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

2012

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

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**

ORIENTADOR: RANILSON DE SOUZA BEZERRA

CO-ORIENTADOR: CAIO RODRIGO DIAS DE ASSIS

RECIFE

2012

Silva, Kaline Catiely Campos
Caracterização de Acetilcolinesterase cerebral de tucunaré, *Cichla ocellaris* (BLOCH & SCHNEIDER, 1801): efeito de íons e pesticidas organosforados e carbamatos sobre sua atividade/ Kaline Catiely Campos Silva – Recife: O Autor, 2012.

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

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

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

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.**

BANCA EXAMINADORA

Prof. Dr. Ranilson de Souza Bezerra
Departamento de Ciências Biologicas – CCB-UFPE

Profa. Dra. Maria Tereza dos Santos Correia
Departamento de Ciências Biologicas – CCB-UFPE

Dr. Ian Porto Gurgel do Amaral
Departamento de Bioquímica – DBIOq - UFPE

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 Juscy 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 Km e Vmax. 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 kinetica 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 Km and Vmax 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 IC50. 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*.

LISTA DE FIGURAS

Revisão bibliográfica

Artigo

LISTA DE TABELAS

Revisão Bibliográfica

Artigo

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
Vmax	Velocidade maxima de catálise atingida por uma enzima
WHO	World Health Organization

SUMÁRIO

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, neuróglias, 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 (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). Seu mecanismo de ação se dá através da ligação com o sítio esterásico 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, 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 lêntico, 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 lênticos (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 aqüicultura 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, consequentemente, 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., 1 997).

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 Rancágua, 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, 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)

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 gastrintestinal, 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).

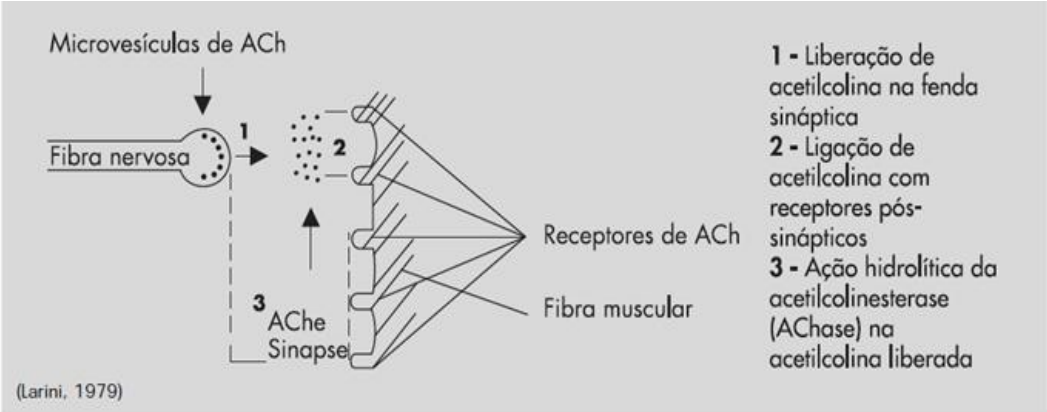


Figura 01 – Transmissão do Impulso Nervoso pela Acetilcolina

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).

3. REFERÊNCIAS

ALDRIDGE, W. N. Some properties of specific cholinesterase with particular reference to mechanism of inhibition by diethyl p-nitrophenyl thiophosphate (E605) and analogues. **Biochemical Journal**, 46, p. 117–124, 1950.

AMARANENI, S.R. & PILLALA R.R. **Concentrations of pesticide residues in tissues of fish from Kolleru Lake in India**. Environ. Toxicol., v.16, n.6, p.550-556, 2001.

ANWAR, W. A. Biomarkers of human exposure to pesticides. **Environmental Health Perspectives**. v. 105, supl. 4, p. 801-806, 1997

ASSIS, C. R. D.; AMARAL, I. P. G.; CASTRO, P. F.; CARVALHO Jr., L., B.; BEZERRA, R. S. Effect of dichlorvos on the acetylcholinesterase from tambaqui (Colossoma macropomum) brain. **Environmental Toxicology and Chemistry**, v. 26, N. 7, p. 1451–1453. 2007.

ASSIS, C.R.D., BEZERRA, R.S., CARVALHO Jr., L.B., 2011a. Fish cholinesterases as biomarkers of organophosphorus and carbamate pesticides. In: Pesticides in modern world – Book 5. Stoytcheva, M. (Ed). Intech, Rijeka, Croatia.

ATSDR. AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY. Toxicologic information about insecticides used for eradicating mosquitoes (West Nile virus control). Atlanta, 2005.

BEAUVAIS, S. L.; COLE, K. J.; ATCHISON, G. J.; COFFEY, M. **Factors affecting brain cholinesterase activity in Bluegill (*Lepomis macrochirus*)**. Water, Air, and Soil Pollution, 135, p. 249–264, 2002.

BOCQUENÉ, G.; GALGANI, F.; TRUQUET, P. Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring. Marine Environmental Research, v. 30, p. 75-89, 1990

BONSALL, J. L.; GOOSE, J. The safety evaluation of bendiocarb, a residual insecticide for vector control. **Toxicology Letters**. v. 33, p. 45-59, 1986.

BRASIL. Lei Federal nº 7.802 de 11/07/89. Dispõe sobre o tratamento de embalagens e resíduos de agrotóxicos e dá outros tratamentos. Disponível em: http://www.planalto.gov.br/ccivil_03/leis/L9974.htm. Acessso em: 20 de janeirode 2012.

BRASIL. Conselho Nacional de Meio Ambiente. Resolução no 302/2002. Brasília, 2004.

CALDAS,L.Q.de A;2000 Centro de Controle de Intoxicaçoes. **Intoxicações Exógenas Agudas por Carbamatos, Organofosforados, Compostos Bipiridílicos e Piretróides**. Niterói-RJ. Disponível em http://www.uff.br/ccin/. Acessado em 10/09/2010.

CAIRNS, J.Jr., PRATT, J.R. A history of biological monitoring using benthic macroinvertebrates. In: ROSENBERG, D.M, RESH, V.H. **Freshwater biomonitoring and benthic macroinvertebrates.** New York: Chapman & Hall; p. 10-27. 1993.

CHANDRASEKARA, H. U.; PATHIRATNE, A. Influence of low concentrations of Trichlorfon on haematological parameters and brain acetylcholinesterase activity in common carp, Cyprinus carpio L. Aquaculture Research, 36, p. 144-149, 2005.

CONDEPE/FIDEM, 2001. Diagnóstico sócio, econômico, político e cultural de Petrolândia-PE.

ECOBICHON, D. J. Toxic Effects of Pesticides. In: CASARETT, L. J.; KLASSEN, L.; DOULLS, P. **Toxicology – The Basic Science of Poisons**. 5 ed.: McGraw-Hill. p. 763-810, 1996.

ECOBICHON, D. J. Toxic effects of pesticides. In: AMDUR, M. O.; DOULL, J.; KLAASSEN, C. D., editores. **Casarett and Doll's toxicology: the basic science of poisons**. 4. ed. Nova York: Mc Graw Hill; 1993. p. 565-622.

FAO. 2007. Pesticides in food report 2007. FAO plant production and protection paper 191. Food and Agriculture Organization, Rome, IT.

GOTTI, C., CLEMENTI, F. **Neuronal nicotinic receptors: from structure to pathology**. *Prog Neurobiol.* (74): 363-396, 2004.

HE, F. Biological monitoring of occupational pesticides exposure. **International Archives of Occupational & Environmental Health**. v. 65, p. 569-576. 1993.

HEEREN, G.A., TYL ER, J. & MANDEYA, A. **Agricultural chemical exposures and birth defects in the Eastern cape province , SouthAf rica a case – control study**. Environ mental Health: A Global Access Science Source 2: 11. 2003

IBAMA. INSTITUTO BRASILEIRO DO MEIO AMBIENTE E RECURSOS NATURAIS RENOVÁVEIS. **GEO Brazil 2002**. Brazil Environment Outlook. Brasília, 2002.

JAGA, K. et al.**What are the implications of the interaction bet ween ddt and estrogen receptors in the body?** Medical Hypotheses 54:18-25. 2000

JARRARD, H. E.; DELANEY, K. R.; KENNEDY, C. J. Impacts of carbamate pesticides on olfactory neurophysiology and cholinesterase activity in coho salmon (*Oncorhynchus kisutch*). **Aquatic Toxicology**, 69, p. 133–148, 2004.

JEYARATNAM, J.; MARONI, M. Organophosphorus compounds. **Toxicology**. v. 91, p. 15-27, 1994.

KEIZER, J.; D'AGOSTINO, G.; NAGEL, R.; VOLPE, T.; GNEMID, P.; VITOZZI, L. Enzymological differences of AChE and diazinon hepatic metabolism: correlation of in vitro data with the selective toxicity of diazinon to fish species. **Science of the Total Environment**, v. 171, p. 213-220, 1995.

KELLAR, K. J. Overcoming inhibitions. **Proceedings of The National Academy of Sciences**. v. 103, p. 13263–13264, 2006.

LARINI, L. **Toxicologia dos Inseticidas**. p. 57-86, Sarvier, São Paulo; 1979. 281 p.

