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DISSERTAÇÃO DE MESTRADO

Caracterização da acetilcolinesterase cerebral do ciclídio jaguar (*Parachromis managuensis*) e seu potencial como biomarcador de pesticidas e íons metálicos

MARLYETE CHAGAS DE ARAÚJO

RECIFE
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Dissertação apresentada ao Programa **de Pós-Graduação em Ciências Biológicas,** da **Universidade Federal de Pernambuco,** como parte dos requisitos à obtenção do título de **Mestre em Ciências Biológicas**.

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A minha querida mãe Edileusa Araújo, pela dedicação, amor e exemplo de vida.

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“Porque para Deus nada é impossível.”

(Lucas 1: 37)

RESUMO

Acetilcolinesterase (AChE; 3.1.1.7) é uma enzima do grupo das serino-esterases que atua na hidrólise do neurotransmissor acetilcolina garantindo a intermitência dos impulsos nervosos responsáveis pela comunicação neuronal. A inibição deste mecanismo ocorre devido aos efeitos da exposição a pesticidas organofosforados e carbamatos, bem como a íons metálicos. A atividade da acetilcolinesterase de várias espécies de peixes tem sido utilizada como biomarcador em programas de monitoramento de recursos hídricos por ser um método diagnóstico informativo e efetivo, além de economicamente viável, visto que esta enzima está localizada e disponível de forma abundante num tecido descartado do peixe, o cérebro. Este trabalho objetivou caracterizar parcialmente parâmetros cinéticos e físico-químicos da acetilcolinesterase cerebral de *Parachromis managuensis* e investigar o efeito *in vitro* de pesticidas e íons metálicos sobre sua atividade. O pH ótimo e a temperatura ótima foram determinados ensaiando a atividade do extrato em pH de 4,0 a 9,0 e temperaturas de 25 a 80°C. A termoestabilidade foi determinada submetendo o extrato as mesmas temperaturas durante 30 min e, após o equilíbrio, foi mensurada a atividade remanescente. Parâmetros cinéticos; velocidade máxima (V_{max}) e constante de Michaelis - Menten (K_m) foram calculados em concentrações crescentes (0,8 a 20,8 mM) dos substratos iodeto de acetil e butirilcolina. O extrato foi incubado durante 60 min com um pesticidas organoforforado (diclorvós) e dois carbamatos (carbaril e carbofuran) e foi exposto durante 40 min a 10 íons (Al^{3+}, Ba^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Fe^{2+} e Zn^{2+}) em cinco concentrações de 0,001 a 10 mM. A atividade enzimática foi analisada após exposição aos inibidores seletivos BW284c51, Iso-OMPA, neostigmina e eserina nas mesmas concentrações. Estes inibidores confirmaram AChE e BChE como responsáveis pela atividade analisada. O pH ótimo e a tempertura ótima encontrados para a AChE foram 8,0 e 35°C respectivamente. A AChE reteve 70% da atividade após incubação a 40°C por 30 min. Os parâmetros cinéticos, V_{max} e K_m foram respectivamente 0,520 ± 08,98 mU/mg e 0,587 ± 0,95 mM para AChE; e 0,267 ± 5,2 mU/mg e 4,06 ± 0,82 mM para BChE. Todos os pesticidas usados mostraram efeito inibitório na atividade da AChE. Os valores da concentração inibitória média dos pesticidas (IC_{50}) foram: 1,68 μM (diclorvós), 4,35 μM (carbaril) e 0,28 μM (carbofuran). A maioria dos íons em 1 mM não mostraram efeito significativo ($p = 0.05$), enquanto Zn^{2+}, Cd^{2+}, $Cu^{2+,}$ $Hg^{2+,}$ inibiram a atividade da enzima em 17%, 25%, 53% e 100% respectivamente. AChE de *P. managuensis* mostrou potencial como biomarcador para todos os pesticidas usados, principalmente para o carbamato carbofuran, além do íon mercúrio (Hg^{2+}). Sendo, portanto, considerada uma ferramenta útil no monitoramento ambiental.

Palavras – chave: Inseticidas, Metais Pesados, Biomarcador, Meio Ambiente.

ABSTRACT

Acetylcholinesterase (AChE; 3.1.1.7) is an enzyme from the group of serine esterases acting in the hydrolysis of the neurotransmitter acetylcholine ensuring the burst of nerve impulses responsible for neuronal communication. The mechanism of this inhibition is due to the effects of exposure to organophosphorus and carbamate pesticides, as well as metal ions. The acetylcholinesterase activity of several species of fish has been used as a biomarker for monitoring water resources programs to be a method informative diagnosis and effective, and economically viable, since this enzyme is localized and available in abundance in a discarded tissue fish, the brain. This study aimed to partially characterize physicochemical and kinetic parameters of the brain acetylcholinesterase from Parachromis managuensis and investigate the in vitro effects of pesticides and metal ions on its activity analyzing its potential role as biomarker. Optimal pH and temperature were estimated exposing the enzyme extracts to a pH variation between 4.0 and 9.0 and a temperatures ranging from 25 to 80°C. The thermostability was determined submitting the extracts to the temperatures decribed above during 30 min and assaying the residual activity. Kinetic parameters such as maximum velocity (Vmax) and Michaelis-Menten constant (Km) were calculated using increasing concentrations (from 0.8 to 20.8 mM) of the substrates acetyl and butyrylcholine iodide. The extract was incubated during 60 min with one organophosphate (dichlorvos) and two carbamate (carbaryl and carbofuran) pesticides and was exposed for 40 min to 10 ions (Al3+, Ba2+, Cd2+, Cu2+, Hg2+, Mg2+, Mn2+, Pb2+, Fe2+ and Zn2+) in five concentrations from 0.001 to 10 mM. The enzymatic activity was analyzed after exposure to the selective inhibitors BW284c51, Iso-OMPA, neostigmine and eserine in the same concnetrations. These inhibitors confirmed AChE and BChE as responsible for the analyzed activity. Optimal pH and temperature found were 8.0 and 35°C, respectively. AChE retained 70% of its activity after incubation with 40°C for 30 min. The kinetic parameters Vmax and Km were, respectively, 520 ± 8.98 mU/mg and 0.587 ± 0.95 mM for AChE; and 267 ± 5.2 mU/mg and 4.06 ± 0.82 mM for BChE. All the used pesticides showed inhibitory effect on ChE acitivities. The mean inhibitory concentration (IC50) values were: 1.68 μM (dichlorvos); 4.35 μM (carbaryl) and 0.28 μM (carbofuran). Most of the analyzed ions did not show significant effect at 1 mM ($p = 0.05$), whereas Zn2+, Cd2+, Cu2+ and Hg2+ inhibited the enzyme activity in 17%, 25%, 53% and 100%, respectively at the same concentration. P. managuensis AChE showed potential as biomarker for all the pesticides under study, mainly for the carbamate carbofuran as well as for the mercury ion (Hg2+) and being, therefore, considered a promising tool for environmental monitoring.

Keywords: Pesticides, heavy metals, biomarker, Environment.

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LISTA DE ABREVIAÇÕES E SIGLAS

ChEs - Colinesterases

AChE - Acetilcolinesterase

BChE - Butirilcolinesterase

ACh - Acetilcolina

OPs - Organofosforados

CBs - Carbamatos

POPs - Poluentes orgânicos persistentes

IC_{50} – Concentração que inibi 50% da atividade enzimática

IC_{20} - Concentração que inibi 20% da atividade enzimática

K_i - Constante de inibição

K_m- Constante de Michaelis-Menten

V_{max} - Velocidade maxima de catálise atingida por uma enzima

DMSO - Dimetilsulfóxido

DTNB - Ácido 5,5’ Ditiobis (2-nitrobenzóico)

Iso-OMPA - Tetraisopropil pirofosforamida

Tris - Tris-hidróximetil-aminometano

OMS - Organização Mundial da Saúde

CONAMA - Conselho Nacional do Meio Ambiente

WHO - World Health Organization

USEPA - National Primary Drinking Water Standards

ROS – Reactive Oxygen Species

UFRPE - Universidade Federal Rural de Pernambuco

CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CNPq - Conselho Nacional de Pesquisa e Desenvolvimento Científico

SUMÁRIO

1. INTRODUÇÃO

Nas últimas décadas o uso intensivo de compostos tóxicos em atividades humanas tem sido um dos principais problemas responsáveis pela contaminação dos ecossistemas aquáticos. Esta prática tem provocado expressiva queda da qualidade da água, perda de biodiversidade aquática, além de alterações em diferentes níveis ecológicos (ARAÚJO e OLIVEIRA, 2013). Frente a essa problemática, grande atenção tem sido despendida com relação ao monitoramento das ações antropogênicas em recursos hídricos.

Atualmente, uma urgência maior tem sido dada ao desenvolvimento de técnicas diagnósticas e prognósticas que sejam economicamente viáveis, bem como informativas e efetivas. Nesse contexto, as substâncias conhecidas como biomarcadores surgem como uma alternativa, visto que são medidas de fluidos corporais, células ou tecidos que indicam, em termos bioquímicos, celulares, fisiológicos ou comportamentais, a presença de contaminantes no organismo (COIMBRA et al., 2013). Entre 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 biomarcadores baseia-se na interferência inibitória ou indutora, causada pelas substâncias-alvo, em sua atividade catalítica (ASSIS et al., 2011). As colinesterases (ChEs; EC 3.1.1.x) são enzimas amplamente utilizadas como biomarcadores da presença de alguns metais pesados e um grupo restrito de pesticidas (carbamatos e organofosforados). São enzimas hidrolases, do grupo das serino-esterases, com alta afinidade por ésteres de colina (ASSIS et al., 2014). Na literatura, distingue-se dois tipos de ChEs: 1) a acetilcolinesterase (AChE, EC 3.1.1.7), que tem como principal e clássica função, a modulação da transmissão dos impulsos nervosos responsáveis pela comunicação neuronal mediante a desativação do neurotransmissor acetilcolina e a 2) butirilcolinesterase (BChE, EC 3.1.1.8), também conhecida como pseudocolinesterase ou acilcolina hidrolase, de função ainda não totalmente elucidada (ASSIS et al., 2014). A AChE predomina nos eritrócitos, neurônios, gânglios do sistema nervoso autônomo e placas motoras terminais, a BChE, por sua vez, predomina no plasma, fígado, neuróglias, pâncreas e paredes do tubo digestivo (SILVA et al., 2013).

