dos compostos organoclorados (ECOBICHON, 1993; ATSDR, 2002; MUKHERJEE e GOPAL, 2002), por serem menos tóxicos, porém com maior bioacumulação no meio ambiente (NUNES e TAJARA, 1998; ATSDR, 2002).

Tabela 1 – Percentual do mecanismo alvo dos 100 inseticidas/acaricidas mais vendidos no mundo e sua participação no mercado mundial

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 – Fonte: Nauen e Bretschneider, 2002

Os pesticidas OPs compreendem um elevado número de substâncias classificadas quimicamente como ésteres derivados de ácidos fosfóricos pentavalentes (JEYARATNAM e MARONI, 1994), cujas propriedades pesticidas foram evidenciadas a partir de 1937 por Gerhard Schrader, na Alemanha, com a síntese, neste mesmo ano, do Tabun e do Sarin. Em seguida, do OMPA (octametil-pirofosforamida) em 1941, do TEPP (tetraetil-pirofosfato) em 1943 e do Paration em 1944. Posteriormente, tais compostos sofreram modificações na sua estrutura química, com o objetivo de reduzir a toxicidade, onde só então foram empregados como pesticidas, com a vantagem de não serem estáveis na natureza como os organoclorados (ECOBICHON, 1993). Os pesticidas pertencentes à classe dos CBs são representados por um grupo de substâncias derivadas quimicamente de ésteres do ácido carbâmico. Ambos apresentam baixa solubilidade em água e são, em geral, facilmente hidrolizáveis em ambientes alcalinos. Em geral, os OPs necessitam de biotransformação (dessulfuração por ação das monoxigenases do complexo citocromo P450) para se tornarem toxicologicamente ativos, enquanto os CBs já são bioativos (WHO, 1986a). Esses

pesticidas são inibidores típicos das enzimas colinesterases (ALDRIDGE, 1950; ALDRIDGE e DAVIDSON, 1952; WHO, 1986a).

2.2.2 Mecanismo de ação e toxicidade

O mecanismo de ação tóxica de pesticidas OPs reside em sua capacidade de inibirem de forma irreversível a enzima AChE no cérebro, na junção neuromuscular e nas sinapses dos nervos periféricos, resultando em acúmulo de acetilcolina nesses locais, determinando um aumento da atividade colinérgica, a qual é responsável por toda a sintomatologia da intoxicação por esses compostos (Tabela 2). A inibição da AChE pelos compostos OPs é a causa principal de anormalidades da transmissão neuromuscular, sendo os sinais e os sintomas da intoxicação proporcionais ao nível de exposição da enzima. A interação entre a acetilcolinesterase e seu inibidor OP parece envolver somente o sítio esterásico, formando um complexo bastante estável. A estabilidade do complexo formado está relacionada fundamentalmente com a estrutura química do composto OP. A ação anticolinesterásica dos compostos OPs não está restrita à AChE do tecido nervoso central e periférico, ocorrendo de forma paralela a inibição da BChE plasmática e a AChE eritrocitária (MUTCH, BLAIN e WILLIAMS, 1992).

Casos de câncer foram evidenciados em 1992, em adultos jovens indígenas que viviam em uma aldeia na Amazônia. Nestes jovens foram encontrados níveis elevados de OPs 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 Rancagua, no Chile, são a Síndrome de Down, espinha bífida e hidrocefalia (ROJAS, OJEDA e BARRAZA, 2000).

Tabela 2 - Sinais e sintomas dos envenenamentos 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, diarreia, 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, câibras, enfraquecimento muscular generalizado.

Fonte: Larini (1999)

Segundo Moreira (2002), a contaminação por agrotóxicos pode ocorrer por três vias: a ocupacional, no preparo e utilização dos agrotóxicos; a ambiental, que ocorre pela dispersão dessas substâncias em diferentes componentes do meio ambiente, atingindo um maior número de pessoas; e a alimentar, que, apesar de resultar em impacto individual, atinge uma ampla parcela da população. Diversos compostos OPs, em face da elevada lipossolubilidade que apresentam, são absorvidos pelo organismo humano através de todas as vias possíveis, incluindo o trato gastrointestinal, a via respiratória, a via dérmica e as membranas mucosas. A absorção pela via oral ocorre nas intoxicações acidentais, particularmente em crianças. Em adultos, muitas vezes as intoxicações são intencionais, como as de natureza suicida. A intoxicação por esta via é também observada nos indivíduos que durante o manuseio dos pesticidas fumam inadvertidamente ou levam as mãos sujas à boca, especialmente durante a alimentação. A absorção dérmica é a principal via de penetração nos envenenamentos ocupacionais, naqueles indivíduos que aplicam produtos sob a

forma de pulverização ou naqueles que lidam nas plantações após a aplicação dos compostos OPs, para fazer a colheita, catações e podas. A absorção dérmica dos compostos OPs é grandemente aumentada nos ambientes de temperatura elevada e quando da existência de dermatites. Pela via respiratória pode ocorrer absorção, especialmente em indivíduos que trabalham nas indústrias de formulação, naqueles que trabalham na aplicação dessas substâncias sob a forma de pulverização, operando contra o vento e em situações de uso inadequado dos equipamentos de proteção individual, e também no uso doméstico sob a forma de aerossóis. Depois da absorção, os compostos OPs são distribuídos no organismo concentrando-se especialmente nos tecidos adiposos, no fígado, rins, glândulas salivares, tireóide, pâncreas, pulmões e paredes do estômago e intestinos e, em menor proporção, no sistema nervoso central e músculos. A excreção do OP ocorre predominantemente pela urina e em pequenas proporções pelas fezes, quase sempre nas primeiras 48 horas. O tratamento mais freqüente de intoxicações por agentes anticolinesterásicos, sobretudo os organofosforados, é feito através do uso de atropina em combinação com oximas. A primeira 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).

À semelhança dos compostos OPs, os CBs agem inibindo a AChE, diferenciando-se pelo fato da combinação se processar de uma maneira mais reversível, em função da estrutura química dos mesmos, similar à da acetilcolina. Os compostos CBs são considerados inibidores reversíveis da AChE, apresentando a enzima carbamilada uma regeneração mais rápida do que a fosforilada. A inibição da acetilcolinesterase determina o acúmulo da acetilcolina nas junções colinérgicas, resultando no aparecimento de uma sintomatologia grave e polimorfa, como relatado para os inseticidas OPs. Esses compostos, por inibirem de forma reversível a AChE apresentam uma larga margem entre a dose requerida para causar os sintomas precoces de toxicidade, e a dose necessária para causar efeitos severos (BONSALL e GOOSE, 1986). Além desta ação, decorrente da inibição da AChE, pesticidas CBs exibem outros efeitos bioquímicos e fisiológicos, incluindo o decréscimo da atividade metabólica do fígado, o decréscimo na síntese cerebral de fosfolipídeos, alteração dos níveis de serotonina sanguínea e um decréscimo na atividade da tireóide. Os CBs assim como os OPs, são rapidamente decompostos no organismo de mamíferos sem que haja acúmulo excessivo, produzindo, assim, efeitos de baixa toxicidade crônica (MACHEMER e PICKEL, 1994). Entretanto, os CBs possuem elevada toxicidade aguda, pois, ao contrário de diversos compostos OPs, são inibidores diretos da AChE não necessitando de ativação metabólica (FUKUTO, 1990). A reversibilidade da inibição enzimática exibida por carbamatos torna extremamente difícil o

diagnóstico clínico de intoxicações não recentes por esses agentes. Enquanto a sintomatologia de envenenamento por compostos OPs pode persistir por 1 ou 2 semanas, os sintomas da intoxicação por CBs podem desaparecer dentro de 24 horas, porém podem ser mais severos (O'MALLEY, 1997). Os compostos CBs, especialmente quando em formulações do tipo pó ou pó-molhável, são pouco absorvidos pelo organismo humano. Entretanto, os compostos CBs são rápida e eficazmente absorvidos pelo trato digestivo. A excreção dos CBs e de seus produtos de biotransformação é bastante rápida.