LARINI, L. **Toxicologia dos Praguicidas**. São Paulo: Editora Manole, 1999.

LANSAC-TÔHA, F. A.; VELHO, L. F. M.; BONECKER, C. C.; 1999. **Estrutura da Comunidade Zooplanctônica Antes e Após a Formatação do reservatório de Corumbá-GO** IN: Ecologia de reservatórios: Estrutura, função e Aspectos sociais (Ed. Henry, R.), FABESP, São Paulo.

MACHEMER, L.H.; PICKEL, M. Carbamates Insecticides. **Toxicology**. v. 91, p. 29-36, 1994.

MATOS, E. L.; LORIA, D. J.; ALBIANO, N.; SOBEL, N.; BUJÁN, E. C. Efectos de los plaguicidas en trabajadores de cultivos intensivos. Boletim de la **Oficina Sanitária Panamericana**. v. 104, n. 2, p. 160-170, 1988.

MELO, G. L. de.; 2006. Estudo da qualidade da água no reservatório de Itaparica localizado na bacia do rio São Francisco. Recife,. Dissertação de mestrado – Universidade Federal de Pernambuco. Programa de Pós Graduação em Engenharia Civil. p. 15-16.

MOREIRA, J. C. et al. Avaliação integrada do impacto do uso de agrotóxicos sobre a saúde humana em uma comunidade agrícola de Nova Friburgo, RJ. Ciência Saúde Coletiva;7(2):299-311. 2002

MUKHERJEE, I.; GOPAL, M. Organochlorine insectricide residues in drinking and groundwater in and around Delhi. Environmental Monitoring and Assessment, 76, p. 185–193, 2002.

MUTCH, E.; BLAIN, P. G.; WILLIAMS, F. M. Interindividual variations in enzymes controlling organophosphate toxicity in man. **Human & Experimental Toxicology**. v. 11, p. 109-116, 1992.

NAUEN, R.; BRETSCHNEIDER, T. New modes of action of insecticides. **Pesticide Outlook, 13, 241-245, 2002.**

NIMMO, D. R. Pesticides. In: RAND, G. M.; PETROCELLI, S.R., eds. **Fundamentals of aquatic toxicology: methods and applications**, New York: Hemisphere, p. 335-373, 1985.

NUNES, M. V.; TAJARA, E. H. Efeitos tardios dos praguicidas organoclorados no homem. **Revista de Saúde Pública**, 3, 4, p. 372-383, 1998.

O'MALLEY, M. Clinical evaluation of pesticide exposure and poisonings. **Lancet**. v. 349, p. 1161-1166, 1997.

PEROCCO, P. et al. Cytotoxic and cell transforming activities of the fungicide emethyl thiophana teon BALBRC 3T3 cells in vitro.**Mutation Research** 394:29-35. 1997

QUINN, D. M. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. **Chemical Reviews**, 87, p. 955-979, 1987.

RODRÍGUEZ-FUENTES, G.; GOLD-BOUCHOT, G. Environmental monitoring using acetylcholinesterase inhibition in vitro. A case study in two Mexican lagoons. Marine Environmental Research, v. 50, p. 357-360, 2000.

RODRÍGUEZ-FUENTES, G.; ARMSTRONG, J.; SCHLENK, D. Characterization of muscle cholinesterases from two demersal flatfish collected near a municipal wastewater outfall in Southern California. Ecotoxicology and Environmental Safety, v. 69, p. 466–471. 2008.

ROJAS R., A.; OJEDA B., M. E.; BARRAZA O., Ximena. Malformaciones congénitas y exposición a pesticidas. **Revista Médica de Chile**, v. 128, n. 4, p. 399-404, 2000.

SÁNCHEZ-HERNÁNDEZ, J. C.; MORENO-SÁNCHEZ, B. Lizard cholinesterases as biomarkers of pesticide exposure: enzymological characterization. Environmental Toxicology and Chemistry, 21, p. 2319-2325, 2002.

SCOTT, G. R..; SLOMAN, K. A. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquatic Toxicology, 68, p. 369–392, 2004.

SILMAN, I.; SUSSMAN, J. L. Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. Current Opinion in Pharmacology, 5, p. 293–302, 2005.

SILVA, J.W.B.; CHACON, J.O.; SANTOS, E.P. Curva de rendimento do Tucunaré pinima Cichla temensis (Humboldt,1833), do açude público "Estevam Marinho"(Curemas, Paraíba, Brasil) (Pisces, actinopterygl Cichlidae). Revista Brasileira de Biologia, v.40, p.203-206, 1980.

SOBRAL, M D.; CARVALHO, R. M. C. M. O., SILVA, M. M. da. MELO, G. L.; 2006. **Uso e ocupação dos solos no entorno de reservatórios no semi-árido brasileiro como fator determinante da qualidade da água**. In: Congresso Interamericano de Ingeniería Sanitária y Ambiental. Punta del Leste, Anais.

STURM, A.; DA SILVA DE ASSIS, H. C.; HANSEN, P. Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. Marine Environmental Research, 47, 389–398, 1999.

SUNGUR, M. & GÜVEN, M.I.. Intensive care managem entof organophosphate insecticide poisoning. **Criti cal Care** 5:211-215. 2001

TAYLOR, P.; RADIC, Z.; HOSEA, N. A.; CAMP, S.; MARCHOT, P. BERMAN, H. A. Structural basis for the specificity of cholinesterase catalysis and inhibition. **Toxicology Letters**, 82/83, p. 453-458. 1995.

TÕUGU, V. Acetylcholinesterase: mechanism of catalysis and inhibition. **Current Medicinal Chemistry - Central Nervous System Agents**, 1, p. 155-170, 2001.

TUNDISI. J.G.; 2003. **A água no século XXI: enfrentando a escassez**. São Carlos: RIMA, 2003. p.256.

UFF/CCIn. UNIVERSIDADE FEDERAL FLUMINENSE/CENTRO DE CONTROLE DE INTOXICAÇÕES. **Intoxicações Exógenas Agudas por Carbamatos, Organofosforados, Compostos Bipiridílicos e Piretróides**, Niterói: UFF, 2000.

USDA/USEPA/ATSDR. U. S. DEPATMENT OF AGRICULTURE/U. S. ENVIRONMENT PROTECTION AGENCY/AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY. Toxicological Profile for DDT, DDE and DDD. Toxicological Profile 35, Atlanta, 2002.

YOUNES, M & Galal-Gorchev, H. **Pesticides in drinking water – a case study**. Food Chem Toxicol., v.38; p.S87-S90, 2000.

WAICHMAN, A.V.; 2008. Uma proposta de avaliação integrada de risco do uso de agrotóxicos no estado do Amazonas, Brasil. **Acta Amazônica**.vol. 38(1) 2008: 45 – 51

WHO. WORLD HEALTH ORGANIZATION. Organophosphorus insecticides: a general introduction. **Environmental Health Criteria 63**, Genebra, 1990.

WHO/UNEP/ILO/IPCS. The WHO recommended classification of pesticides by hazard and guidelines to classification: 2004. **The WHO recommended classification of pesticides by hazard**. World Health Organization, Geneva, Switzerland. 2006.

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;

- Analizar 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*.

Corresponding author:

Ranilson de Souza Bezerra.

Laboratório de Enzimologia – LABENZ, Departamento de Bioquímica and Laboratório de Imunopatologia Keizo Asami,

Universidade Federal de Pernambuco, Campus Universitário,

50670-901 Recife, Pernambuco, Brazil

Tel.: + 55 81 21268540;

Fax: + 55 81 21268576.

E-mail: ransoube@uol.com.br

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 Verônica Matoso Maciel Carvalho, Luiz Bezerra Carvalho Jr and Ranilson Souza Bezerra*

Departamento de Bioquímica and Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Recife-PE, Brazil.

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 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*

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 (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 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 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.

2.2. Methods

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.

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-Mentem 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 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 at 25ºC (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: A^{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 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 ± standard deviation were compared using ANOVA and Tukey test ($\rho < 0.05$).

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 (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 2.2.5. All assays were carried out at room temperature (25ºC).

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 ($\rho < 0.05$) 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 (Ki) using the equation of Cheng and Prussoff (1973):

$$Ki = \frac{\text{IC50}}{1+\ [\text{S}]/\text{Km}}$$, were [S] corresponds to the substrate concentration.

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 is observed a 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. Kinetic and catalytic efficiency parameters of brain AChE from *C. ocellaris* and other species.

Species	**K_m [mM]**	**V_{max} [U/mg protein]**	**References**
Cichla ocellaris	0.769 ± 0.27	0.189 ± 0.04	Present work
Colossoma macropomum	0.430 ± 0.02	0.129 ± 0.05	Assis et al., 2010
Oreochromis niloticus	0.10 ± 0.03	0.229 ± 0.014	Rodríguez-Fuentes and Gold-Boucht, 2004
Pleuronectes vetulus	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**.