O estudo da atividade da acetilcolinesterase de várias espécies de peixes tem sido proposto para serem utilizados como biomarcadores em programas de monitoramento

ambiental (BOTTÉ et al., 2012; GHAZALA et al., 2014; WANG ET AL., 2015), uma vez que esta enzima está disponível de forma abundante num tecido descartado do peixe, o cérebro, órgão extremamente sensível capaz de fornecer rápida resposta biológica. Entretanto, essas enzimas são altamente polimórficas tanto interespecífica quanto intraespecíficamente com alta variabilidade de formas entre as diferentes espécies e diferentes tecidos, sendo necessário caracterizá-la em condições de exposição e normalidade (ASSIS et al., 2010).

A espécie escolhida para esse estudo foi o Jaguar, *Parachromis managuensis* (Teleostei: Actinopterygii), um ciclídeo originário da América Central, que foi recentemente introduzido no Brasil. A primeira referência sobre a sua ocorrência no país encontra-se em Barbosa e Leitão (2003). Incluído entre as principais espécies de grande importância para a pesca esportiva é um peixe que prefere água turva, eutrófica e com baixo teor de oxigênio (BARBOSA et al., 2006). Tem hábito alimentar carnívoro, ocupando o topo da cadeia alimentar no seu habitat, sendo assim submetido a bioacumulação. Pouco se conhece ainda da sua biologia e não há referências na literatura que reportem informações sobre seu comportamento bioquímico, o que sugere a necessidade destes estudos.

Este trabalho objetivou caracterizar parcialmente parâmetros físico-químicos e cinéticos da AChE do cérebro de *P. managuensis* e investigar o efeito *in vitro* de pesticidas e íons metálicos sobre sua atividade, a fim de verificar seu potencial como biomarcador dessas substâncias.

2. REVISÃO BIBLOGRÁFICA

2.1. Biomarcadores Enzimáticos

Nos últimos anos, o nível de contaminantes aumentou de forma alarmante nos ecossistemas aquáticos como resultado das atividades antropogênicas e desta forma a biota aquática se tornou uma ferramenta importante para a detecção do grau de impacto, uma vez que este meio está constantemente exposto a um grande número de substâncias tóxicas oriundas de diversas fontes de emissão (COSTA et al., 2008, GRUPTA et al., 2014). Estes organismos são fontes de biomoléculas biologicamente ativas que são úteis na determinação desses contaminantes. Entre essas biomoléculas, as enzimas destacam-se devido ao seu alto grau de especificidade e rapidez de respostas às alterações pertinentes aos organismos.

Estudos recentes demonstram grande interesse por biomarcadores enzimáticos como alternativa de uso no monitoramento de ambientes impactados (NIGAN et al., 2012; SILVA et al., 2013; GHAZALA et al., 2014, WANG et al., 2015). A utilização da atividade enzimática como biomarcador deve-se ao fato dos compostos tóxicos, que apresentam uma meia-vida relativamente longa, possuírem alta afinidade por pares de elétrons encontrados nos aminoácidos que formam as enzimas, como o grupamento sulfidril - SH (IVANINA et al. 2008).

O uso de enzimas como biomarcadores baseia-se na interferência negativa (inibitória) ou indutora, causada pelas substâncias química, em sua atividade catalítica (ASSIS et al., 2011). Dentre as principais enzimas utilizadas extensivamente para esta finalidade destacam-se as enzimas envolvidas na detoxificação de xenobióticos e de seus metabólitos, tais como as enzimas de biotransformação e de defesa antioxidante, catalase, glutationa redutase e superóxido dismutase, e as enzimas moduladoras tais como as colinesterases (COGO et al., 2009 ; EL-BASSYOUNI, et al., 2012; ASSIS et al. 2014).

Um bom exemplo da utilização de atividade enzimática como método de monitoramento pode ser observado no trabalho realizado por Ghazala et al (2014), no qual foram utilizados enzimas colinesterases como biomarcadores de efeitos subletais de pesticidas carbamatos e organofosforados em tecidos da espécie *Labeo rohita*. Neste estudo foi testado o efeito de três concentrações subletais de profenofos e carbofuran sobre a atividade da acetilcolinesterase (AChE) e butirilcolinesterase (BChE) no cérebro, guelras, músculo, rim, fígado e sangue dessa espécie de peixe. Como resultado foi observado que a exposição a ambos os tipos de pesticidas afetou as funções destes órgãos, incluindo metabolismo e neurotransmissão, em graus diferentes a depender da concentração de exposição, sugerindo que a espécie analisada é sensível a estes pesticidas e que os mesmos devem ser monitorados adequadamente no ambiente, para reduzir seus efeitos tóxicos sobre organismos não-alvo.

2.1.1. Colinesterases

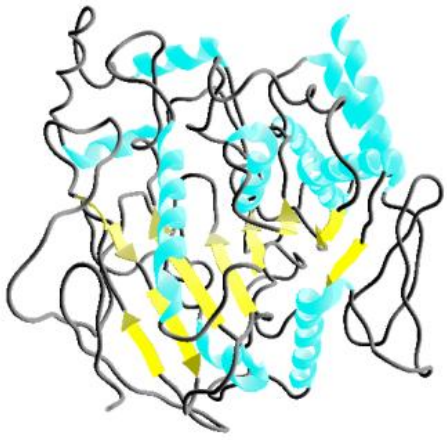
As colinesterases (ChEs; EC 3.1.1.x) são enzimas do grupo da superfamília das α/β hidrolases (NARDINI; DIJKSTRA, 1999). As α/β hidrolases têm uma habilidade de fornecer um arcabouço estável de sítios ativos de uma ampla variedade de enzimas, sendo que a tríade catalítica, altamente conservada, é formada por um nucleófilo composto por resíduos de serina, cisteína ou ácido aspártico, um resíduo ácido e um resíduo de histidina (NARDINI;

DIJKSTRA, 1999). São aceitos, na literatura, dois tipos de ChEs: 1) a acetilcolinesterase (AChE, EC 3.1.1.7), que tem como principal e clássica função, a modulação da transmissão dos impulsos nervosos responsáveis pela comunicação neuronal, mediante a desativação do neurotransmissor acetilcolina, e a 2) butirilcolinesterase (BChE, EC 3.1.1.8), de função não totalmente elucidada, apontada principalmente como uma enzima detoxificadora e eventual substituta da AChE (ASSIS et al., 2014). Ambas são inibidas por pesticidas das classes dos organofosforados e carbamatos e alguns metais pesados.

2.1.2. Acetilcolinesterase

A AChE ou acetil-hidrolase (Figura 1), é uma enzima regulatória responsável pela finalização da transmissão dos impulsos nervosos. Está presente nos sistemas nervosos central e periférico, promovendo a hidrolise da ACh nas junções neuromusculares e nas sinapses colinérgicas. É a enzima mais eficiente em hidrolisar ésteres de colina e sua eficiência catalítica bem como sua alta reatividade com vários inibidores covalentes e não covalentes são determinadas pela arquitetura funcional única do seu sítio ativo (MATOS, 2012).

Figura 1. Estrutura tridimensional da AChE.

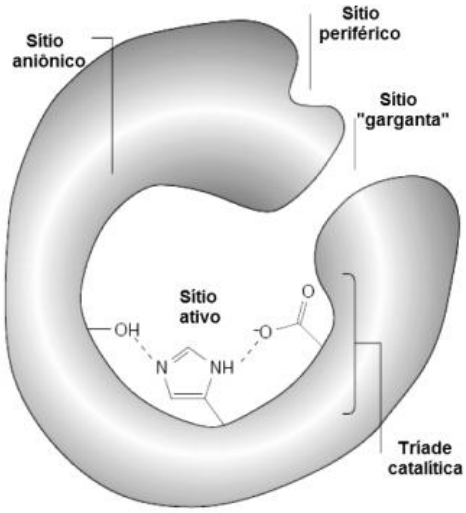


Fonte : Matos, 2012

Cada monômero da AChE contém um centro catalítico composto por dois compartimentos: o subsítio catalítico contendo a tríade catalítica e o subsítio aniônico que acomoda o compartimento quaternário positivo de ACh (GONÇALVES, 2009). Nessas regiões há quatro domínios (Figura 2): no primeiro encontram-se os resíduos de histidina e serina da tríade catalítica (Ser203, His447, e Glu334), a qual é encontrada no fundo da

"garganta" do sítio ativo; o segundo é o próprio subsítio aniônico, carregado negativamente, onde o grupo amônio quaternário da ACh interage, eletrostaticamente; o terceiro domínio é constituído por uma região hidrofóbica importante para a ligação com substratos cíclicos; e o quarto domínio, denominado sítio aniônico periférico no qual interagem ligantes catiônicos e alguns outros ligantes neutros (GONÇALVES, 2009).

Figura 2. Sítio ativo da Acetilcolinesterase.



Fonte : Matos, 2012.

Devido à sua função chave no controle da transmissão sináptica, esta enzima se torna um dos alvos moleculares mais vulneráveis à ação de agentes neurotóxicos, tais como íons metálicos e pesticidas.