2.3 Acetilcolinesterase como bioindicador

A AChE é freqüentemente descrita como uma enzima perfeita porque suas propriedades catalíticas se conjugam para aproximar sua atividade do limite máximo de velocidade permitido pela própria difusão do substrato no meio circundante (TÕUGU, 2001; SILMAN e SUSSMAN, 2005). Uma molécula de acetilcolinesterase é capaz de degradar 300 mil moléculas de acetilcolina por minuto.

De acordo com Caldas (2000), para que haja a transmissão sináptica é necessário que a acetilcolina seja liberada na fenda sináptica e se ligue a um receptor pós-sináptico. Em seguida, a ACh disponível é hidrolisada pela acetilcolinesterase (Fig.01).

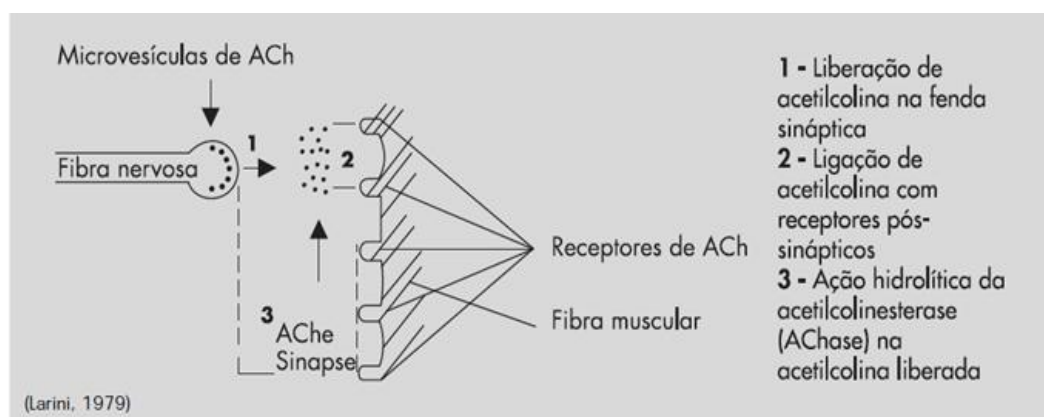


Figura 01 – Transmissão do Impulso Nervoso pela Acetilcolina

A enzima AChE tem 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 AChE, capazes de tornar rotineiros os procedimentos de biomonitoramento de recursos hídricos (BOCQUENÉ, GALGANI e TRUQUET, 1990).

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4. OBJETIVOS

4.1. Geral

Caracterizar a enzima acetilcolinesterase do Tucunaré (*Cichla ocellaris* BLOCH e SCHNEIDER) e avaliar o efeito de íons e pesticidas organofosforados e carbamatos sobre sua atividade.

4.2. Específicos

- Definir as propriedades físico-químicas, cinéticas e parâmetros de eficiência catalítica da acetilcolinesterase do tucunaré;
- Analisar o efeito de cinco pesticidas organofosforados (diclorvós, clorpirifós, diazinon, temefós e TEPP) e 02 carbamatos (carbofuran e carbaril) sobre a atividade da enzima em questão, em diferentes concentrações;
- Analisar o efeito de 14 íons catiônicos: Mn^{2+} ; Cu^{2+} ; Zn^{2+} ; Al^{3+} ; Ca^{2+} ; Pb^{2+} ; Cd^{2+} ; Hg^{2+} ; Fe^{2+} ; Ba^{2+} ; Mg^{2+} ; K^{+} ; As^{3+} ; Li^{+} e 1 íon complexo aniônico quelante: $EDTA^{2-}$ sobre a atividade da enzima estudada.

5. ARTIGO CIENTÍFICO

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

Este artigo será submetido à revista internacional Aquatic Toxicology



Qualis: A1

ISSN: 0166-445x

Fator de Impacto: 3.333

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

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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 K_m and V_{max} were determined as 0.769 mM and 0.189 U/mg protein, respectively. Selective inhibitors such 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 45°C. The enzyme retained approximately 45 % of the activity after incubation at 50°C for 30 min. All the employed pesticides showed inhibitory effects on *C. ocellaris* AChE. However, the strongest effects were observed with carbofuran ($\text{IC}_{50} = 0.21 \mu\text{M}$ and $\text{Ki} = 2.57 \times 10^{-3} \mu\text{M}$). 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*

1. Introduction

Organophosphate (OP) and carbamate (CB) compounds are the most widely used insecticides in the world. In 2007, only OPs accounted for 35% of all insecticides used in the United States (USEPA, 2011).

OP and CB 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 (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 by strong interaction with the esteratic site of AChE, preventing the binding of the substrate through virtual irreversible phosphorylation for OP and reversible carbamoylation in the case of CB (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 pesticides at biochemical level

can specifically detect the presence of contaminants in the environment before they reach higher organizational levels (Montserrat *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 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 resulting inhibition rates. This study aims to investigate physicochemical and kinetic properties 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.

2. Materials and Methods

2.1. Materials

Acetylthiocholine iodide, S-butyrylthiocholine iodide, tetraisopropyl pyrophosphoramidate (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.

2.2. Methods

2.2.1. Enzyme extraction

The fishes were sacrificed in an ice bath (0°C). 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 $1,000 \times g$ (4°C) and the supernatants (crude extracts) were frozen at -20°C for further assays.

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.

2.2.3. Kinetic parameters

The kinetic parameters Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), 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).

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 alkaline 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 80°C for 180 s. Thermal stability of fish AChE was evaluated by exposing crude extract samples for 30 min at temperatures ranging from 25 to 80°C and assaying the remaining activity after 15 minutes at 25°C (room temperature) equilibration.

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 inhibitors) 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 absorbance was followed at 405 nm for 180s under the same conditions of 2.2.2. The respective residual activities were determined, considering the absence of inhibitors as 100% activity.

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 $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$), Zn^{2+} (ZnCl_2) and the complex chelating ion EDTA^{2-} as $\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8$. 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 the pesticides. The ions solutions (10 μL) were incubated with crude extract (10 μL) for 40 min (Bocquené *et al.*, 1990) at 25°C and the residual activity was determined according to 2.2.2 and 2.2.5. Means $\pm$ standard deviation were compared using ANOVA and Tukey test ($p < 0.05$).

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 ($\mu\text{g/mL}$). 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 2.2.5. All assays were carried out at room temperature (25°C).