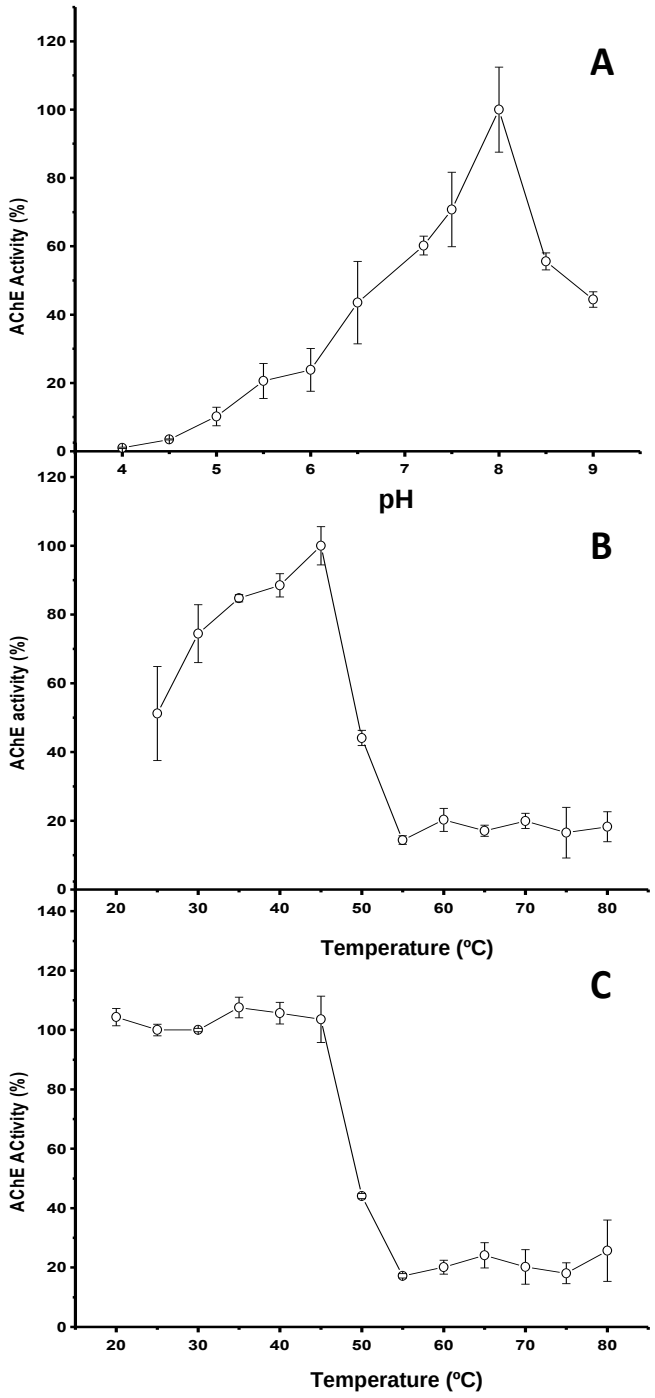


Figure 1 – (A) Effect of pH on the AChE activity from brain of *C. ocellaris*. The pH range was attained by using citrate-HCl, citrate-phosphate and tris-HCl buffers; (B) Effect of temperature on

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

Figure 1B displays the optimum temperature for *C. ocellaris* AChE estimated as 45 ºC, the same for *C. macropomum* (**Table 2**). For the same parameter, was found 33 ºC for *Pleuronectes platessa*, while 25 and 35ºC for *Lepomis macrochirus* and *Carassius auratus*, respectively. The enzyme retained around 45 % of the activity after incubation at 50ºC 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 [ºC]**	**References**
Cichla ocellaris	8.0	45	present work
Colossoma macropomum	7.5-8.0	45	Assis et al., 2010
Lepomis macrochirus	-	25	Beauvais et al., 2002
Solea solea	7,5	-	Bocquene, Galgani e Truquet, 1990
Pleuronectes platessa	8,5	33	Bocquene, Galgani e Truquet, 1990
Scomber scomber	8,0	-	Bocquene, Galgani e Truquet, 1990
Carassius auratus	-	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 Ki related to each selective inhibitor are presented in **Table 3**.

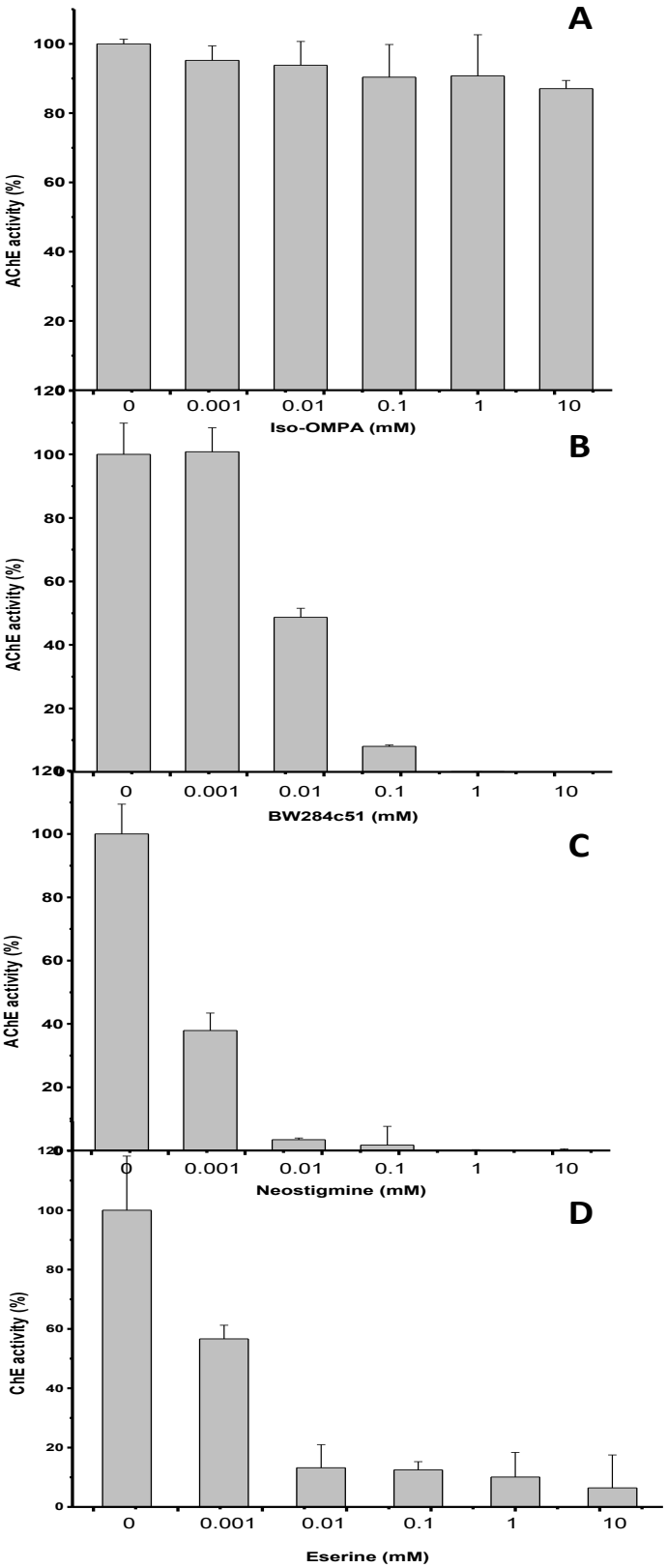


Figure 2 – Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-10 mM) of the selective inhibitors: (A) Iso-OMPA; (B) BW284c51; and the total ChEs inhibitors: (C) neostigmine; (D) eserine.

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

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

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+}**	**$EDTA^{2-}$**	**Fe^{2+}**	**Hg^{2+}**	**K^{+}**	**Li^{+}**	**Mg^{2+}**	**Mn^{2+}**	**Pb^{2+}**	**Zn^{2+}**
-	75%	-	-	-	35%	-	-	100%	-	-	-	-	-	18%

– No effect at 1 mM

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)

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 = 2.57×10^{-3} µM), 0.37 µM (Ki = 4.53×10^{-3} µM) and 4.41 µM (Ki = 5.4×10^{-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.

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	**IC_{20} (µM)**	**IC_{50} (µM)**	**K_i (µM)**
Dichlorvos			
Cichla ocellaris	4.02	5.52	6.76×10^{-2}
Colossoma macropomum	-	0.04	1.37×10^{-4}
Diazinon			
Cichla ocellaris	182.57	2.9×10^{3}	36.3
Colossoma macropomum	NE	NE	NE
Chlorpyrifos			
Cichla ocellaris	2.17	10.13	1.21×10^{-1}
Colossoma macropomum	-	7.6	2.61×10^{-2}
Temephos			
Cichla ocellaris	NE	NE	NE
Colossoma macropomum	NE	NE	NE
TEPP			
Cichla ocellaris	0.32	0.37	4.53×10^{-3}
Colossoma macropomum	-	3.7	1.27×10^{-2}
Carbaryl			
Cichla ocellaris	1.18	4.41	5.4×10^{-2}
Colossoma macropomum	-	33.8	1.16×10^{-1}
Carbofuran			
Cichla ocellaris	0.082	0.21	2.57×10^{-3}
Colossoma macropomum	-	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 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 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 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 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; 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 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 (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., 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 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 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 Aqüicultura 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.