2.2. Pesticidas organofosforados e carbamatos

São considerados pesticidas os produtos e os componentes de processos físicos, químicos ou biológicos destinados ao uso nos setores de produção, armazenamento e beneficiamento de produtos agrícolas, bem como em ambientes urbanos, cuja finalidade seja a preservação da ação danosa de seres vivos considerados nocivos (Lei Federal nº 7.802 de 11/07/89). De acordo com a espécie que se pretende eliminar, esses compostos são classificados como inseticidas, fungicidas, herbicidas, acaricidas, rodenticidas, moluscicidas, entre outros. De acordo com a toxicidade oral e dérmica são classificados em extremamente tóxicos (classe I a), altamente tóxicos (classe I b), moderadamente tóxicos (classe II) e

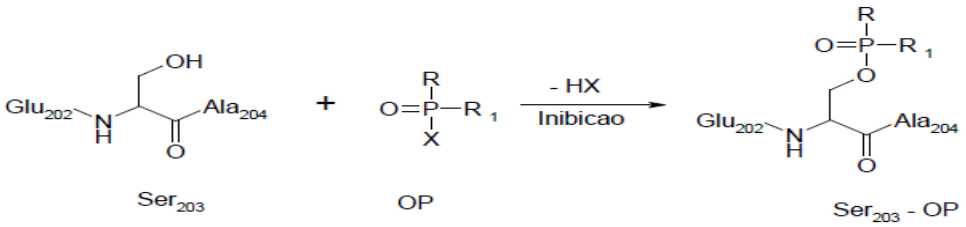
discretamente tóxicos (classe III). Conforme a classe química são agrupados em piretróides, organoclorados, organofosforados, carbamatos, entre outros (WHO/UNEP/ILO/IPCS, 2006).

Nas últimas décadas, os ecossistemas aquáticos receberam de forma alarmante grandes quantidades desses compostos, liberados pelas comunidades urbanas, propriedades rurais e indústrias. No início da década de 1960 a sociedade começou a se preocupar com os efeitos adversos dessas substâncias e o risco potencial que representam para a saúde humana e o meio ambiente, e, em vários países, a produção, comercialização e utilização de muitos desses compostos, em especial aqueles considerados poluentes orgânicos persistentes (POPs), tais como os organoclorados, foram proibidos (FRANÇA et al., 2010). Com a proibição da maioria dos compostos organoclorados (compostos menos tóxicos, porém com maior bioacumulação no meio ambiente), após a II guerra mundial, os pesticidas carbamatos e organofosforados tiveram seu uso intensificado. Atualmente estes são os pesticidas mais utilizados em todo mundo e juntos respondem por mais de 50% do que é comercializado (SILVA et al., 2013). São largamente utilizados nos países em desenvolvimento, de economia predominantemente agrícola.

Os pesticidas organofosforados (OPs) compreendem um elevado número de substâncias classificadas quimicamente como ésteres, amidas ou derivados de ácidos fosfóricos pentavalentes. Os carbamatos (CBs) são ésteres ou derivados N-substituídos do ácido carbâmico (monoamida do ácido carbâmico). Ambos apresentam baixa solubilidade em água e são, em geral, facilmente hidrolisáveis em ambientes alcalinos (GHAZALA et al., 2014). Em geral, os OPs necessitam de biotransformação para se tornarem toxicologicamente ativos, ao contrário dos CBs que já são bioativos. Esses pesticidas são inibidores típicos das enzimas colinesterases (NIGAN et al., 2011; WANG et al., 2015).

A inibição do pesticida a enzima se dá pela interação com o sítio esterásico da AChE, diferindo apenas no tipo de ligação – fosforilação para organofosforado e carbamilação para carbamatos (ASSIS et al., 2011). Os OPs se ligam covalentemente ao resíduo catalítico Ser203, impedindo a ligação do substrato (Figura 3).

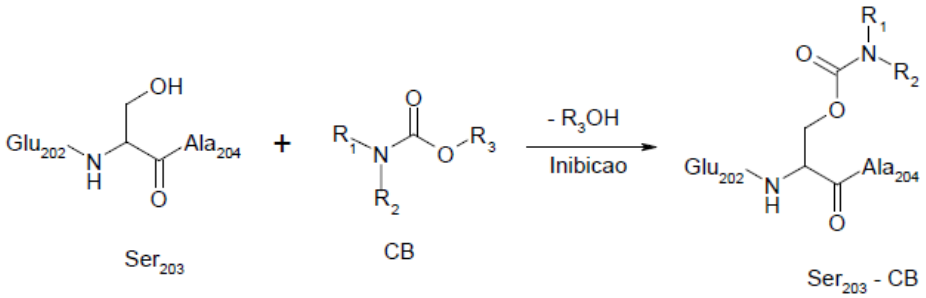
Figura 3. Inibição da AChE por organofosforado.



Fonte: Hörnberg et al., 2007.

Semelhante aos OPs, os CBs também se ligam ao sítio ativo da enzima, entretanto, o grupo hidroxil do resíduo de serina promove um ataque nucleofílico ao grupo carbonil do CB (Figura 4). Por este motivo, a inibição por carbamatos é reversível e a enzima pode rapidamente ser regenerada numa fração de minutos ou horas. Já a inibição por organofosforado tende a irreversibilidade, uma vez que a enzima é progressivamente fosforilada por uma ligação covalente, processo que normalmente leva 24 a 48hs, impedindo a enzima de ser regenerada (MATOS, 2012). A ação anticolinesterásica desses compostos não está restrita à AChE do tecido nervoso central e periférico. Ocorre de forma paralela a inibição da BChE plasmática e a AChE eritrocitária (ASSIS et al., 2010).

Figura 4. Inibição da AChE por carbamato.

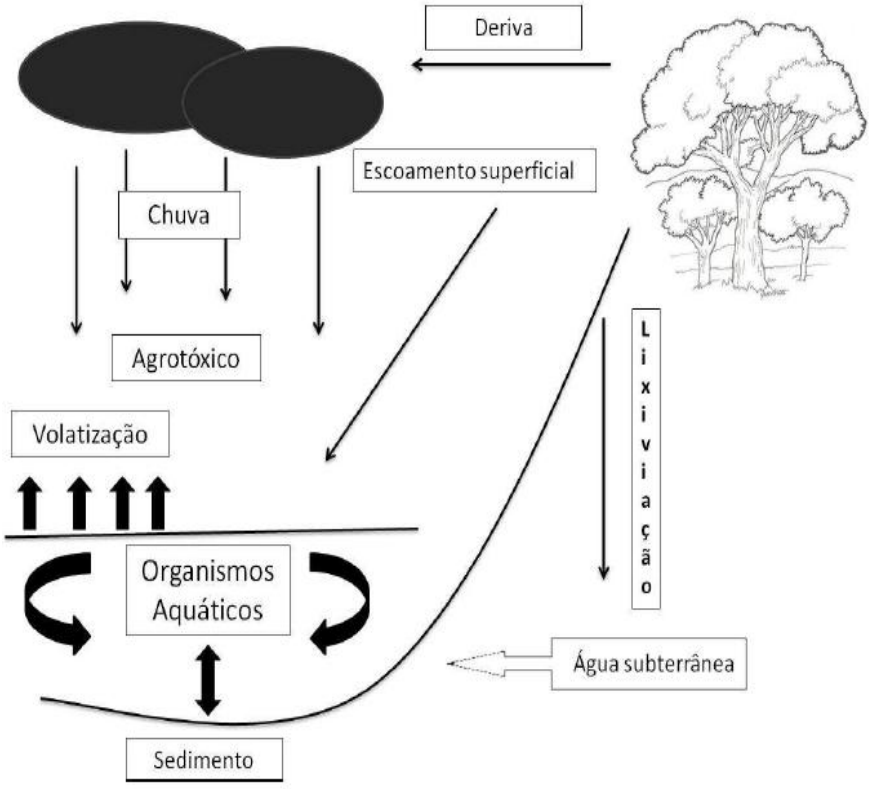


Fonte: Matos, 2012.

OPs e CBs podem atingir os ecossistemas aquáticos, e uma vez presentes neste ambiente tendem a ser absorvidos pelos organismos, que por sua vez tendem a sofrer bioacumulação. Essas substâncias são transportadas para esse meio carreadas por escoamento superficial e lixiviação das águas da chuva, irrigação e drenagem ou através de pulverizações (Figura 5), e podem penetrar nos organismos aquáticos através de diversas vias: por via oral –

através da ingestão de alimento contaminado, respiratória – por meio das brânquias e dérmica – através da superfície do corpo. A exposição a esses compostos pode provocar inúmeras alterações fisiológicas, por influência direta sobre determinadas estruturas celulares, como na membrana lisossomal, a qual pode ser degradada e provocar reações adversas no organismo, gerando grave desequilíbrio ecológico (COGO et al., 2009).

Figura 5. Principais rotas dos pesticidas em ecossistemas aquáticos.



Fonte: Tomita e Beyruth, 2002.

2.3. Metais pesados

Os metais pesados são compostos quimicamente reativos que são introduzidos nos ecossistemas aquáticos naturalmente através dos processos químicos e do intemperismo. Enquanto a contribuição atribuída à atividade humana é um reflexo do lançamento direto de grandes quantidades de efluentes industriais e urbanos sem tratamento adequado (COIMBRA et al., 2013). Uma vez lançados no ambiente esses compostos tendem a se distribuir nos diferentes compartimentos: água sedimento e biota. Essas substâncias não são biodegradáveis

e desta forma, tendem a se acumular, nesses compartimentos, onde manifestam sua toxicidade (NUNES et al., 2014). Por este motivo, os metais pesados são alvos de preocupação por parte dos programas de monitoramento e legislação ambiental (MANSOURI et al., 2012).