2.2.8. Estimation of IC_{50} , IC_{20} and K_i

Data from curves generated in the inhibition assays were statistically analyzed by linear and non-linear regression fitted to sigmoidal (Boltzmann) or exponential decay ($p < 0.05$) modelling 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 (K_i) using the equation of Cheng and Prusoff (1973):

$$K_i = \frac{IC_{50}}{1 + [S]/K_m}, \text{ where } [S] \text{ corresponds to the substrate}$$

concentration.

3. Results

The kinetic parameters K_m and V_{max} 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 is observed a variation for K_m from 0.1 (*O. niloticus*) to 1.69 mM (*P. Vetulus*) while for V_{max} values ranged from 0.129 (*C. macropomum*) to 0.482 U/mg protein (*P. Vetulus*).

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

Species	K_m [mM]	V_{max} [U/mg protein]	References
<i>Cichla ocellaris</i>	0.769 ± 0.27	0.189 ± 0.04	Present work
<i>Colossoma macropomum</i>	0.430 ± 0.02	0.129 ± 0.05	Assis et al., 2010
<i>Oreochromis niloticus</i>	0.10 ± 0.03	0.229 ± 0.014	Rodríguez- Fuentes and Gold- Boucht, 2004
<i>Pleuronectes vetulus</i>	1.69 ± 0.26	0.482 ± 0.034	Rodríguez- Fuentes et al., 2008
- not determined			

Optimum pH for *C. ocellaris* enzyme was found to be 8.0 (**Fig. 1A**). These results are close to the values found for some studies showed in **Table 2**.

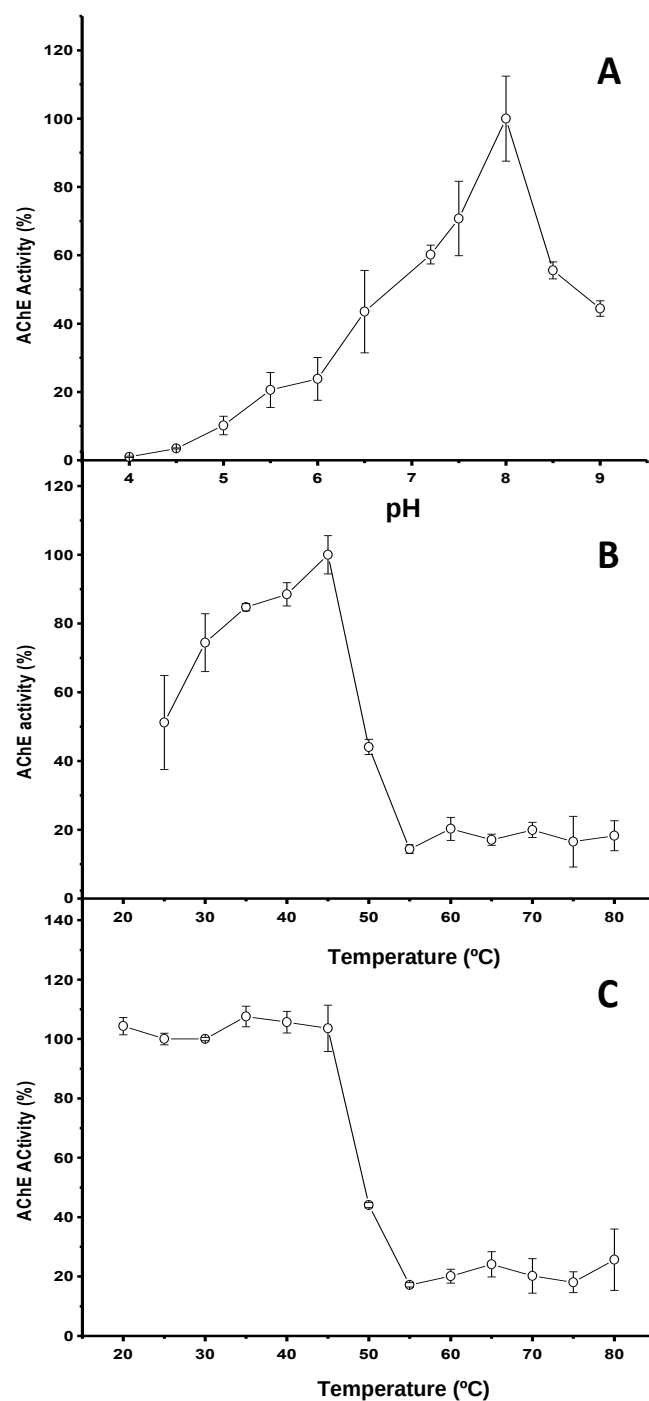


Figure 1 – (A) Effect of pH on the AChE activity from brain of *C. ocellaris*. The pH range was attained by using citrate-HCl, citrate-phosphate and tris-HCl buffers; (B) Effect of temperature on

the activity of brain AChE from *C. ocellaris*. The activity was assayed in a range from 25 to 80°C; (C) AChE thermal stability assayed from 20 to 80°C on the enzyme preparation for 30 min and after 15 min equilibrium at 25°C.

Figure 1B displays the optimum temperature for *C. ocellaris* AChE estimated as 45 °C, the same for *C. macropomum* (**Table 2**). For the same parameter, was found 33 °C for *Pleuronectes platessa*, while 25 and 35°C for *Lepomis macrochirus* and *Carassius auratus*, respectively. The enzyme retained around 45 % of the activity after incubation at 50°C for 30 min and 15 min equilibration in room temperature (**Fig. 1C**).

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

Species	pH optimum	Optimum Temperature [°C]	References
<i>Cichla ocellaris</i>	8.0	45	present work
<i>Colossoma macropomum</i>	7.5-8.0	45	Assis et al., 2010
<i>Lepomis macrochirus</i>	-	25	Beauvais et al., 2002
<i>Solea solea</i>	7,5	-	Bocquene, Galgani e Truquet, 1990
<i>Pleuronectes platessa</i>	8,5	33	Bocquene, Galgani e Truquet, 1990
<i>Scomber scomber</i>	8,0	-	Bocquene, Galgani e Truquet, 1990
<i>Carassius auratus</i>	-	35	Hazel, 1969

- not determined

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. 2A**), while the results for exposition to BW284c51 was a sudden drop in its activity after 0.001 mM of this inhibitor (**Fig. 2B**). Under neostigmine and

eserine exposure, activity decreased sharply at 0.001 mM (**Figs. 2C, 2D**). The IC_{50} and K_i related to each selective inhibitor are presented in **Table 3**.