6. References

Abou-Donia, M.S., Menzel, D.B., 1967. Fish brain cholinesterase: its inhibition by carbamates and automatic assay. Comp. Biochem. Physiol. 21, 99-108.

Antwi, L.A.K., 1987. Fish head acetylcholinesterase activity after aerial application of temephos in two rivers in Burkina Faso, West Africa. Bull. Environ. Contam. Toxicol. 38, 461-466.

ANVISA. Agência Nacional de Vigilância Sanitária. Indice monográfico C06: Carbofurano. Available in: http://portal.anvisa.gov.br/wps/wcm/connect/b5fd6a004

119a559bbc1bf65e55ae87b/C06++Carbofurano.pdf?MOD=AJPERES. Access in: jun 2011. updated in: set 2006.

Assis, C.R.D., Amaral, I.P.G., Castro, P.F., Carvalho Jr, L.B., Bezerra, R.S. 2007. Effect of dichlorvos on the acetylcholinesterase from tambaqui (*Colossoma macropomum*) brain. Environ. Toxicol. Chem. 26, 1451–1453.

Assis, C.R.D., Castro, P.F., Amaral, I.P.G., Maciel Carvalho, E.V.M., Carvalho Jr, L.B., Bezerra, R.S., 2010. Characterization of acetylcholinesterase from the brain of the Amazonian tambaqui (*Colossoma macropomum*) and *in vitro* effect of organophosphorus and carbamate pesticides. Environ. Toxicol. Chem. 29, 2243–2248.

Assis, C.R.D., Bezerra, R.S., Carvalho Jr., L.B., 2011. Fish cholinesterases as biomarkers of organophosphorus and carbamate pesticides. In: Pesticides in modern world – Book 5. Stoytcheva, M. (Ed). Intech, Rijeka, Croatia.

Beauvais, S.L., Cole, K.J., Atchison, G.J., Coffey, M., 2002. Factors affecting brain cholinesterase activity in Bluegill *(Lepomis macrochirus)*. Water Air Soil Pollut. 135, 249–264.

Bocquené, G., Galgani, F., Truquet, P., 1990. Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring. Mar. Environ. Res. 30, 75-89.

Chandrasekara, H.U., Pathiratne, A., 2005. Influence of low concentrations of Trichlorfon on haematological parameters and brain acetylcholinesterase activity in common carp, *Cyprinus carpio* L. Aquac. Res. 36, 144-149.

Cheng, Y., Prusoff, W.H. 1973. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol*. 22 (23): 3099–108.

Çokugras, A. N. Butyrylcholinesterase: Structure and Physiological Importance. Turk. J. Biochem. 2003; 28 (2); 54-61.

Cunha Bastos, V.L.F., Silva Filho, M.V., Rossini, A., Cunha Bastos, J., 1999. The Activation of Parathion by Brain and Liver of a Brazilian Suckermouth Benthic Fish Shows Comparable in Vitro Kinetics. Pest. Biochem. Physiol. 64, 149–156.

EFSA. European Food Safety Agency. 2004. Draft Assessment Report (DAR): Carbofuran vol. 1, Brussels, BE.

Fairbrother, A., Bennett, J. K., 1988. The usefulness of cholinesterase measurement, J. Wildl. Dis. 24, 587-590.

FAO. 2007. Food and Agriculture Organization. Pesticides in food report 2007. FAO plant production and protection paper 191. Food and Agriculture Organization, Rome, Italy.

Gill, T.S., Tewari, H., Pande, J., 1990. Use of the fish enzyme system in monitoring water quality: effects of mercury on tissue enzymes. Comp. Biochem. Physiol. 97C, 287-292.

Hazel, J., 1969. The effect of thermal acclimation upon brain acetylcholinesterase activity of *Carassius auratus* and *Fundulus heteroclitus*. Life Sci. 8, 775–784.

Hughes, R.J., Bennett, J., 1985. Effect of metal ions on the activity of acetylcholinesterase. Biochem. Soc. Trans. 13, 219-220.

Jarrard, H.E.; Delaney, K.R.; Kennedy, C.J. 2004. Impacts of carbamate pesticides on olfactory neurophysiology and cholinesterase activity in coho salmon (*Oncorhynchus kisutch*). Aquat. Toxicol. 69, p. 133–148.

Jung, J.-H., Addison, R.F., Shim, W.J., 2007. Characterization of cholinesterases in marbled sole, *Limanda yokohamae*, and their inhibition in vitro by the fungicide iprobenfos. Mar. Envir. Res. 63, 471–478.

Monserrat, J.M., Geracitano, L.A., Bianchini, A., 2003. Current and future perspectives using biomarkers to assess pollution in aquatic ecosystems. Comments. Toxicol. 9, 255–269.

Nicolet, Y.; Lockridge, O.; Masson, P.; Fontecilla-Camps, J. C.; Nachon, F. 2003. Crystal Structure of Human Butyrylcholinesterase and of Its Complexes with Substrate and Products. J. Biol. Chem. 278: 41141–41147.

Olson, D.L., Christensen, G.M., 1980. Effects of water pollutants and other chemicals on fish acetylcholinesterase (*in vitro*). Environ. Res. 21, 327-335.

Payne, J. F., Mathieu, A., Melvin, W., Fancey, L.L., 1996. Acetylcholinesterase, an old biomarker with a new future? field trials in association with two urban rivers and a paper mill in Newfoundland. Mar. Pollut. Bull. 32, 225-231.

Pezzementi, L., Chatonnet, A., 2010. Evolution of cholinesterases in the animal kingdom. Chem. Biol. Interact. 187, 27–33.

Quinn, D.M., 1987. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. Chem. Rev. 87, 955-979.

Reddy, G.R., Basha, M.R., Devi, C.B., Suresh, A., Baker, J.L., Shafeek, A., Heinz, J., Chetty, C.S., 2003. Lead induced effects on acetylcholinesterase activity in cerebellum and hippocampus of developing rat. Int. J. Devl. Neuroscience. 21, 347–352.

Rendón-von Osten, J., Ortíz-Arana, A., Guilhermino, L., Soares, A.M.V.M., 2005. In vivo evaluation of three biomarkers in the mosquitofish (*Gambusia yucatana*) exposed to pesticides. Chemosphere. 58, 627-636.

Rodríguez-Fuentes, G., Gold-Bouchot, G., 2000. Environmental monitoring using acetylcholinesterase inhibition in vitro. A case study in two Mexican lagoons. Mar. Env. Res. 50, 357-360.

Rodríguez-Fuentes, G., Gold-Bouchot, G., 2004. Characterization of cholinesterase activity from different tissues of Nile tilapia (*Oreochromis niloticus*). Mar. Environ. Res. 58, 505-509.

Rodríguez-Fuentes, G., Armstrong, J., Schlenk, D., 2008. Characterization of muscle cholinesterases from two demersal flatfish collected near a municipal wastewater outfall in Southern California. Ecotox. Environ. Safe. 69, 466–471.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392.

Sedmak, J.J., Grossberg, S.E., 1977. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. 79, 544-552.

Shaoguo, R., Xuanhui, W., Ming, J., Yongqi, L. 2003. In vivo and in vitro inhibitions of red drum (*Sciaenops ocellatus*) brain acetylcholinesterase and liver carboxylesterase by monocrotophos at sublethal concentrations. Water Air Soil Poll. 149, 17–25.

Soreq H, Zakut H. 1990. Cholinesterase genes: multileveled regulation. In Monographs in Human Genetics, ed. RS Sparkes, 13:1-102. Basel: Karger.

Taylor, P., Radic, Z., Hosea, N.A., Camp, S., Marchot, P., Berman, H.A. 1995. Structural basis for the specificity of cholinesterase catalysis and inhibition. Toxicol. Lett. 82/83, p. 453-458.

Tham, L.G., Perumal, N., Syed, M.A., Shamaan, N.A., Shukor, M.Y., 2009. Assessment of *Clarias batrachus* as a source of acetylcholinesterase (AChE) for the detection of insecticides. J. Environ. Biol. 30, 135-138.

Tomlinson, G., Mutus, B., McLennan, I., 1980. Modulation of acetylcholinesterase activity by peripheral site ligands. Mol. Pharmacol. 18, 33-39.

Tomlinson, G., Mutus, B., McLennan, I., 1981. Activation and inactivation of acetylcholinesterase by metal ions. Can. J. Biochem. 59, 728-735.

Tõugu, V., 2001. Acetylcholinesterase: mechanism of catalysis and inhibition. Curr. Med. Chem. 1, 155-170.

USEPA. United States Environmental Protection Agency. 2011. Pesticides Industry Sales and Usage - 2006 and 2007 Market Estimates. U.S. Environmental Protection Agency. Washington, DC. February 2011. Available at: http://www.epa.gov/opp00001/pestsales/07pestsales/market_estimates2007.pdf.

USEPA. United States Environmental Protection Agency. 1984. Chemical Fact Sheet number 24: Carbofuran. Insecticide/Rodenticide Branch. Registration Division (TS-767). Washington, DC, US.