No Brasil, a resolução 357/2005 do CONAMA no Ministério do Meio Ambiente estabelece limites máximos desses metais tóxicos em corpos de água (Tabela 1). Entretanto, ainda não se exige a determinação das espécies químicas, relevante à biodisponibilidade produzida nas diferentes matrizes, nem sobre os efeitos subletais, avaliados pelos biomarcadores, destes metais nos organismos aquáticos. Por outro lado, a Resolução Conama 421/2010 relacionada ao material a ser dragado em águas jurisdicionais brasileiras dispõe sobre as análises de metais em material oriundo do sedimento (sedimentos totais, ou suas frações - elutriato, água intersticial, interface água-sedimento) e recomenda a realização de ensaios ecotoxicológicos como complementar as análises físico-químicas.

Tabela 1. Padrões de lançamento de metais pesados em corpos de água doce (classes 1 e 2)

Parâmetros inorgânicos – metais	**Valor máximo**
Alumínio	0,1 mg/L Al
Bário	0,7 mg/L Ba
Cádmio	0,001 mg/L Cd
Chumbo	0,01 mg/L Pb
Cobre	0,009 mg/L Cu
Ferro	0,3 mg/L Fe
Manganês	0,1 mg/L Mn
Mercúrio	0,0002mg/L Hg
Zinco	0,18 mg/L Zn

Fonte: Conselho Nacional do Meio Ambiente – CONAMA (357/2005)

Embora alguns metais pesados tais como ferro, cobre, zinco e cobalto sejam essenciais para a manutenção da vida, todos os metais são tóxicos em concentrações elevadas, pois causam estresse oxidativo pela formação de espécies reativas de oxigênio (ROS – *Reactive Oxygen Species*), tais como o íon superóxido (O^{2-}), peróxido de hidrogênio (H_2O_2), radical hidroxila (OH) e oxigênio livre (O^1) (KHAYATZADEH, 2010). Os radicais livres produzidos pela presença de compostos tóxicos no organismo reagem com lipídeos, proteínas ou ácidos nucléicos e resultam em diversas injúrias bioquímicas ou genéticas (COGO et al.,

2009). Diversos estudos têm informado sobre efeitos inibitórios e algumas vezes também efeitos ativadores de metais sobre atividades enzimáticas. A ligação dos metais a enzima ocorre através de ligações com grupamentos tióis proteicos, alterando o estado de hidratação do sítio ativo (MARQUES et al., 2011).

Silva et al., 2013 testaram, *in vitro*, as condições de ensaio da acetilcolinesterase cerebral de tucunaré (*Cichla ocellaris*) na presença de alguns metais e verificou que os íons Hg^{2+}, As^{3+}, Cu^{2+} e Zn^{2+} reduziram significativamente a atividade da enzima na concentração de 1 mM. Os autores observaram ainda que em concentração superior a 1 mM a enzima foi também inibida pelos íons $Cd^{2+,}$ Fe^{2+}, Li^{+} e Pb^{2+} , demonstrando portanto, sensibilidade a maioria dos íons estudados.

Ao contrário do que ocorre com a acetilcolinesterase, algumas enzimas na presença de metais pesados tem sua atividade aumentada, como por exemplo, as enzimas do estresse oxidativo, catalase, superóxido dismutase e glutationa-s-transferase, que utilizam esses compostos como seus substratos específicos. Este fato foi observado no trabalho de Atli e Canli (2007) através da exposição da catalase do fígado de *Oreochromis niloticus* a concentrações (5, 10 e 20 µM) de cádmio e chumbo.

2.4. Peixes: Bioindicadores aquáticos

Bioindicador é uma espécie ou grupo de espécies que indicam efeito de contaminação no seu habitat. Sua utilização permite a avaliação integrada dos efeitos ecológicos causados por múltiplas fontes de poluição (GODEFROID et al., 2014). São espécies escolhidas por sua sensibilidade ou tolerância a vários parâmetros toxicológicos. Além destas, outras características devem ser consideradas na escolha de um indicador biológico, ou seja, este deve ser taxonomicamente bem definido, ser abundante ou de fácil coleta, dispor de características ecológicas bem conhecidas e ter possibilidade de uso em laboratório (MACEDA et al., 2015).

Organismos pertencentes ao topo da cadeia alimentar são comumente utilizados como bioindicadores por possuírem intrínseca relação com toda a cadeia inferior, indicando respostas de efeitos crônicos, acumulativos e persistentes no nível de cadeia, além de efeitos diretos no nível do indivíduo, fornecendo ampla faixa de respostas frente a diferentes níveis de contaminação ambiental (LINS et al., 2010).

Atualmente é crescente o número de trabalhos nos quais peixes são empregados como bioindicadores da qualidade dos ecossistemas aquáticos, uma vez que são organismos bioacumuladores e muitos contaminantes, mesmo em baixas concentrações, podem afetar sua capacidade de sobrevivência (NIGAN et al., 2012; NUNES et al., 2014; WANG et al., 2015). As alterações observadas nestes organismos podem ser genéticas, bioquímicas, fisiológicas, morfológicas, ecológicas ou comportamentais (MANSOURI et al., 2012). Desta forma, a utilização de peixes como bioindicadores, pode ser instrumento de estimável utilidade na avaliação de efeitos, exposição e danos; no monitoramento da qualidade ambiental dos ecossistemas aquáticos.

A presença ou ausência de determinadas espécies no ambiente também é um indicador de alterações ambientais, como relatado no trabalho de Godefroid et al (2014) em que foram utilizados peixes como indicadores da qualidade de um rio urbano alterado. Para isto, foram capturados no total 1061 peixes, dos quais 1012 pertenciam a espécie *Phalloceros sp*., 37 a espécie *Corydoras eharharditi*, 11 a espécie *Rhamdia quelen* e 1 a espécie *Geophagus brasiliensis*. Neste trabalho foi avaliada a diversidade de peixes em três pontos distintos do rio Bacacheri, Curitiba – PR com diferentes graus de impacto e foi verificado que no ponto mais impactado (de acordo com análises físico-químicas) foi menor a diversidade de espécies. Este resultado foi atribuído ao efeito das ações antropogêncicas na região.

2.5. Jaguar (*Parachromis managuensis*)

O Jaguar, *Parachromis managuensis* (Teleostei: Actinopterygii) é um ciclídeo nativo da América Central, mas que foi introduzido para vários outros países adjacentes, tais como El Salvador, Guatemala, Panamá, México, Cuba, Porto Rico, Filipinas e mais recentemente, para o Brasil (BARBOSA e LEITÃO, 2003). É uma espécie de água doce que prefere água turva, eutrófica e com baixo teor de oxigênio. Habita lagos em clima tropical de preferência com pH da água entre 7,0 a 8,7 e temperatura de 25 a 36°C, e geralmente são encontrados em profundidades de 3 - 10 metros (AGASEN et al., 2006).

São animais bento - pelágicos que vivem e se alimentam tanto próximo ao fundo, bem como perto da superfície. Tem hábito alimentar carnívoro, ocupando o topo da cadeia alimentar no seu habitat, sendo portanto, submetido a bioacumulação. É uma espécie altamente predatória e oportunista, e sua dieta consiste principalmente de pequenos peixes e

macro invertebrados. Está incluído entre as principais espécies de grande importância para a pesca esportiva (BARBOSA et al., 2006).

Estes ciclídeos são peixes morfologicamente atraentes, com cores que variam do bronze ao ouro amarelado e possuem marcações pretas por todo o corpo que se estendem horizontalmente ao longo da área da linha lateral (Figura 6). Os machos tornam-se mais coloridos à medida que envelhecem e podem adquirir um comprimento de até 50 cm, enquanto as fêmeas permanecem um pouco menor em torno de 40 cm (AGASEN et al., 2006).

Figura 6. Jaguar (*Parachromis managuensis)*.

Este peixe tem ampla distribuição e comumente são encontrados em áreas impactadas (água morna, esgotada de oxigênio), podendo, portanto, ser utilizados com eficiência como indicadores de qualidade ambiental. Entretanto, pouco se conhece ainda da sua biologia e não há referências na literatura que reportem informações sobre seu comportamento bioquímico, bem como seu potencial como bioindicador de toxicidade, o que sugere a necessidade destes estudos.

3. REFERÊNCIAS

AGASEN, E.V.; CLEMENTE, J.P.; ROSANA, M.R.; KAWIT, N.S. 2006. Biological Investigation of Jaguar Guapote *Parachromis managuensis* (Gunther) in Taal Lake, Philippines. **Journal of Environmental Science and Management**.Vol. 9. No. 2. p. 20-30, 2006.

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. **Environmental Toxicology and Chemistry**, v. 29, p. 2243–2248, 2010.

ASSIS, C. R. D.; BEZERRA, R. S.; CARVALHO Jr, L. B. **Fish cholinesterases as biomarkers of organophosphorus and carbamate pesticides.** In: Pesticides in modern world, Book 5 - Pests control and pesticides exposure and toxicity assessment. Stoytcheva, M. (Ed). Intech, Rijeka, Croatia, 2011.

ASSIS, C.R.D., LINHARES, A.G., OLIVEIRA, V.M., FRANÇA, R.C.P., SANTOS, J.F., MARCUSCHI, M., CARVALHO, E.V.M., BEZERRA, R.S., CARVALHO JR, L.B., 2014. Characterization of catalytic efficiency parameters of brain cholinesterases in tropical fish. **Fish Physiol Biochem** DOI 10.1007/s10695-014-9956-1.

ARAÚJO , M.C. ; OLIVEIRA, M.B.M. Monitoring of water quality of a stream at the Federal University of Pernambuco, Brazil. **Rev. Ambient.Água**. Vol.8 no.3 Taubaté Sept./Dec. 2013**.** http://dx.doi.org/10.4136/ambi-agua.1192.