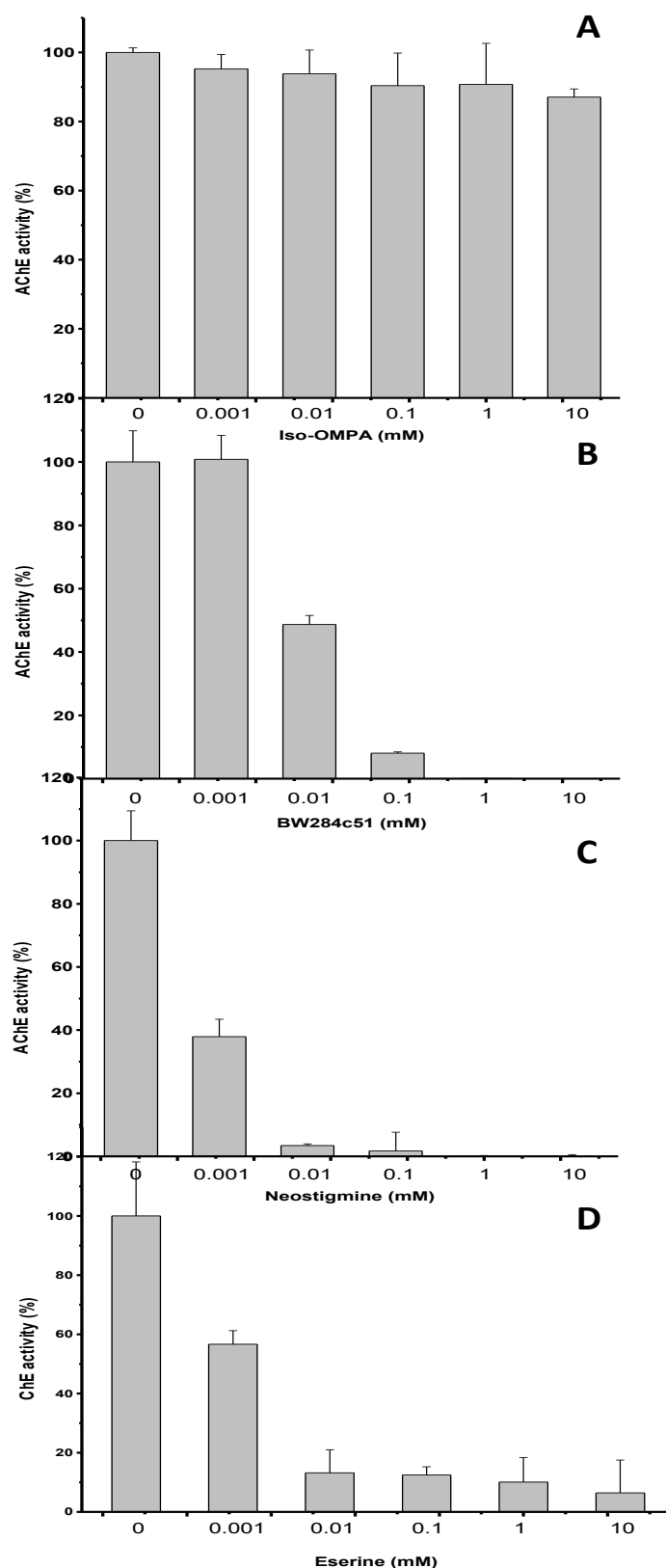


Figure 2 – Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-10 mM) of the selective inhibitors: (A) Iso-OMPA; (B) BW284c51; and the total ChEs inhibitors: (C) neostigmine; (D) eserine.

Table 5. IC₅₀ and Ki values estimated for AChE from *C. ocellaris* and IC₅₀ for *Pimephales promelas** in presence of some metal ions.

Species	<i>C. ocellaris</i>		<i>P. promelas</i>
Ions	IC ₅₀ (mM)	K _i (mM)	IC ₅₀ (mM)
As ³⁺	0.1	0.59 x 10 ⁻³	0.03
Cd ²⁺	6.14	36.4 x 10 ⁻³	0.57
Cu ²⁺	2.1	12.5 x 10 ⁻³	0.16
Hg ²⁺	0.22	1.34 x 10 ⁻³	1.60
Zn ²⁺	2.57	15.3 x 10 ⁻³	10.0

*From Olson and Christensen (1980)

The degree of AChE inhibition by pesticides is represented by the IC₅₀ 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 IC₅₀ 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 IC₅₀ in the concentration range analyzed. **Table 6** also shows the IC₂₀ of the pesticides considering that 20% of AChE inhibition is an important point to develop threshold limits by several international regulations.

Table 6. IC₅₀ and K_i *in vitro* estimated for *C. ocellaris* and *C. macropomum* in presence of some organophosphorus and carbamate pesticides.

Species	IC ₂₀ (μ M)	IC ₅₀ (μ M)	K _i (μ M)
Dichlorvos			
<i>Cichla ocellaris</i>	4.02	5.52	6.76×10^{-2}
<i>Colossoma macropomum</i>	-	0.04	1.37×10^{-4}
Diazinon			
<i>Cichla ocellaris</i>	182.57	2.9×10^3	36.3
<i>Colossoma macropomum</i>	NE	NE	NE
Chlorpyrifos			
<i>Cichla ocellaris</i>	2.17	10.13	1.21×10^{-1}
<i>Colossoma macropomum</i>	-	7.6	2.61×10^{-2}
Temephos			
<i>Cichla ocellaris</i>	NE	NE	NE
<i>Colossoma macropomum</i>	NE	NE	NE
TEPP			
<i>Cichla ocellaris</i>	0.32	0.37	4.53×10^{-3}
<i>Colossoma macropomum</i>	-	3.7	1.27×10^{-2}
Carbaryl			
<i>Cichla ocellaris</i>	1.18	4.41	5.4×10^{-2}
<i>Colossoma macropomum</i>	-	33.8	1.16×10^{-1}
Carbofuran			
<i>Cichla ocellaris</i>	0.082	0.21	2.57×10^{-3}
<i>Colossoma macropomum</i>	-	0.92	3.15×10^{-3}

- not determined; NE – Negligible effect in the concentration range analyzed.

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. In the case of enzymes, this characterization is the determination of kinetic and physicochemical parameters of their activity.

The k_m and V_{max} 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 comparable with *Colossoma macropomum* data from Assis *et al.* (2010). Moreover, they are in the range value for these parameters according to a recent review (Assis *et al.*, 2011) and not far from the values for another Cichlid (Nile tilapia) reported by Rodríguez-Fuentes and Gold-Bouchot (2004).

Optimum temperature of enzymes is not the same as the temperatures commonly found in habitat of the species. They work in a 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 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 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.

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 site, modifying the rate of hydrolysis of 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, 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. Value discrepant in relation to Olson and Christensen (1980) who found for *Pimephales promelas* 50% inhibition at 1.6 mM. Gill *et al.* (1990) using AChE from *Puntius conchoni* 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 carbamylation 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 by 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 chelating agent to be a protective measure 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; $\text{P}=\text{O}$) and the phosphorothioates (thion form; $\text{P}=\text{S}$) implies in important differences in the power of inhibition. The first group directly inhibits the cholinesterases since the higher electronegativity of the double-bonded oxygen 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 ($\text{P}=\text{O}$) inhibited more intensely the AChE from *C. ocellaris* (and *C. macropomum* for comparison) than chlorpyrifos and diazinon ($\text{P}=\text{S}$). 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., 2011). The enzyme of a given species can provide the best monitoring capabilities 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 IC₂₀ and IC₅₀ 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 comparison 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 ($IC_{20} = 0.32 \mu M \sim 94.84 \mu g/L$ and $IC_{50} = 0.37 \mu M \sim 107.37 \mu g/L$). 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 was 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 advantages of using *C. ocellaris* AChE as a biomarker for anticholinesterase pesticides, particularly carbofuran.