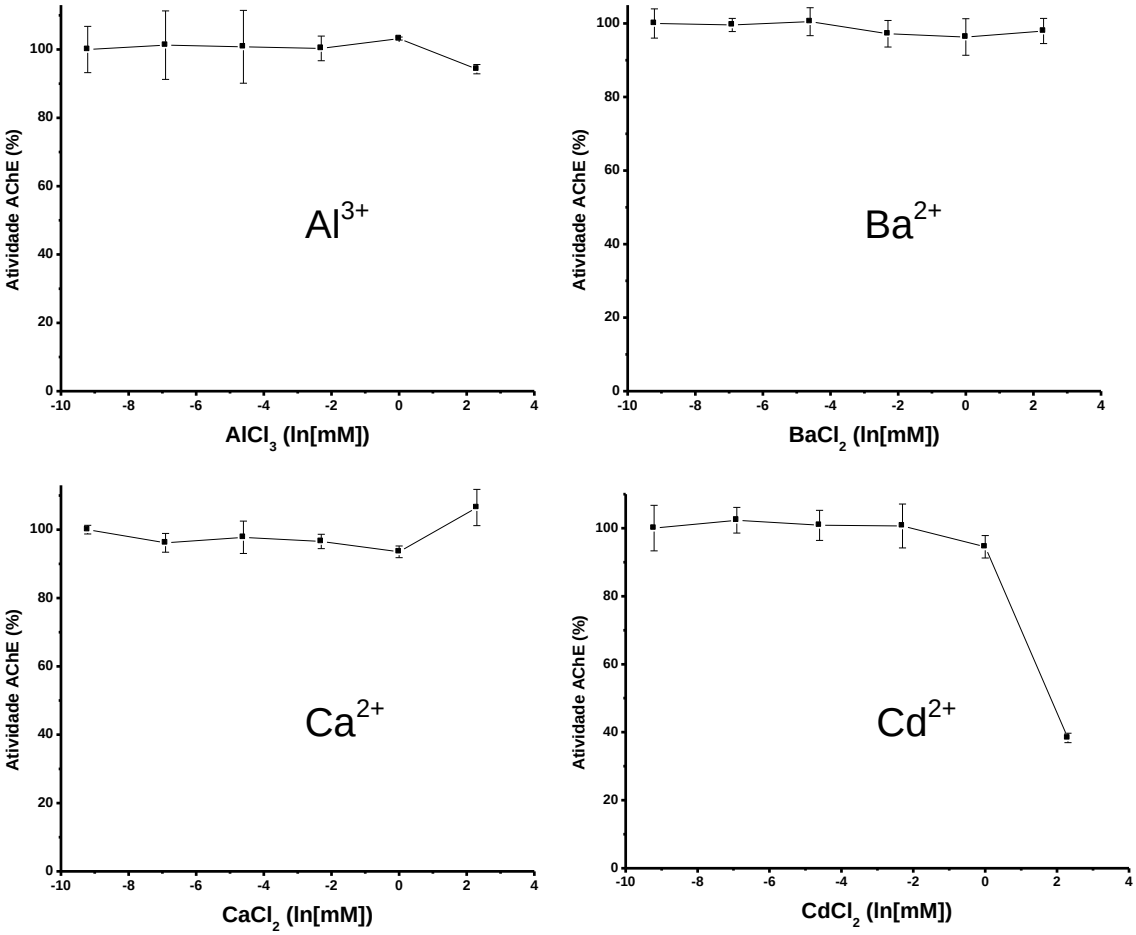
Valle, B.L., Ulmer, D.D. 1972. Biochemical effects of mercury, cadmium and lead. Ann. Rev. Biochem. 41, 91-128.

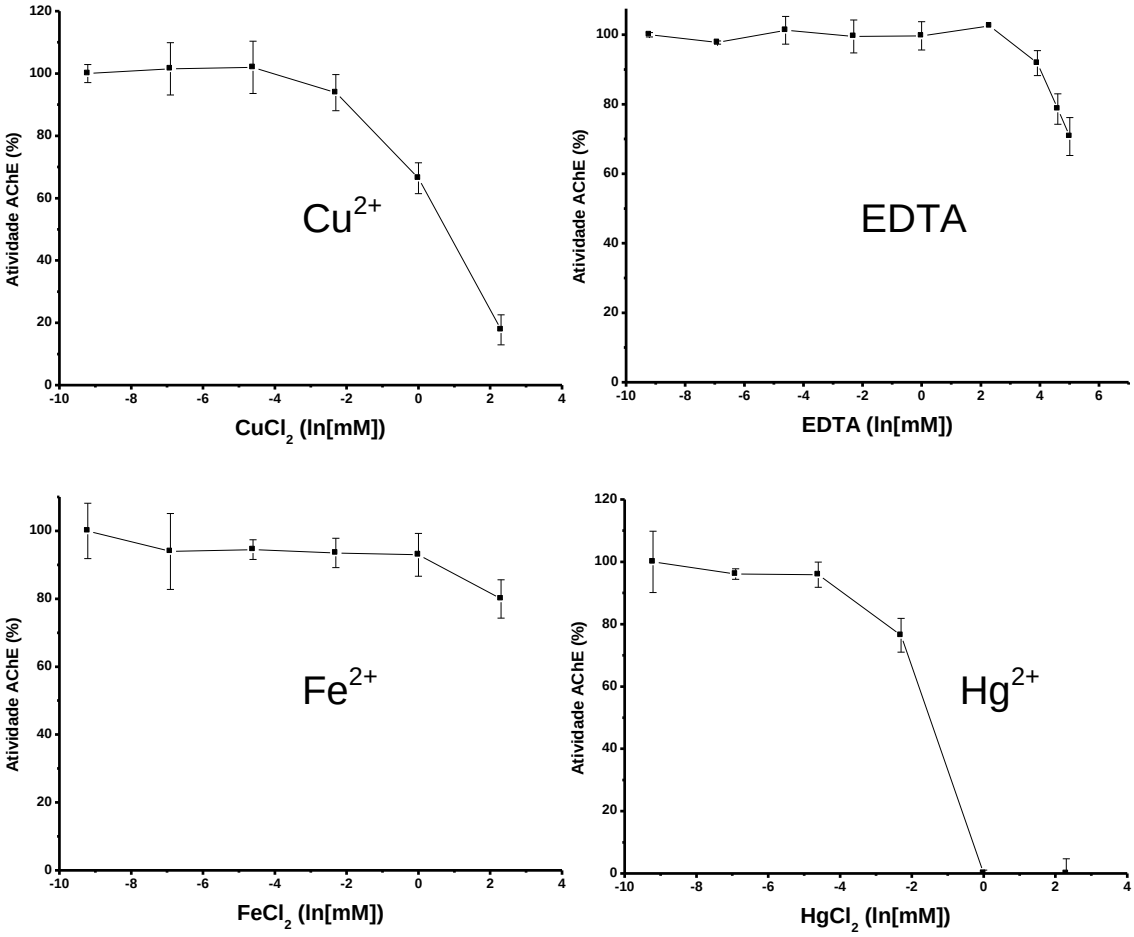
Vale, J.A., 1998. Toxicokinetic and toxicodynamic aspects of organophosphorus (OP) insecticide poisoning. Toxicol. Lett. 102-103, 649-652.

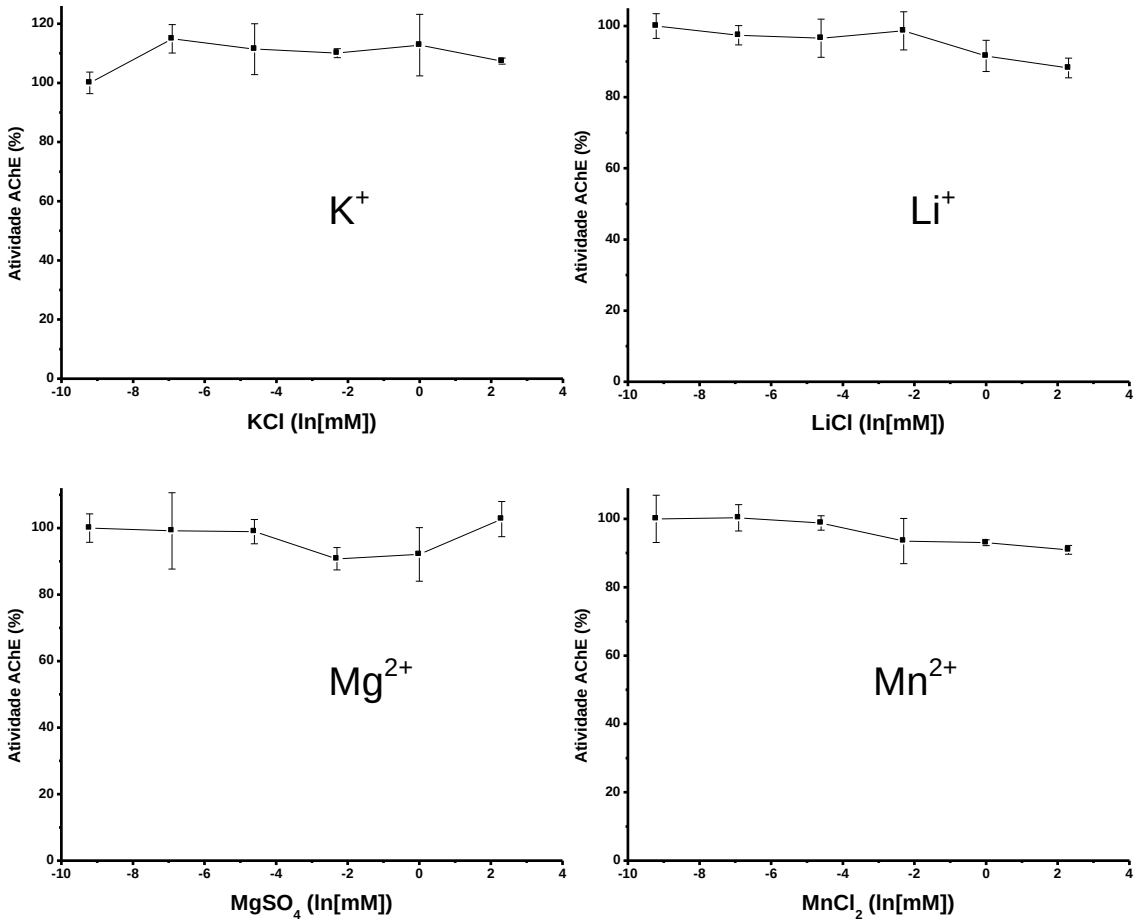
WHO. World Health Organization. 1986. Organophosphorus insecticides: a general introduction. Environmental Health Criteria 63, Geneva, CH.