ATLI, G.; CANLI, M.(2007) Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. **Comparative Biochemistry and Physiology Part C** 145:282-287.

BRASIL. Ministério do Desenvolvimento Urbano e Meio Ambiente. Conselho Nacional do Meio Ambiente – CONAMA. Resolução nº 357, de 17 de março de 2005. Dispõe sobre a classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, bem como

estabelece as condições e padrões de lançamento de efluentes, e dá outras providências. **Diário Oficial [da] República Federativa do Brasil**, Brasília, DF, 2005.

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: 22 de Julho de 2015.

BARBOSA, J. M. & LEITÃO, S. S., 2003, ***Parachromis managuensis*: Um ciclídeo introduzido no Brasil**, pp.100-1. *In*: P. P. MENDES (ed.), Congresso Brasileiro de Engenharia de Pesca, 13, *Anais*...Porto Seguro, CDROM.

BARBOSA, J.M.; MENDONÇA, I.T.L.; PONZI JÚNIOR, M.; Comportamento social e crescimento em *parachromis managuensis* (Günter, 1867) (pisces, cichlidae): uma espécie introduzida no Brasil. **Rev. Bras. Eng. Pesca** 1(1), ago. 2006.

BOTTÉ, E.S., JERRY, D.R., CODI KING, S. SMITH – KEUNE, C., NEGRI, A.P., 2012. Effects of chlorpyrifos on cholinesterase activity and stress markers in the tropical reef fish *Acanthochromis polyacanthus*. **Mar. Pollut. Bull**. 65 (4-9), 384-393.

COGO AJD, SIQUEIRA AF, RAMOS AC, CRUZ ZMA & SILVA AG (2009) Utilização de enzimas do estresse oxidativo como biomarcadoras de impactos ambientais. **Natureza on line** 7 (1): 37-42. [on line] http://www.naturezaonline.com.br

COIMBRA, R.S.C.; SANTOS, C.R.S.; SARAIVA, V.B.; OLIVEIRA, M.M. **Biomarcadores como ferramentas na avaliação da qualidade do pescado contaminado com metais traço.** Boletim do Observatório Ambiental Alberto Ribeiro Lamego, Campos dos Goytacazes/RJ, v. 7 n. 1, p. 153-172, jan. / jun. 2013.

COSTA, C. R.; OLIVI, P.; BOTTA, C. M. R.; ESPINDOLA, E. I. G. A toxicidade em ambientes aquáticos: discussão e métodos de avaliação. **Química Nova**, v. 31, n. 7, p. 1820-1830, 2008.

EL-BASSYOUNI, H. T., ABDEL MAKSOUD, S. A., SALEM, F. A., BADR EL-DEEN, REEM, ABDEL AZIZ, H., THOMAS, M. M. (2012). Evidence of oxidative stress in peroxisomal disorders. **Singapore Med** J 53(9): 608-614.

FRANÇA, T. C. C.; SILVA, G. R.; CASTRO, A. T. de. Defesa química: uma nova disciplina no ensino de química. **Revista Virtual de Química**, Niterói, v. 2, n. 2, p. 84-104, out. 2010.

GHAZALA.; MAHBOOB, S.; AHMAD, L.; SULTANA, S.; ALGHANIM, K.; AL-MISNED, F.; AHMAD, Z. 2014. Fish Cholinesterases as Biomarkers of Sublethal Effects of Organophosphorus and Carbamates in Tissues of *Labeo Rohita*. **J Biochem Molecular Toxicology** DOI 10.1002/jbt.

GODEFROID, R.S.; FONSECA, A.S.; SILVA, R.C. Utilização dos peixes do rio bacacheri como indicadores da qualidade ambientaL. **Revista Meio Ambiente e Sustentabilidade** | Ed. Especial, vol. 7, n. 3, p. 675 - 690 | jul – dez 2014.

GONÇALVES, A. S. **Estudo da reativação da acetilcolinesterase humana inibida pelo organofosforado tabun através de métodos híbridos clássicos quanto-mecânicos**. 2009. 207 p. Tese (Doutorado em Biofísica) – Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2009.

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.

HÖRNBERG, A.; TUNEMALM, A.; EKSTRÖM, F. Crystal structures of acetylcholinesterase in complex with organophosphorus compounds suggest that the acyl pocket modulates the aging reaction by precluding the formation of the trigonal bipyramidal transition state. **Biochemistry**, New York, v. 46, n. 16, p. 4815-4825, Feb. 2007.

IVANINA AV, HABINCK E & SOKOLOVA IM (2008) Differential sensitivity to cadmium of key mitochondrial enzymes in the eastern oyster, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). **Comparative Biochemistry and Physiology Part C** 148:72-79.

LINS, J.A.P.N.; KIRSCHNIK. P.G.; QUEIROZ, V.S.; CIRIO, S.M. Uso de peixes como bioindicadores para monitoramento ambiental aquático. **Rev. Acad., Ciênc. Agrár. Ambient**., Curitiba, v. 8, n. 4, p. 469-484, out./dez. 2010.

MACEDA, E.B.; GRISOLIA, A.B.; VAINI, J.O. ; CANDIDO, L.S. Uso de biomarcadores para monitoramento das águas do Córrego Arara no município de Rio Brilhante, MS, Brasil. **Rev. Ambient. Água** vol. 10 n. 1 Taubaté – Jan. / Mar. 2015.

MARQUES SM, ANTUNES SC, NUNES B, GONÇALVES F, PEREIRA R., 2011. Antioxidant response and metal accumulation in tissues of Iberian green frogs (Pelophylax perezi) inhabiting a deactivated uranium mine. **Ecotoxicology** 20:1315–1327.

MATOS, K.S. **Aspectos moleculares da reativação da acetilcolinesterase inibida por ciclosarin e carbofurano / Karina Silvia Matos. – Lavras**: UFLA, 2012. 148 p. : il. Dissertação (mestrado) – Universidade Federal de Lavras, 2012.

MANSOURI, B.; EBRAHIMPOUR, M.; BABAEI, H. Bioaccumulation and elimination of nickel in the organs of black fish (Capoeta fusca). **Toxicology and Industrial Health**, v. 28, n. 4, p. 361-368, 2012. http://dx.doi.org/10.1177/0748233711412425.

NARDINI, M.; DIJKSTRA, B. W. α/β hydrolase fold enzymes: the family keeps growing. **Current Opinion in Structural Biology**, London, v. 9, n. 6, p. 732- 737, June 1999.

NUNES, B., 2011. **The use of cholinesterases in ecotoxicology**. Reviews of Environmental Contamination and toxicology. Springer, New York. PP. 29-59, vol.212.

NUNES, B., BARBOSA, A.R., ANTUNES, S.C., GONÇALVEZ, F., 2014. Combination effects of anticholinesterasics in acetylcholinesterase of a fish species: effects of a metallic compound, an organophosphate pesticide, and a pharmaceutical drug. **Environ Sci Pollut Res** (2014) 21:6258–6262.

NIGAM, A.K., SRIVASTAVA, N., RAI, A.K., KUMARI, U., MITTAL, A.K., MITTAL, S., 2012. The fist evidence of cholinesterase in skin muçus of carps and its applicability as biomarker of organophosphate exposure. **Environmental Toxicology**. Doi 10.1002/tox.

RODRIGUES SR, CALDEIRA C, CASTRO BB, GONÇALVES F, NUNES B, ANTUNES SC., 2011. Cholinesterase (ChE) inhibition in pumpkinseed (*Lepomis gibbosus*) as environmental biomarker: ChE characterization and potential neurotoxic effects of xenobiotics. P**estic Biochem Physiol** 99:181–188.

SILVA, K.C.C.; ASSIS, C. R. D.; OLIVEIRA, V. M.; CARVALHO JR, L. B.; BEZERRA, R. S. Kinetic and physicochemical properties of brain acetylcholinesterase from the peacock bass (Cichla ocellaris) and in vitro effect of pesticides and metal ions. **Aquatic Toxicology**, Amsterdã, v. 126, p. 191-197, jan. 2013.

TOMITA, R.Y.; BEYRUTH, Z. Toxicologia de agrotóxico em ambiente aquático**. O Biológico**, v. 64, n. 2, p.135-142, 2002.

WANG. Y., CHEN, C., ZHAO, X., WANG. Q., QIAN. Y., 2015. Assessing joint toxicity of four organophosphate and carbamate insecticides in common carp (*Cyprinus carpio*) using acetylcholinesterase activity as an endpoint. **Pesticide Biochemistry and Physiology** 122 (2015) 81–85.

4. OBJETIVOS

4.1. Geral

- ✓ Caracterizar físico-química e cineticamente a acetilcolinesterase da espécie *Parachromis managuensis* e investigar o efeito de pesticidas e íons metálicos sobre sua atividade.

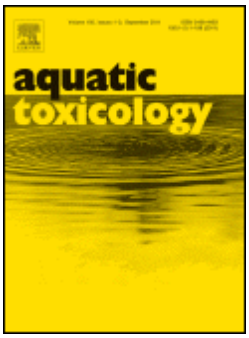
4.2. Específicos

- ✓ Definir as propriedades físico-químicas da acetilcolinesterase presente no cérebro de *P. managuensis*.
- ✓ Definir a velocidade máxima (V_{max}) e constante de Michaelis - Menten (K_m) das ChEs de *P. managuensis.*
- ✓ Analisar o efeito de pesticidas (organofosforado e carbamato) sobre a atividade da AChE do Jaguar.
- ✓ Analisar o efeito de íons metálicos sobre a atividade da AChE.
- ✓ Calcular a constante de inibição (K_i), o IC_{20}, ou seja, concentração que inibe 20% da atividade enzimática e a concentração inibitória média (IC_{50}) dos pesticidas e íons que apresentarem inibição significativa.