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 chelating agent to be used in protection measures 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.

Acknowledgement — The authors would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Financiadora de Estudos e Projetos (FINEP/RECARCINE), Petróleo do Brasil S/A (PETROBRAS), Secretaria Especial de Aquicultura e Pesca (SEAP/PR), Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq) and Fundação de Apoio à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for financial support.

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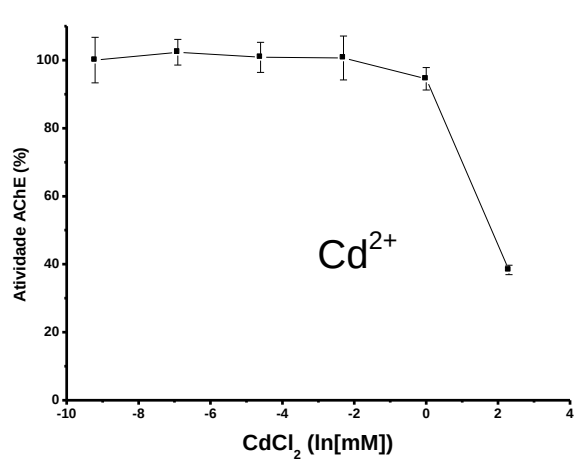
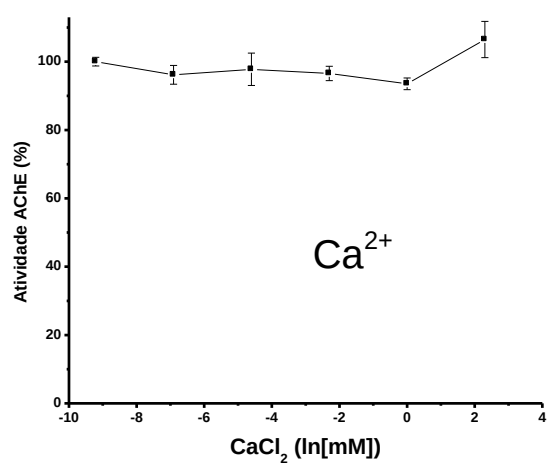
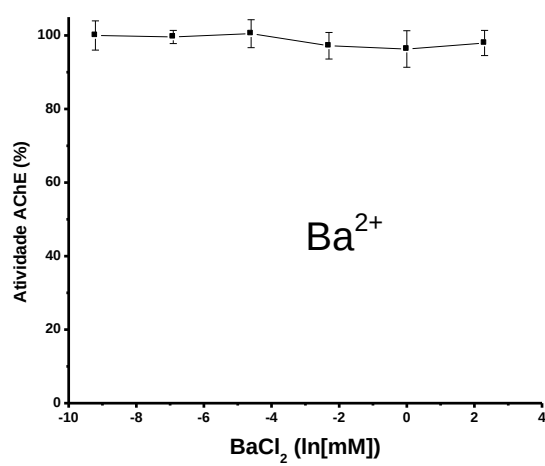