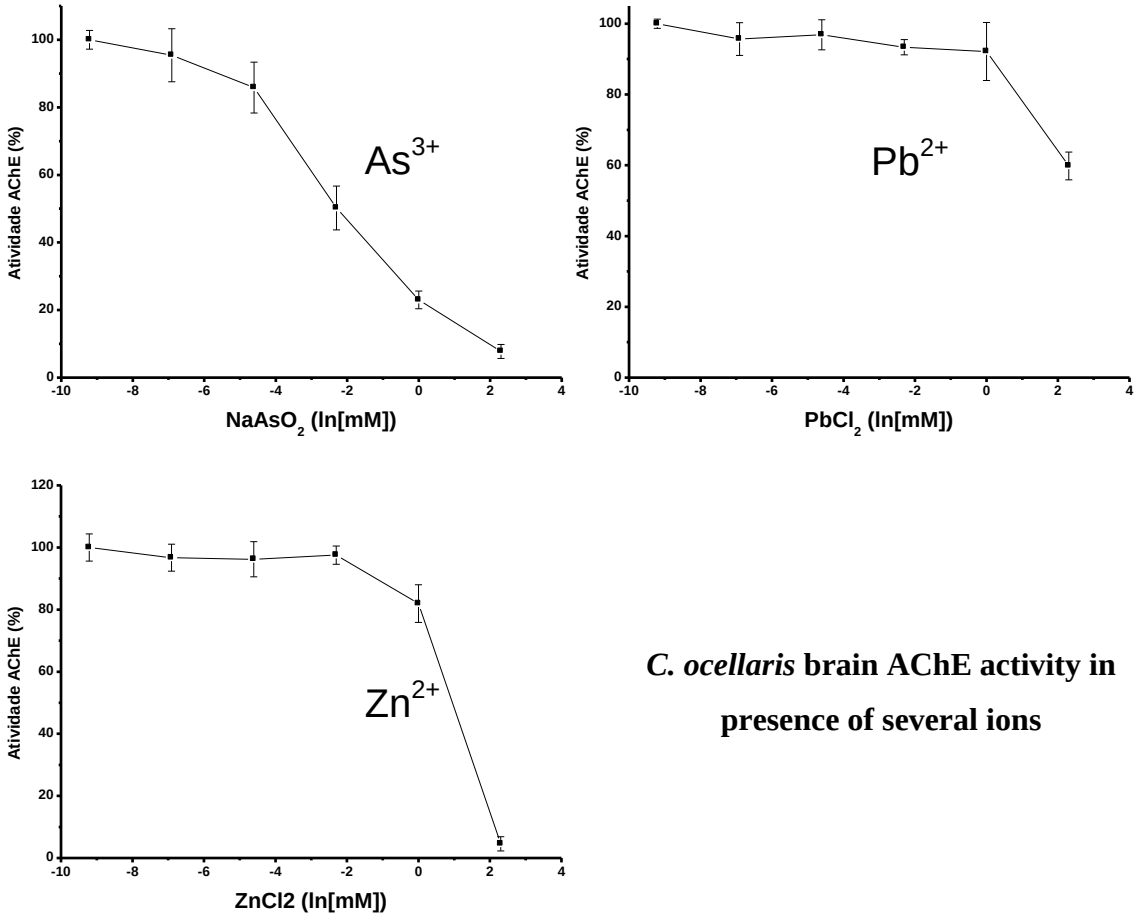
WHO/FAO. World Health Organization/Food and Agriculture Organization. 2004. Pesticide Residues in Food 2003: Evaluations For Food and Agriculture Organization of the United Nations. World Health Organization. JMPR 2003. Rome, IT.

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



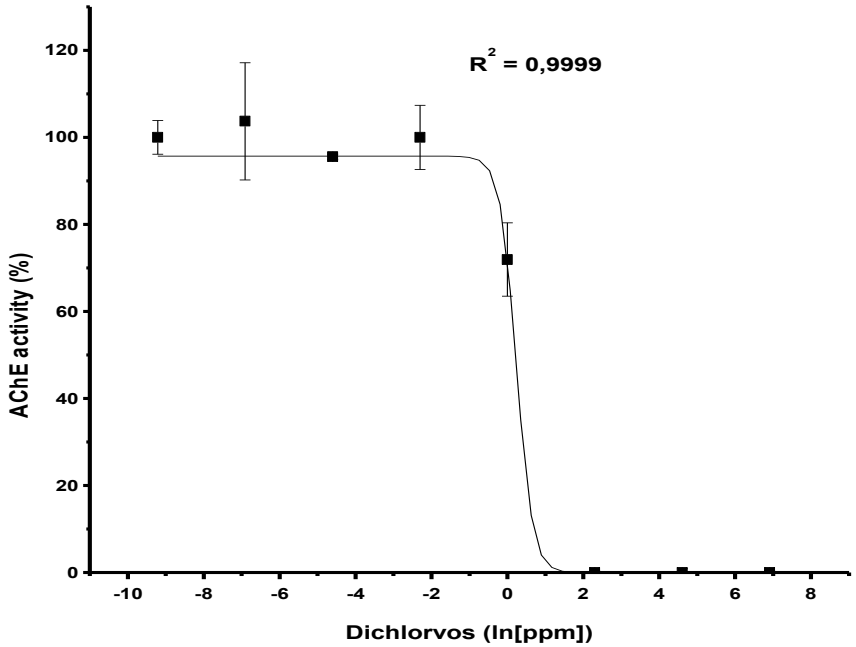

Atividade AChE (%)
Cu2+
CuCl2 (ln[mM])
EDTA
EDTA (ln[mM])
Fe2+
FeCl2 (ln[mM])
Hg2+
HgCl2 (ln[mM])


Atividade AChE (%)
K+
KCl (ln[mM])
Atividade AChE (%)
Li+
LiCl (ln[mM])
Atividade AChE (%)
Mg2+
MgSO4 (ln[mM])
Atividade AChE (%)
Mn2+
MnCl2 (ln[mM])