5. ARTIGO CIENTÍFICO

Brain acetylcholinesterase of jaguar cichlid (*Parachromis managuensis*): from physicochemical and kinetic properties to its potential as biomarker of pesticides and metal ions

Este artigo será submetido à revista internacional Aquatic Toxicology



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Brain acetylcholinesterase of jaguar cichlid (*Parachromis managuensis*): from physicochemical and kinetic properties to its potential as biomarker of pesticides and metal ions

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ABSTRACT

This contribution aimed to partially characterize physicochemical and kinetic parameters of the brain acetylcholinesterase from *Parachromis managuensis* and investigate the *in vitro* effects of pesticides and metal ions on its activity analyzing its potential role as biomarker. Optimal pH and temperature were estimated exposing the enzyme extracts to a pH variation between 4.0 and 9.0 and a temperatures ranging from 25 to 80°C. The thermostability was determined submitting the extracts to the temperatures decribed above during 30 min and assaying the residual activity. Kinetic parameters such as maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) were calculated using increasing concentrations (from 0.8 to 20.8 mM) of the substrates acetyl and butyrylcholine iodide. The extract was incubated during 60 min with one organophosphate (dichlorvos) and two carbamate (carbaryl and carbofuran) pesticides and was exposed for 40 min to 10 ions (Al^{3+}, Ba^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Fe^{2+} and Zn^{2+}) in five concentrations from 0.001 to 10 mM. The enzymatic activity was analyzed after exposure to the selective inhibitors BW284c51, Iso-OMPA, neostigmine and eserine in the same concnetrations. These inhibitors confirmed AChE and BChE as responsible for the analyzed activity. Optimal pH and temperature found were 8.0 and 35°C, respectively. AChE retained 70% of its activity after incubation with 40°C for 30 min. The kinetic parameters V_{max} and K_m were, respectively, 520 ± 8.98 mU/mg and 0.587 ± 0.95 mM for AChE; and 267 ± 5.2 mU/mg and 4.06 ± 0.82 mM for BChE. All the used pesticides showed inhibitory effect on ChE acitivities. The mean inhibitory concentration (IC_{50}) values were: 1.68 μM (dichlorvos); 4.35 μM (carbaryl) and 0.28 μM (carbofuran). Most of the analyzed ions did not show significant effect at 1 mM ($p = 0.05$), whereas Zn^{2+}, Cd^{2+}, Cu^{2+} and Hg^{2+} inhibited the enzyme activity in 17%, 25%, 53% and 100%, respectively at the same concentration. *P. managuensis* AChE showed potential as biomarker for all the pesticides under study, mainly for the carbamate carbofuran as well as for the mercury ion (Hg^{2+}) and being, therefore, considered a promising tool for environmental monitoring. The present data about AChE activity in *P. managuensis* are important to shed more light on possible further applications of AChE from this species.

keywords: pesticides, heavy metals, biomarker, *Parachromis managuensis*, environment.

1. INTRODUCTION

Acetylcholinesterase (AChE; 3.1.1.7) is an enzyme from the group of serine esterases that acts on the hydrolysis of the neurotransmitter acetylcholine in the cholinergic synapses, ensuring the intermittence of nerve impulse responsible for neuronal communication (Nunes, 2011; Assis et al., 2014). The inhibition of such mechanism by exposure to organophosphorus and carbamate pesticides as well as by metal ions results in the accumulation of acetylcholine in the synaptic cleft causing cholinergic hyperstimulation (Valbonesi et al., 2011; Nunes et al., 2014). The inhibition of the enzyme activity by pesticides occurs by the interaction with the esteratic subsite of AChE, differing only in the type of binding – phosphorylation for organophosphates and carbamoylation for carbamate pesticides, avoiding the binding of substrate (WHO, 1986a; 1986b ; Quinn, 1987; Tõugu, 2001; Assis et al., 2011). The binding to metals occurs by interaction with peripheral anionic sites and thiol groups possibly affecting, reversibly or not, the hydration state of the active site. (Tomlinson et al., 1980; Olson and Christensen, 1980; Hughes and Bennet, 1985; Marques and Yamanaka, 2008; Marques et al., 2011).

Pesticides and metal ions are widely used in domestic environment, agriculture and industry. Studies pointed that only 0.1% of the applied pesticides reach the target pests. The rest spreads through the environment (Hart and Pimentel, 2002). They can reach aquatic ecosystems and groundwater transported by run-off, leaching of rain water, irrigation, drainage and can be absorbed by living organisms tending to bioaccumulation (Rodrigues et al., 2011). Monitor and control the presence of these compounds in the environment is of great importance since they became a risk to human and environmental health (Assis et al., 2010). Moreover, the previous monitoring allows the identification of contaminations before higher levels of biological organization are affected (Jonsson e Aoyama, 2010). Therefore, compounds known as biomarkers represent a tool capable of determining the magnitude of the effects of such contamination since they are measurements of body fluids, cells or tissues that indicate, in biochemical, cellular, physiological or behavioral terms the presence of contaminants in the target organism (Coimbra et al., 2013).

The activity of AChEs from several species of fish has been proposed to be used as biomarkers in monitoring programs of water resources (Botté et al., 2012; Ghazala et al., 2014; Wang et al., 2015), for providing an effective diagnostic method and being viable since these enzymes are located in a commonly discarded part of fish (Silva et al., 2013). However,

AChEs are highly polymorphics both inter and intra-specificaly with differences depending on the analyzed tissues. Therefore, it is important to characterize them in normal conditions and perform exposure tests (Howcroft et al., 2011; Nigam et al., 2012).

Parachromis managuensis is a cichlid from Central America, recently introduced in Brazil. The first reference to the occurrence of this species in the country is found in Barbosa and Leitão (2003). This species thrives in euthrophic and cloudy waters with low levels of oxygen. They present carnivorous eating habits, generally occupying the top of the food chain in their habitat and therefore, being submitted to bioaccumulation. Little is known about their biology and there are no references in literature reporting biochemical features, suggesting the need for such studies. This contribution aimed to partially characterize physicochemical and kinetic parameters of brain AChE of *P. managuensis* and investigate the *in vitro* effect of pesticides and metal ions on its activity and investigating its potential as biomarker of these substances.

2. MATERIAL AND METHODS

2.1. Specimens obtention and enzyme extraction

Seventeen juvenile specimens (15.7 ± 3.9 cm; 62.4 ± 4.7 g) were collected from the Aquaculture Station Johei Koike in the Universidade Federal Rural de Pernambuco – UFRPE (Recife, PE, Brazil). The brains were excised and homogenized in 0.5 M Tris-HCl pH 8.0, reaching 20 mg of tissue per ml of buffer using a tissue disrupter (IKA RW-20, Staufen, Germany). The homogenate was centrifuged for 10 min at 1,000 x *g* (4°C) and the supernatant (crude extract) was stored at -20°C for further assays.

2.2. Enzyme activity and protein determination

Twenty microliters of crude extract were added to 200 µl of 0.25 mM 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) prepared in 0.5 M Tris-HCl pH 7.4 and the reaction was started by the addition of 20 µL of the substrate 62 mM acetylthiocholine iodide, according to Silva et al (2013). The assays were performed in quadruplicate and the enzyme activity were determined following the increase in absorbance at 405 nm for 180 s using an microplate spectrophotometer (Bio-Rad xMark™; Hercules, CA, EUA). A unit of activity (U) was

defined as the amount of enzyme capable of hydrolyzing 1 µmol of substrate per minute. The protein concentration was determined according to Sedmak e Grossberg (1977) using bovine serum albumin as standard.

2.3. Kinetic parameters

The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were estimated performing the activity determination assay with increasing concentrations of the substrates acetyl or *S*-butyrylthiocholine (0.80 – 20.8 mM final concentration). The activities were fitted to a non-linear regression (hyperbola model), using the software MicroCal™ Origin® Version 8.0 (MicroCal, Northampton, MA, EUA). In order to estimate which type of ChE is predominant in the brain of *P. managuensis* since AChE and BChE can hydrolyze both the mentioned substrates, were calculated the *Relative Efficiency of Hydrolysis* (REH or V_{max} ratio) and the K_m ratio, according to Pezzementi et al. (1991).

2.4. Physicochemical parameters

The optimal pH and temperature were determined assaying the activity for 180s in a pH range of 4.0 – 9.0 using the buffers: citrate-phosphate (4.0 – 7.5) and Tris-HCl (7.2 – 9.0) and in temperatures ranging from 25 to 80°C. Thermal stability was determined submitting the enzyme to the same temperatures for 30 min and, after 15 min of equilibration at 25°C (room temperature), remaining activity was assayed.

2.5. Selective inhibition assay

In order to identify which cholinesterases are present in *P. managuensis* brain, the crude extract was exposed to the selective inhibitors 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51, AChE inhibitor), tetraisopropyl pyrophosphoramide (Iso-OMPA, BChE inhibitor), neostigmine bromide and eserine (total cholinesterase inhibitors) for 1 h at concentrations ranging from 0.001 to 10 mM. The assay was performed in quadruplicate according to Silva et al. (2013) incubating 10 µL of crude extract with 10 µL of inhibitor in a 96-well microplate. Then, 200 µL of 0.25 mM DTNB was added and the reaction started after the addition of 20 µL of 62 mM

acetylthiocholine. The absorbance was followed at 405 nm for 180s and the residual activity was determined using the absence of inhibitor as 100%.