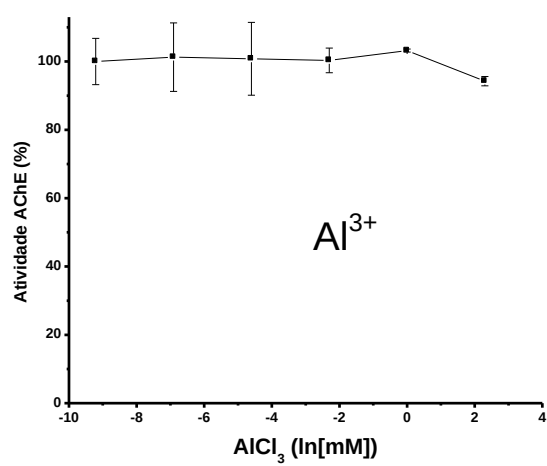
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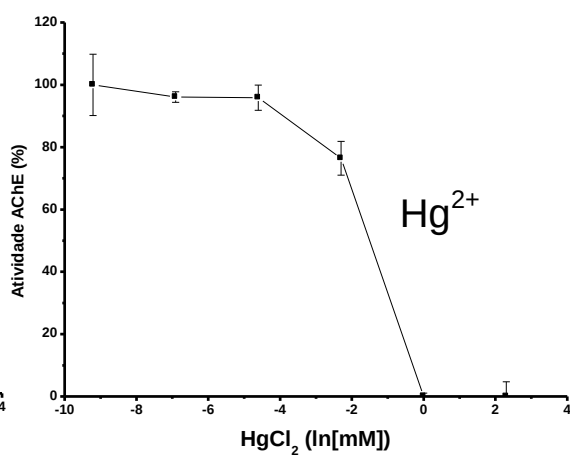
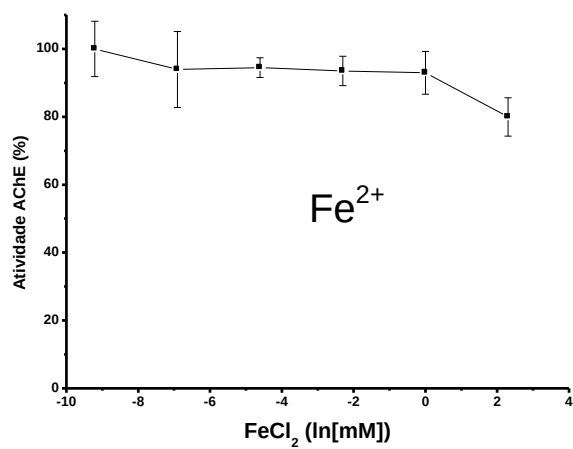
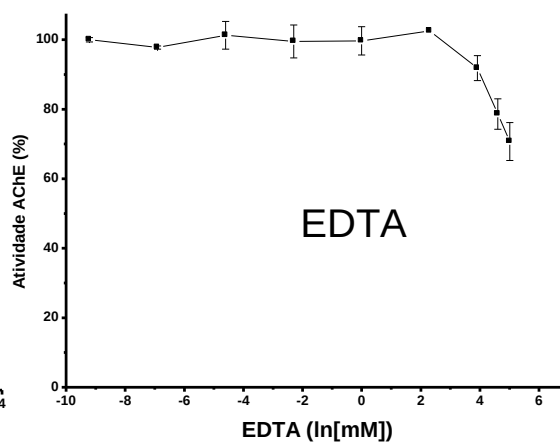
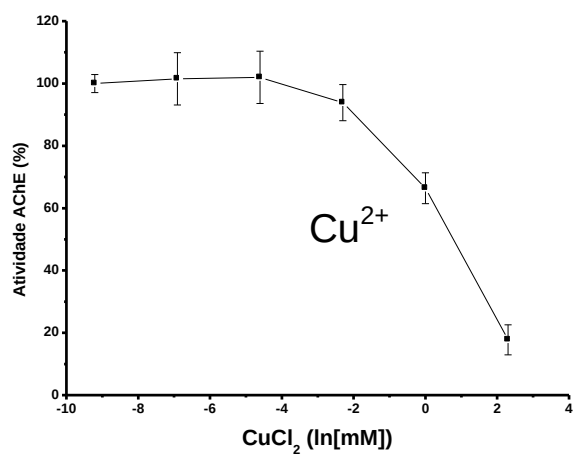
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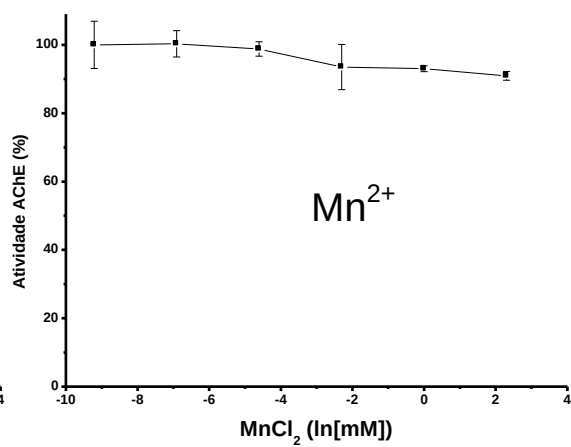
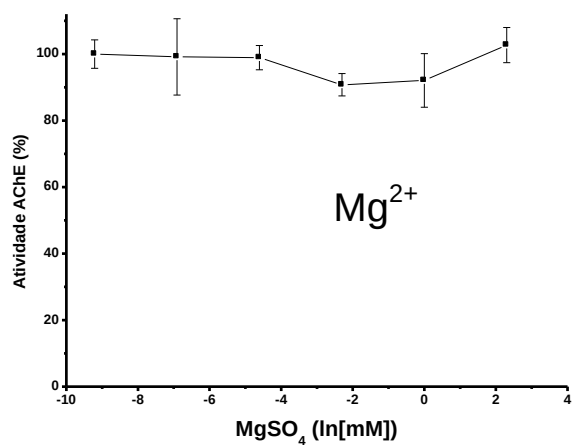
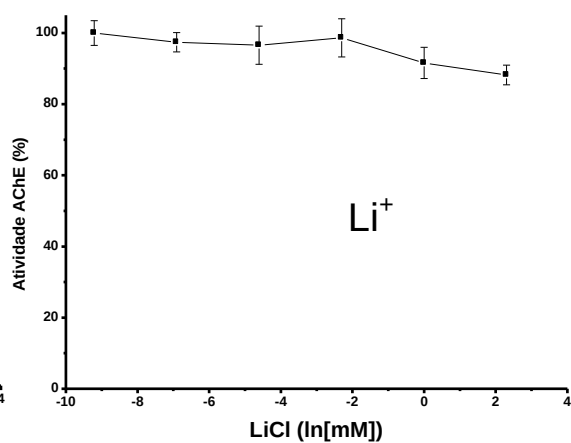
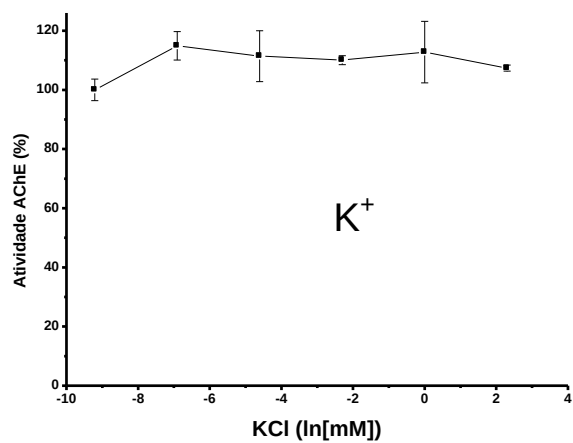
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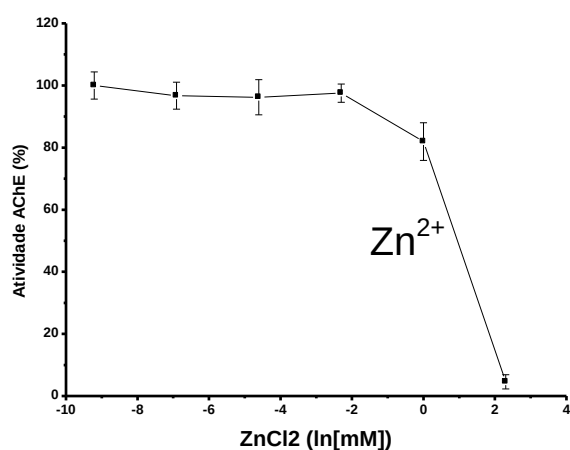
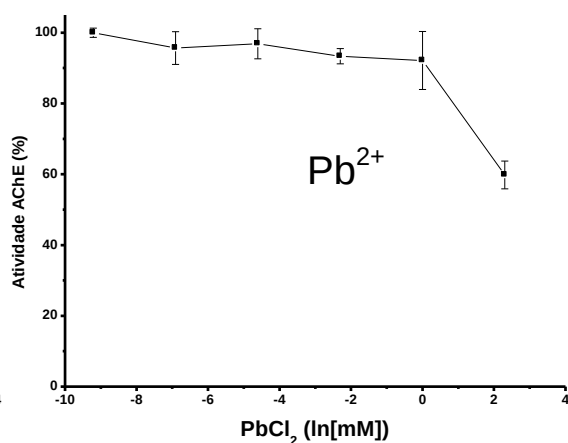
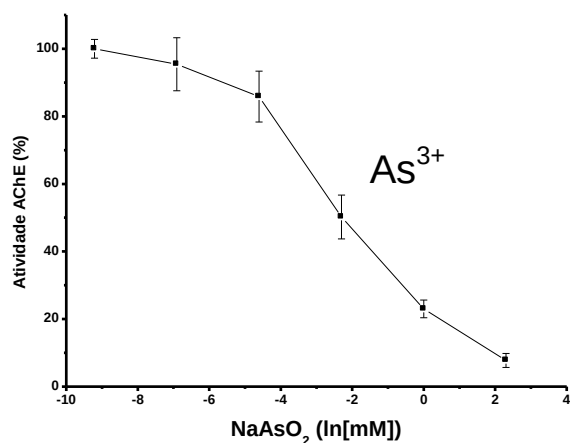
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Supplementary Data – Campos Silva *et al.*, 2011