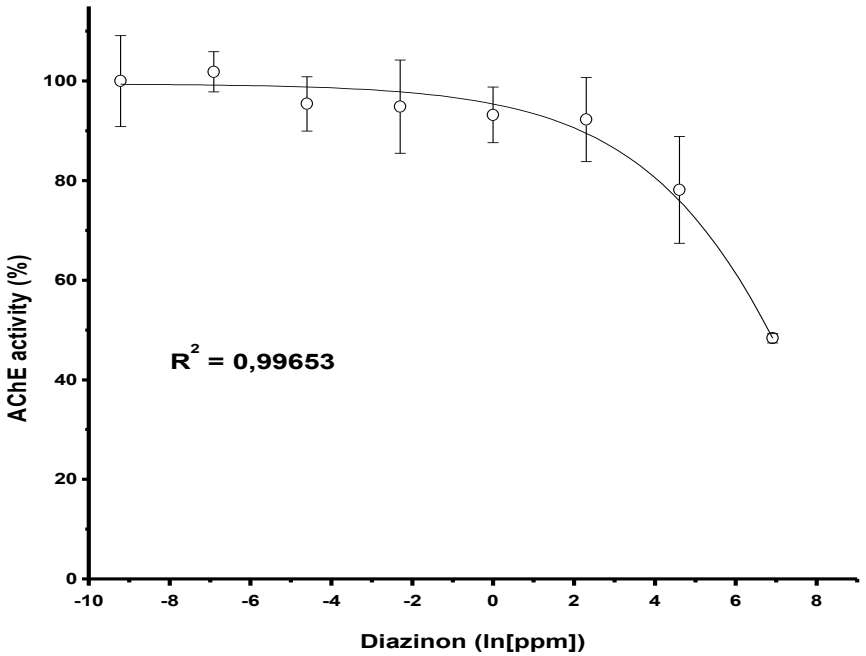


***C. ocellaris* brain AChE activity in presence of several ions**

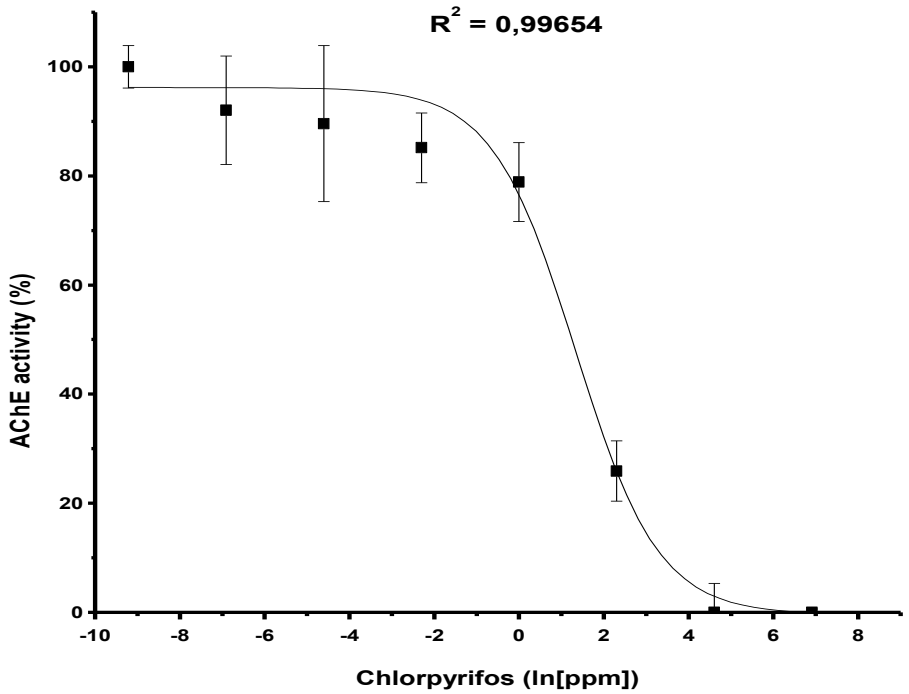
Supplementary Data - Campos *Silva al*., 2011



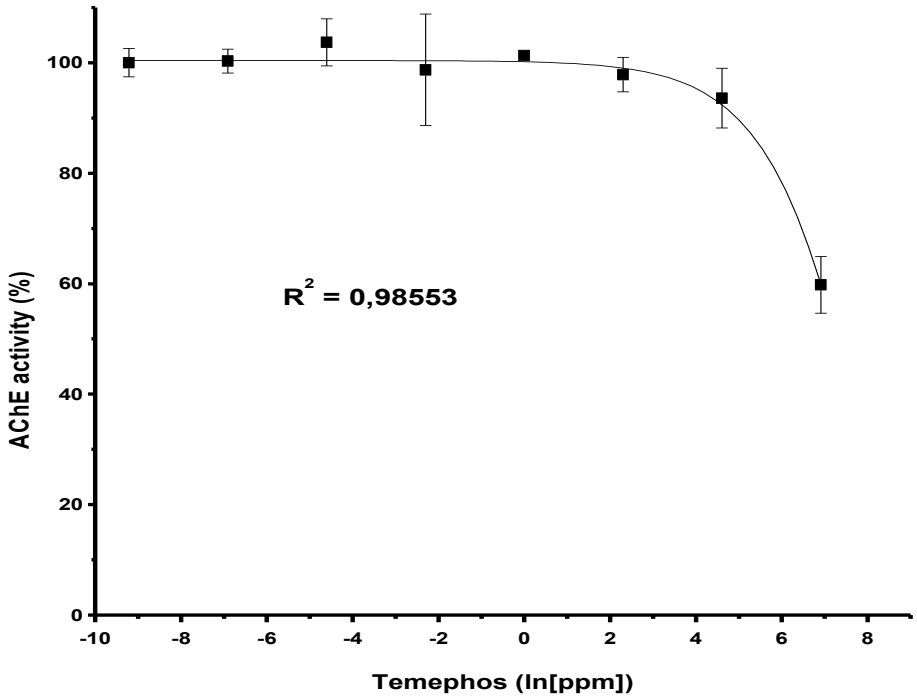
Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus dichlorvos.



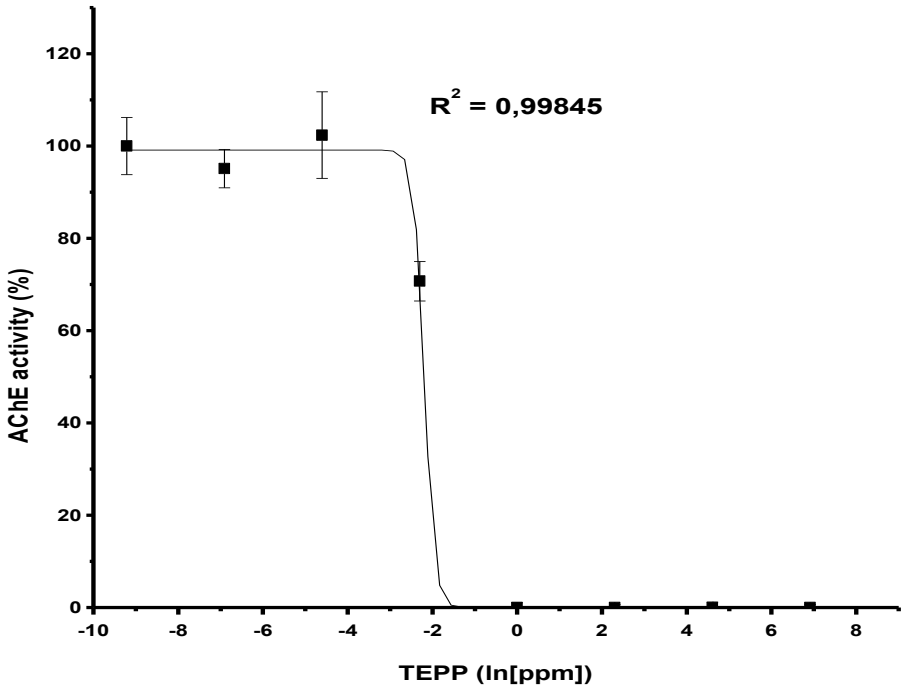
Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus diazinon.



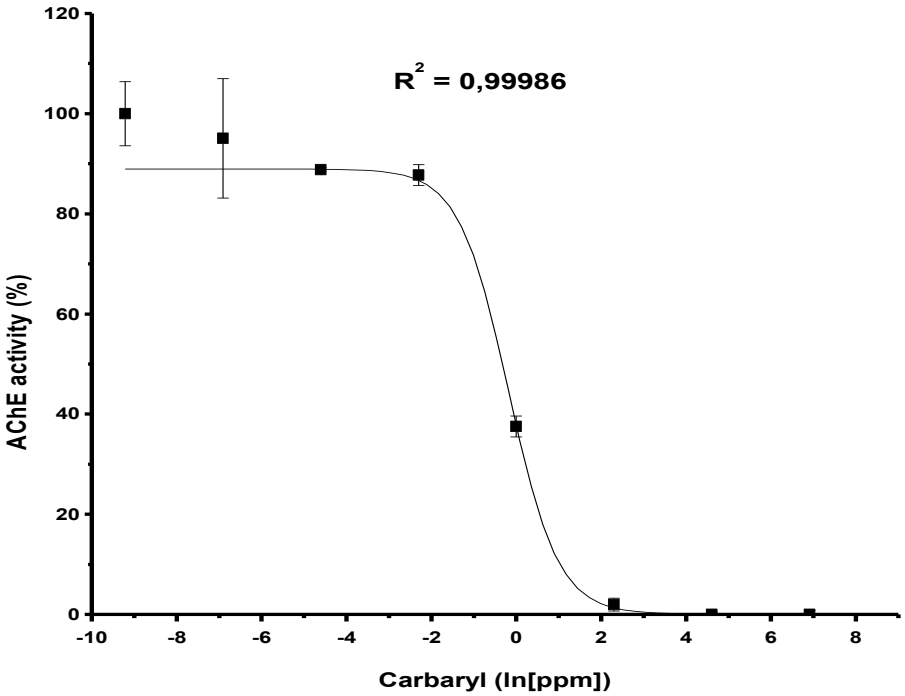
Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus chlorpyrifos.