2.6. Inhibition assays – metal ions

AChE was exposed to ten ions (Al^{3+}, Ba^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Fe^{2+} and Zn^{2+}) for 40 min at 25°C in five concentrations ranging from 0.001 to 10 mM. The enzyme activity was performed according to Bocquené et al. (1990) incubating in 96-well microplates 10 µL of crude extract, 10 µL of ion and 200 µL of 0.25 mM DTNB. The reaction was followed at 405 nm during 180 s after addition of 20 µL of 62 mM acetylthiocholine iodide.

2.7. Inhibition assays – pesticides

The extracts were incubated for 60 min at 25°C with one organophosphate (dichlorvos) and two carbamate (carbaryl and carbofuran) pesticides. The insecticides were firstly dissolved in dimethyl sulfoxide (DMSO) and then diluted in distiled waterreaching five final concentrations ranging from 0.001 to 10 mM, in which each subsequent concentration is 10-fold higher than the previous one. The incubation was performed according to Assis et al. (2010) and the residual activity was determined considering the absence of pesticides as 100%.

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

The concentration able to inhibit in 50% and 20% the enzyme activity (IC_{50} and IC_{20}, respectively) was estimated for each inhibitor (pesticide or ion). These data were required to calculate the inhibition constant (K_i), using the equation of Cheng and Prusoff (1973):

$$Ki = \frac{\mathrm{IC50}}{1 + \ [\mathrm{S}]/\mathrm{Km}}$$

Where [S] corresponds to the substrate concentration.

2.9. Statistical analysis

Data were statistically analyzed by linear and non-linear regression fitted to sigmoidal (Boltzmann), polynomial or exponential decay ($p < 0.05$) modeling using Microcal™ Origin® version 8.0. One-way analysis of variance (ANOVA) followed by Tukey's test were used for mean comparison and differences were reported as statistically significant when $p < 0.05$.

3. RESULTS

The kinetic parameters, maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) were analyzed using the substrates acetylthiocholine and S-butyrylthiocholine in increasing concentrations (0.8 – 20.8 mM). The maximum velocities (V_{max}) of acetylthiocholine and butyrylthiocholine hydrolysis found for *P. managuensis* brain enzymes were 520 ± 8.98 and 267 ± 5.2 mU/mg, respectively. The K_m values, that represent the concentration of substrate required to reach half of the V_{max} of the reaction, were 0.587 ± 0.95 mM for AChE and 4.06 ± 0.82 mM for BChE. These values are similar to those reported in literature for other species (Table 1). The values of Vmax ratio and Km ratio of BChE-like activity are also present in Table 1.

Table 1. Kinetic parameters of brain AChE and BChE-like activity of *P. managuensis* and other species.

Substrate	***P. managuensis***	***O. niloticus****	***C. macropomum****	***C. ocellaris*****	***A. gigas****
ASCh (A)					
K_m [mM]	0.59 ± 0.95	0.39 ± 0.20	0.43 ± 0.02	0.77 ± 0.27	0.42 ± 0.09
V_{max} [mU/mg]	520 ± 8.98	218 ± 7.00	129 ± 0.05	189 ± 0.04	116 ± 2.0
BSCh (B)					
K_m [mM]	4.06 ± 0.82	NA	4.23 ± 4.2	NA	5.14 ± 2.0
V_{max} [mU/mg]	267 ± 5.2	NA	40.96 ± 1.23	NA	54.4 ± 6.5
V_{max} ratio B/A	0.51	-	0.32	-	0.47
K_m ratio B/A	6.88	-	3.74	-	12.24

*Assis et al., 2014; **Silva et al., 2013; NA - no activity in brain.

The optimal pH and temperature found for AChE of *P. managuensis* were 8.0 and 35°C, respectively (Figure 1A and 1B). The enzyme was moderately thermostable retaining approximately 65% of its activity after incubation at 40°C during 30 min and after 15 min equilibration at 25°C (Figure 1C).

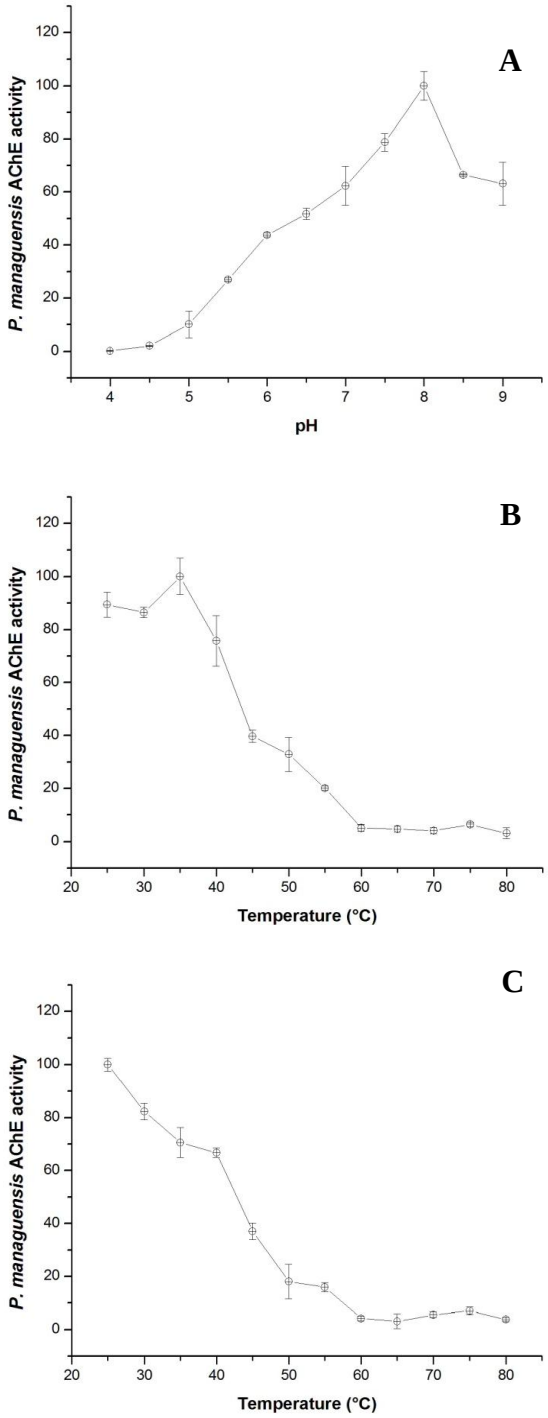


Figure 1 - Effect of pH (A), temperature (B) and thermostability (C) on the brain AChE activity of *P. managuensis.*

The ChE activity of brain of *P. managuensis* was abruptly reduced in presence of the selective inhibitors Iso-OMPA, evidencing the presence of BChE-like activity (Figure 2A) and BW284c51 confirming the presence of AChE activity (Figure 2B). Strong inhibition was also observed in presence of the total ChE inhibitors neostigmine and eserine (Figure 2C and D, respectively). The values of IC_{20} and of the median inhibitory concentration (IC_{50}) related to these inhibitors are shown in table 2.

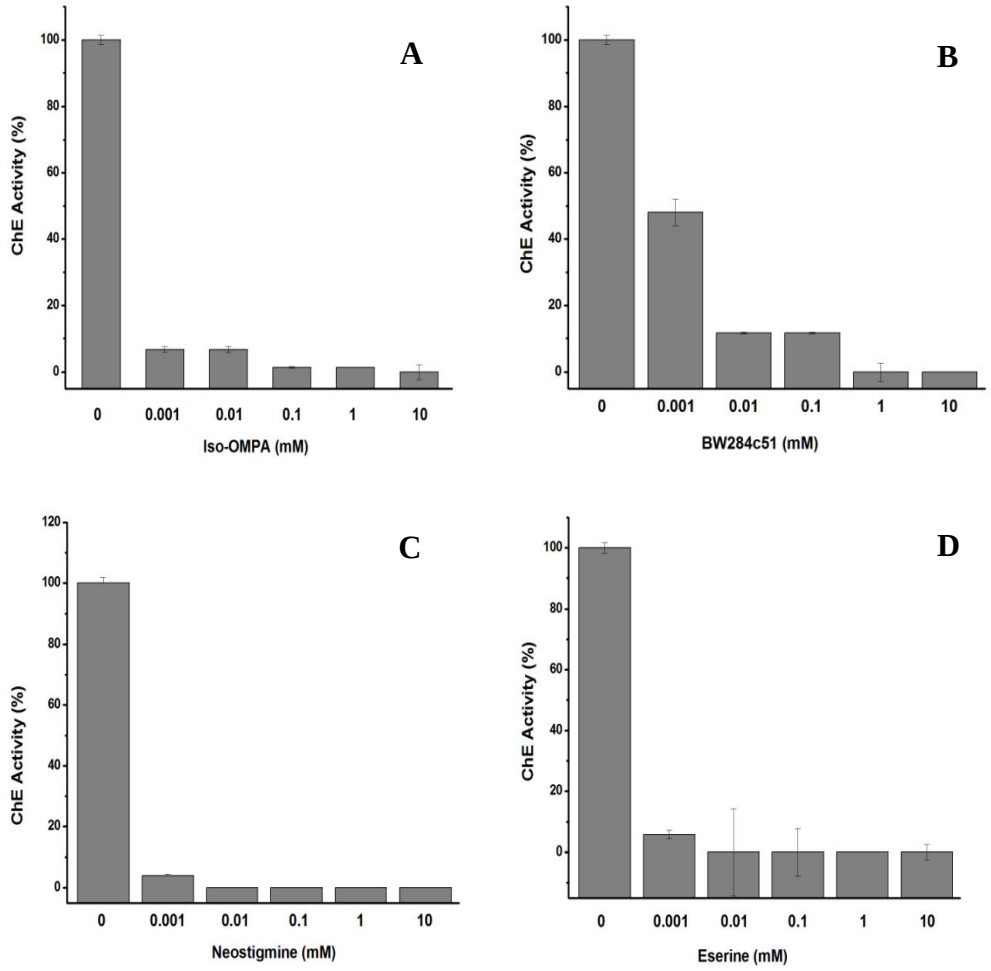
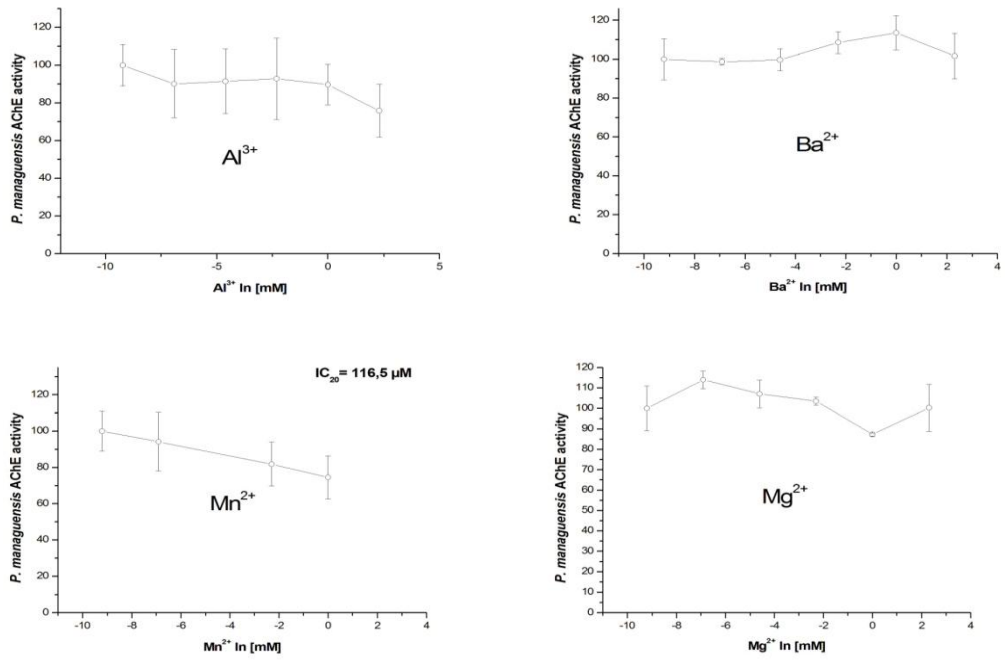