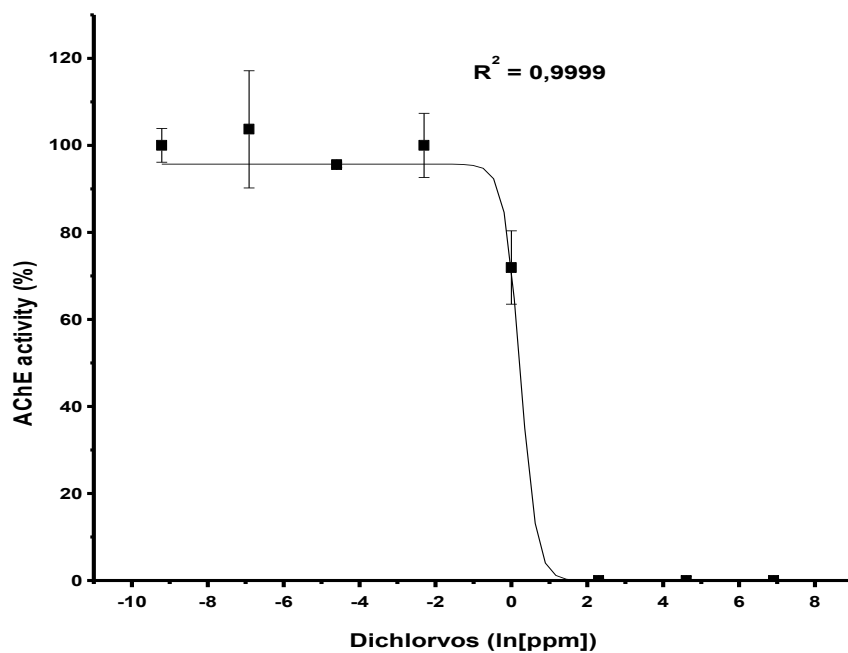




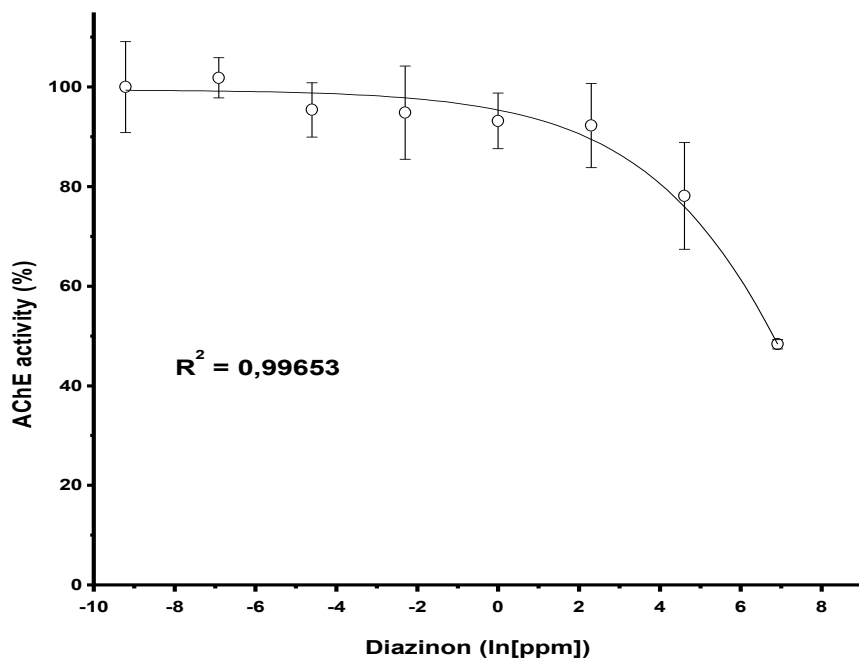


***C. ocellaris* brain AChE activity in
presence of several ions**

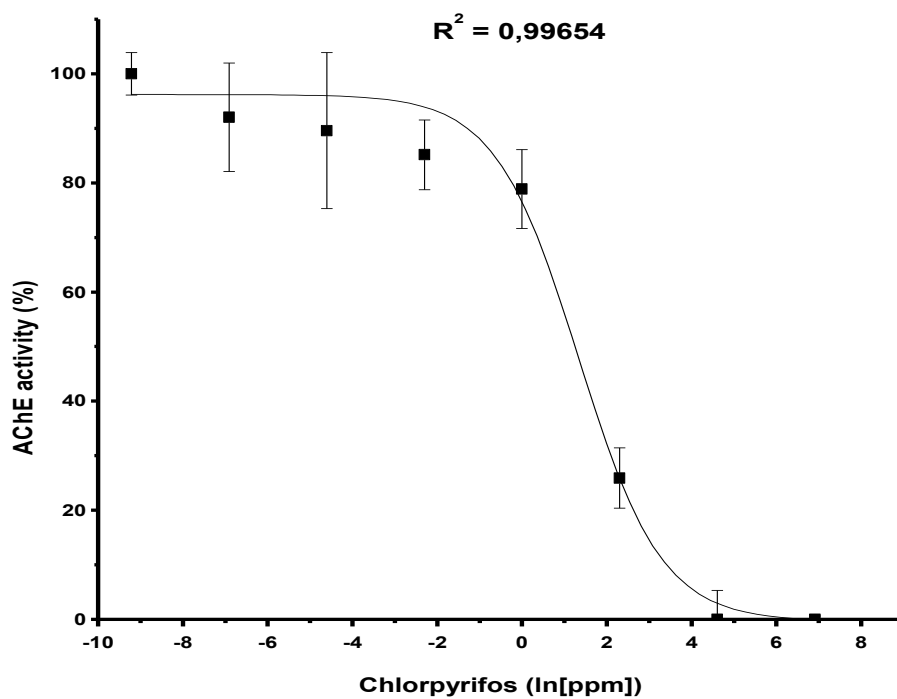
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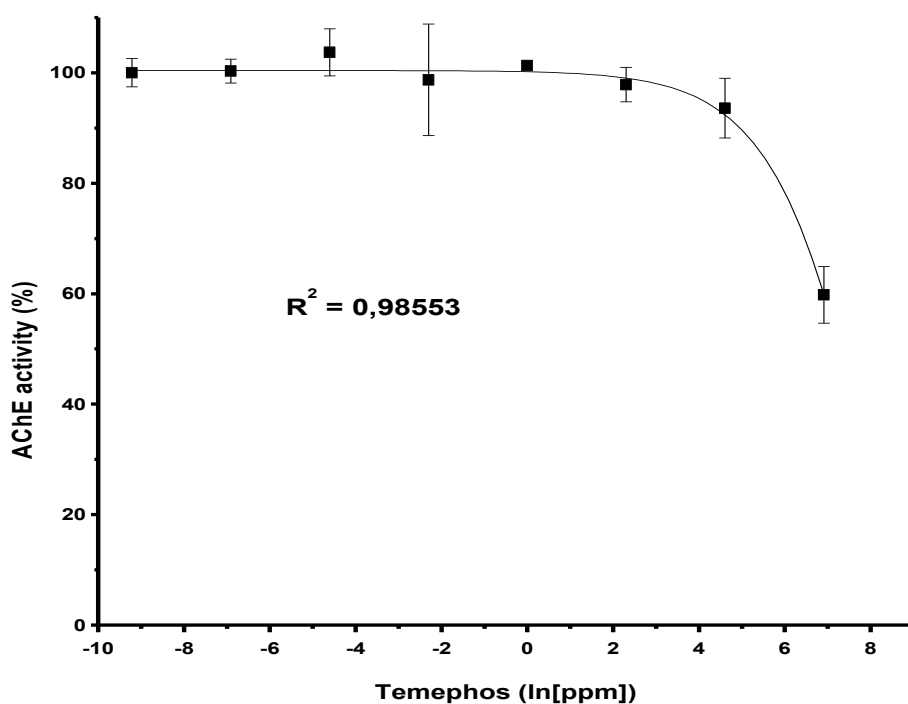
Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus dichlorvos.