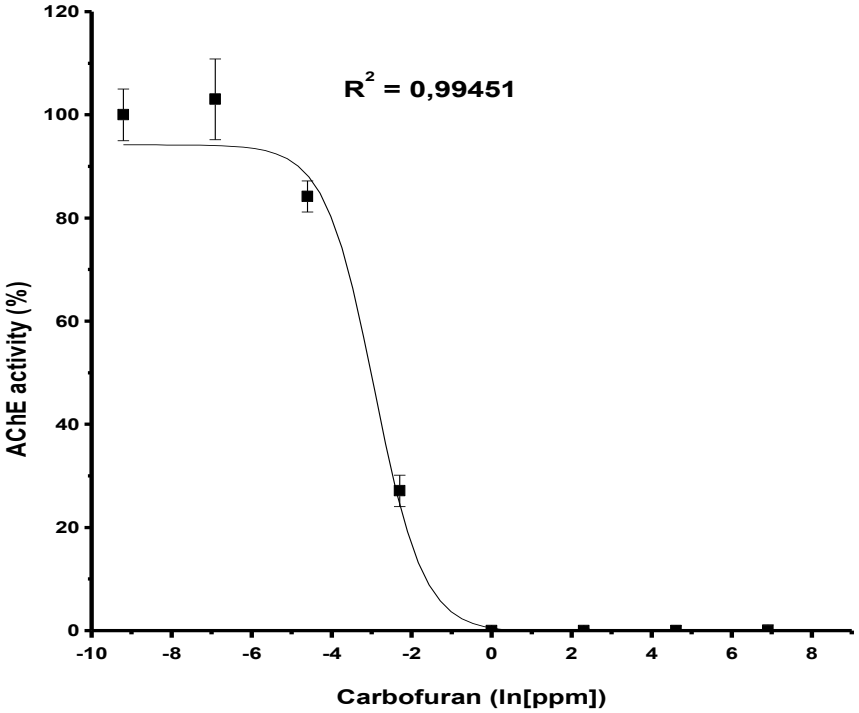
Activity of *C. ocellaris* brain AChE in presence of increasing concentrations (0-1000 mM) of the organophosphorus temephos.



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

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

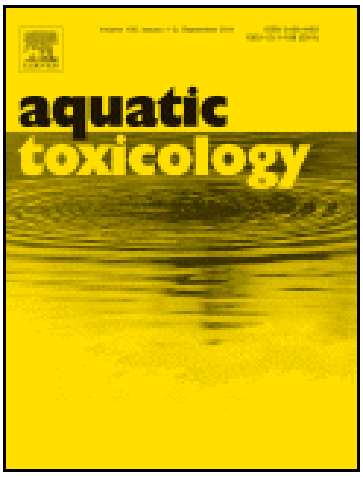
A atividade da acetilcolinesterase proveniente do cérebro de tucunaré Cichla ocellaris é extremamente sensível ao efeito do diclorvós, tetraetil-pirofosfato (TEPP), carbofuran e carbaril podendo assim ser empregada como um biomarcador de inseticidas organofosforado e carbamato.

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:



GUIDE FOR AUTHORS

.

INTRODUCTION

Types of paper

1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communications
4. Letters to the Editor

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.

Review Articles can be divided into three types:

• *Regular reviews* covering subjects falling within the scope of the journal which are of active current interest. These should generally not exceed 12 printed pages (approx. 6000 words).

• *Mini-reviews*. These will be short reviews or overviews (not exceeding 2-3 printed pages, approx. 1000-1500 words) on topics of above-average emerging interest.

• *Commentaries*. This label will be given to mini-reviews which clearly contain the personal opinions of the author concerned. All types of review articles will be solicited by the Reviews Editor, Prof. M.N. Moore, Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth, PL1 3DH, UK. E-mail: mnm@pml.ac.uk. *Short Communications* will be restricted to papers describing short, complete studies. They should not exceed 3 printed pages, including figures and tables (approx. 1500 words), and should be written in a continuous style, without subdivisions of introduction, materials and methods, results, discussion and acknowledgements; they should always begin with a summary. A short communication, although brief, should be a complete and final publication, and figures and tables from the communication should not occur in a later paper.

Letters to the Editor should either offer comment on a paper published in the journal, or comment on any general matter providing that this is relevant to the scope of the journal. In the case of letters commenting on published papers, the author(s) of the latter will be given the opportunity to react to the letter and the two items will subsequently be published together in the journal.

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/ethicalguidelines.

Policy and ethics

The work described in your article must have been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments* http://europa.eu.int/scadplus/leg/en/s23000.htm; *Uniform Requirements for manuscripts submitted to Biomedical journals* http://www.nejm.org/general/text/requirements/1.htm . This must be stated at an appropriate pointin the article.

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest.

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

AUTHOR INFORMATION PACK 4 Oct 2011 www.elsevier.com/locate/aqtox 4

Contributors

Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and/or article preparation, so roles for all authors should be described. The statement that all authors have approved the final article should be true and included in the disclosure.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright see http://www.elsevier.com/copyright). Acceptance of the agreement will ensure the widest possible dissemination of information. An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Retained author rights

As an author you (or your employer or institution) retain certain rights; for details you are referred to: http://www.elsevier.com/authorsrights.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated. Please see http://www.elsevier.com/funding.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit
http://www.elsevier.com/fundingbodies.

Open access

This journal offers you the option of making your article freely available to all via the ScienceDirect platform. To prevent any conflict of interest, you can only make this choice after receiving notification that your article has been accepted for publication. The fee of $3,000 excludes taxes and other potential author fees such as color charges. In some cases, institutions and funding bodies have entered into agreement with Elsevier to meet these fees on behalf of their authors. Details of these agreements are available at http://www.elsevier.com/fundingbodies. Authors of accepted articles, who wish to take advantage of this option, should complete and submit the order form (available at AUTHOR INFORMATION PACK 4 Oct 2011 www.elsevier.com/locate/aqtox 5 http://www.elsevier.com/locate/openaccessform.pdf). Whatever access option you choose, you retain many rights as an author, including the right to post a revised personal version of your article on your own website. More information can be found here: http://www.elsevier.com/authorsrights .

Language Services

Manuscripts should be written in English. Authors who are unsure of correct English usage should have their manuscript checked by someone proficient in the language. Manuscripts in which the English is difficult to understand may be returned to the author for revision before scientific review.
Authors who require information about language editing and copyediting services
pre- and post-submission please visit http://www.elsevier.com/languagepolishing or contact authorsupport@elsevier.com for more information. Please note Elsevier neither endorses nor takes responsibility for any products, goods or services offered by outside vendors through our services or in any advertising. For more information please refer to our Terms & Conditions: http://www.elsevier.com/termsandconditions.

Submission

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.
Please submit your article via http://ees.elsevier.com/aqtox/

Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of three potential referees. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

Page charges

Aquatic Toxicology has no page charges.

PREPARATION

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns.

The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your wordprocessor.

LaTeX

If the LaTeX file is suitable, proofs will be produced without rekeying the text. The article should preferably be written using Elsevier's document class 'elsarticle', or alternatively any of the other recognized classes and formats supported in Elsevier's electronic submissions system, for further information see http://www.elsevier.com/wps/find/authorsview.authors/latex-ees-supported.

The Elsevier 'elsarticle' LaTeX style file package (including detailed instructions for LaTeX preparation) can be obtained from the Quickguide: http://www.elsevier.com/latex. It consists of the file: elsarticle.cls, complete user documentation for the class file, bibliographic style files in various styles,and template files for a quick start.

Article structure

AUTHOR INFORMATION PACK 4 Oct 2011 www.elsevier.com/locate/aqtox 6

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required of no more than 400 words. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separate from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, they must be cited in full, without reference to the reference list. Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

AUTHOR INFORMATION PACK 4 Oct 2011 www.elsevier.com/locate/aqtox 7

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Save text in illustrations as 'graphics' or enclose the font.
• Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Produce images near to the desired size of the printed version.
• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as 'graphics'.
TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) the please supply 'as is'.

Please do not:

• Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication' Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source

Journal names should be abbreviated according to
Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;
List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;
CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions.

Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, highresolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
• E-mail address
• Full postal address
• Telephone and fax numbers All necessary files have been uploaded, and contain:
• Keywords
• All figure captions
• All tables (including title, description, footnotes) Further considerations
• Manuscript has been 'spell-checked' and 'grammar-checked'
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)

• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print
• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes.
For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

AUTHOR INFORMATION PACK 4 Oct 2011 www.elsevier.com/locate/aqtox 10

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. The correct format for citing a DOI is shown as follows (example taken from a document in the journal *Physics Letters B*): doi:10.1016/j.physletb.2010.09.059
When you use the DOI to create URL hyperlinks to documents on the web, the DOIs are guaranteed never to change.

Proofs

One set of page proofs (as PDF files) will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post) or, a link will be provided in the e-mail so that authors can download the files themselves. Elsevier now provides authors with
PDF proofs which can be annotated; for this you will need to download Adobe Reader version 7 (or higher) available free from http://get.adobe.com/reader. Instructions on how to annotate PDF files will accompany the proofs (also given online). The exact system requirements are given at the Adobe site: http://www.adobe.com/products/reader/tech-specs.html.
If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return them to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and return by fax, or scan the pages and e-mail, or by post. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately – please let us have all your corrections within 48 hours. It is important to ensure that all corrections are sent back to us in one communication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.

Offprints

The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail. The PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use. Additional reprints can be ordered on a reprint order form which will be sent to the corresponding author of the accepted article by the publisher.

Author's Discount

Contributors to Elsevier journals are entitled to a 30% discount on most Elsevier books, if ordered directly from Elsevier.

AUTHOR INQUIRIES

For inquiries relating to the submission of articles (including electronic submission) please visit this journal's homepage. Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher. You can track accepted articles at http://www.elsevier.com/trackarticle. You can also check our Author FAQs

(http://www.elsevier.com/authorFAQ) and/or contact Customer Support via http://support.elsevier.com.