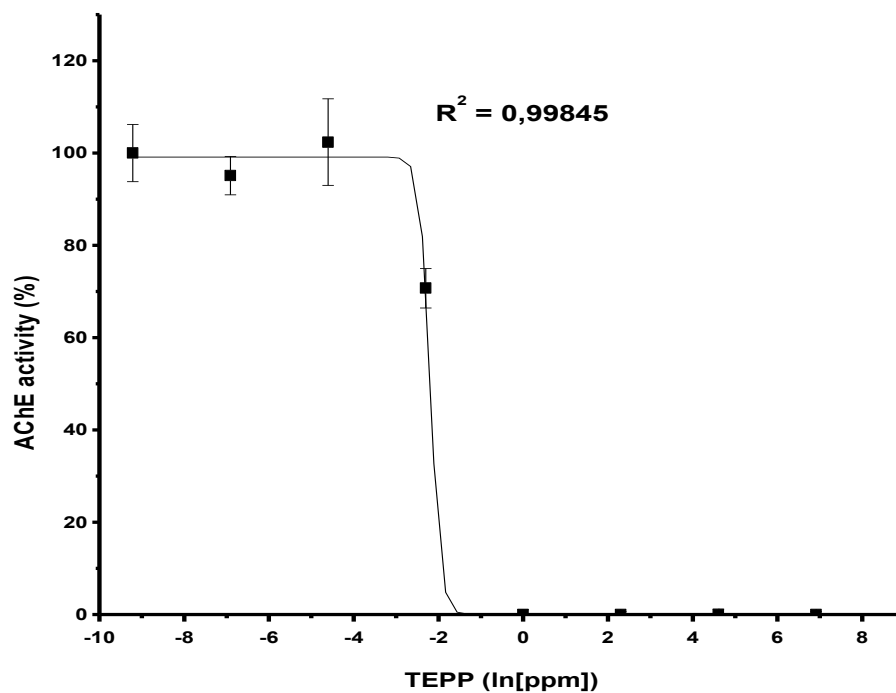
Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus diazinon.



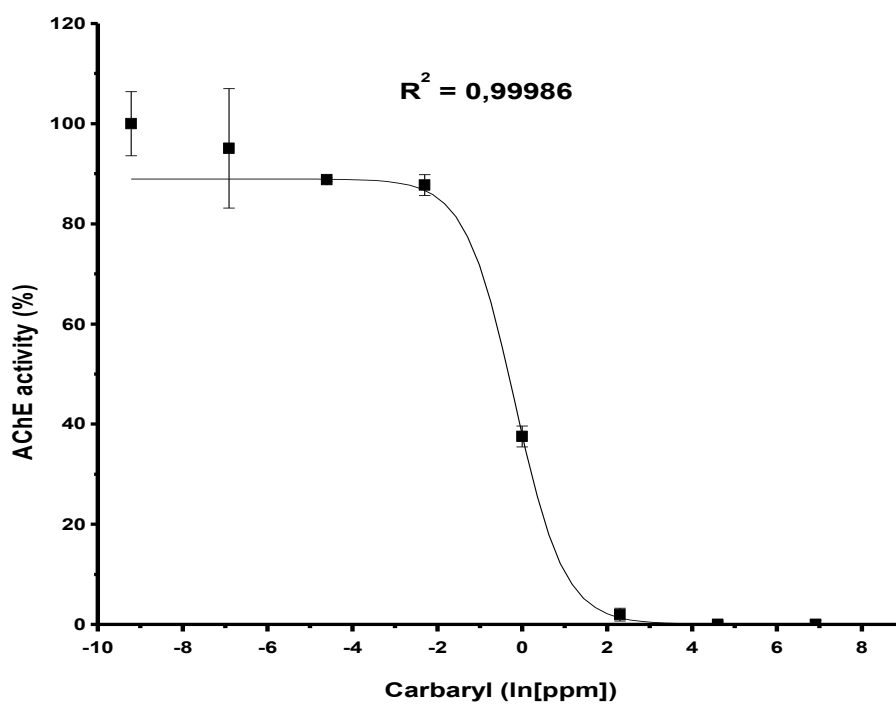
Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus chlorpyrifos.



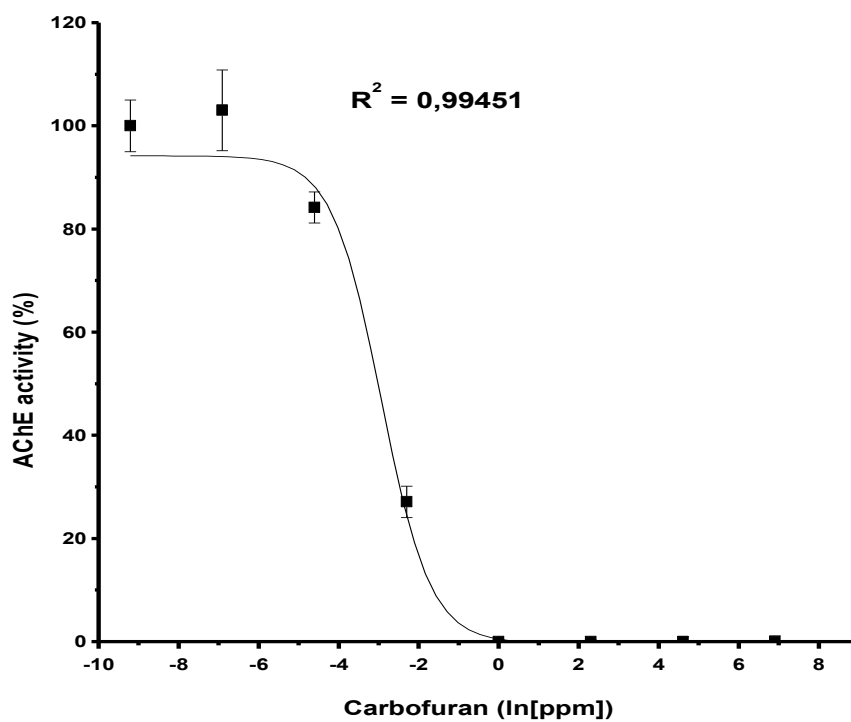
Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus temephos.



Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus tetraethyl pyrophosphate (TEPP).



Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the carbamate carbaryl.



Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the carbamate carbofuran.

Figure captions

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 80°C; (C) AChE thermal stability assayed from 20 to 80°C on the enzyme preparation for 30 min and after 15 min equilibrium at 25°C.

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

6. CONCLUSÕES

A atividade da acetilcolinesterase proveniente do cérebro de tucunaré *Cichla ocellaris* é extremamente sensível ao efeito do diclorvós, tetraetil-pirofosfato (TEPP), carbofuran e carbaril podendo assim ser empregada como um biomarcador de inseticidas organofosforado e carbamato.

O íon complexo EDTA só inibiu a enzima estudada a partir de 10 mM podendo-se então considerar a atividade dessas enzimas como EDTA-resistente.

Desta forma, a inibição in vitro da acetilcolinesterase de tucunaré demonstra ser uma ferramenta promissora para o monitoramento, rotineiro e eficiente, ambiental de recursos hídricos, alimentar e modelo para o monitoramento biológico na avaliação de riscos ocupacionais de exposição a agentes anticolinesterásicos.

7. ANEXOS

Normas para redação de artigo científico para a revista Aquatic Toxicology:



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2. Review Articles
3. Short Communications
4. Letters to the Editor

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