Figure 2. Activity of brain ChEs from *P.managuensis* in presence of increasing concentrations (0-10 mM) of selective inhibitors: (A) Iso-OMPA; (B) BW284c51; (C) neostigmine; (D) eserine.

Table 2. Values of IC_{20} and IC_{50} estimated for *P.managuensis* brain ChEs in the presence selective inhibitors.

Inhibitor	IC_{20} (µM)	IC_{50} (µM)
BW284c51	0.24	0.87
Iso-OMPA	0.12	0.2
Neostigmine	0.16	0.61
Eserine	0.11	0.17

Six ions (Al^{3+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Fe^{2+}) did not show significant inhibitory effect ($p < 0,05$) in the concentration of 1mM (Figure 3) whereas Zn^{2+}, Cd^{2+}, Cu^{2+} and Hg^{2+} inhibited enzyme activity in 17%, 25%, 53% and 100% respectively when submitted to the same concentration. The IC_{20}, IC_{50} and K_i of these ions are presented in Table 3.



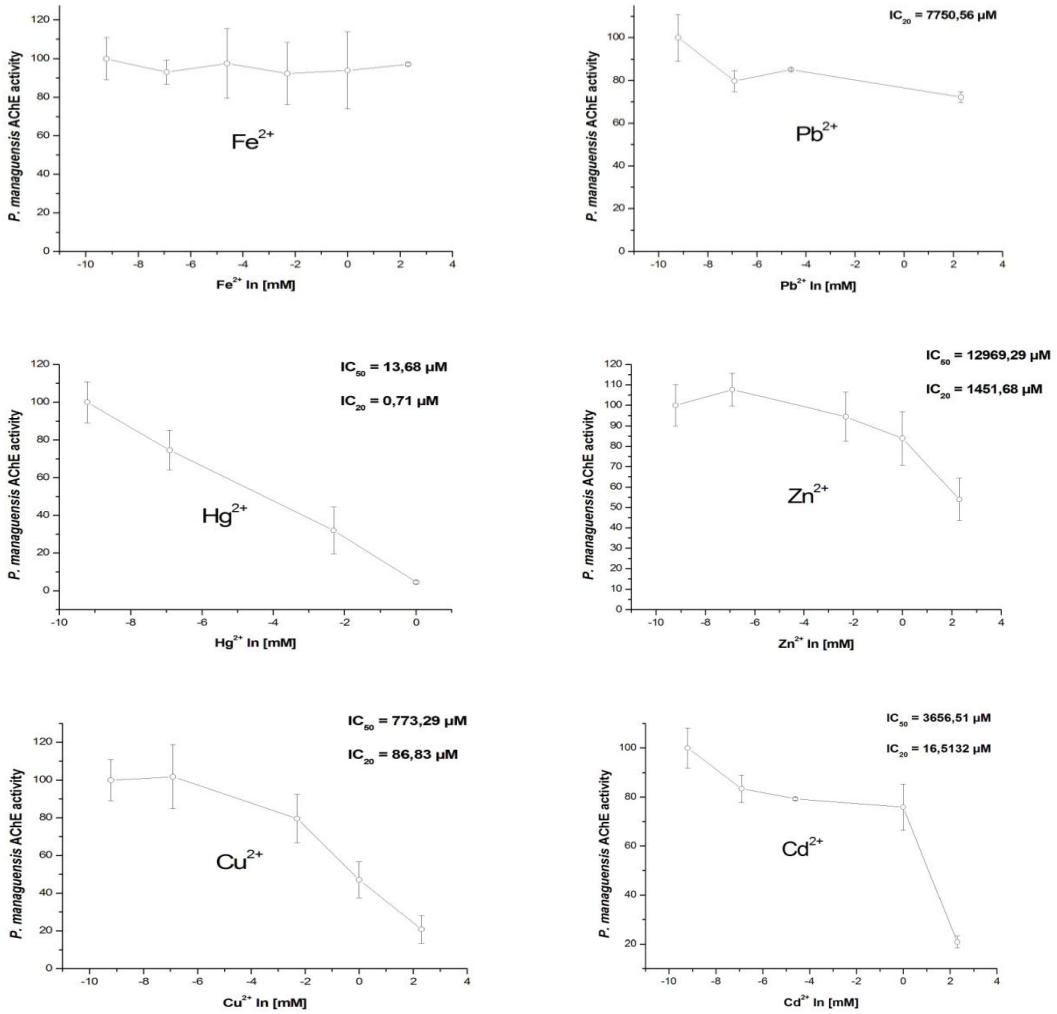
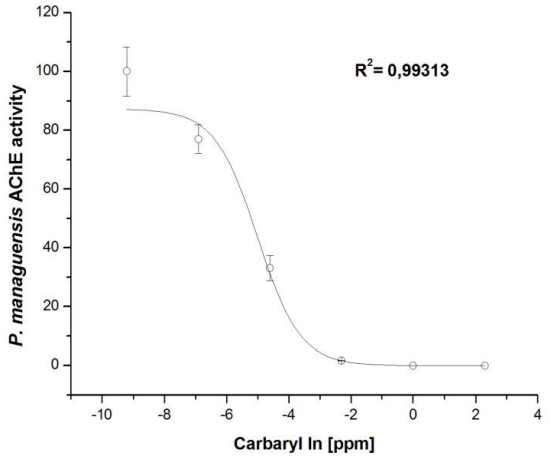


Figura 3 - Effect of metal ions on the activity of brain ChEs of *P. managuensis.*

Table 3. Values of IC_{20}, IC_{50} and K_i estimated for brain ChEs of *P.managuensis* in the presence of some metal ions.

Ion	IC_{20} (mM)	IC_{50} (mM)	K_i (mM)
Cd^{2+}	0.01	3.65	3.43×10^{1}
Cu^{2+}	0.08	0.77	7.25 x 10
Hg^{2+}	0.0007	0.01	1.22×10^{1}
Zn^{2+}	1.45	12.96	1.22×10^{2}

All the pesticides under study showed inhibitory effect on the activity of brain ChEs of *P. managuensis* (Figure 4). The values of the median inhibitory concentration (IC_{50}) for the pesticides were: 1.68 µM (dichlorvos), 4.35 µM (carbaryl) and 0.28 µM (carbofuran). Table 4 shows the inhibition constant (K_i), the IC_{50} and the IC_{20} related to the action of the pesticides under study on the activity of brain ChE of *P.managuensis*. According to the *Food and Agriculture Organization* 20% inhibition of ChE activity is the threshold for considering the presence of anticholinesterasic agent in the sample. Signals and symptoms appear above 50% inhibition and death occurs after 90%.



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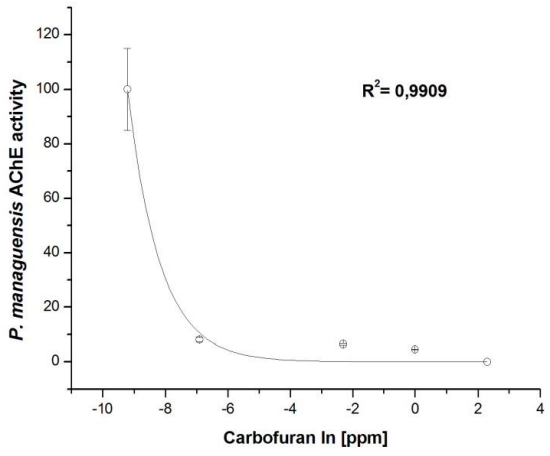

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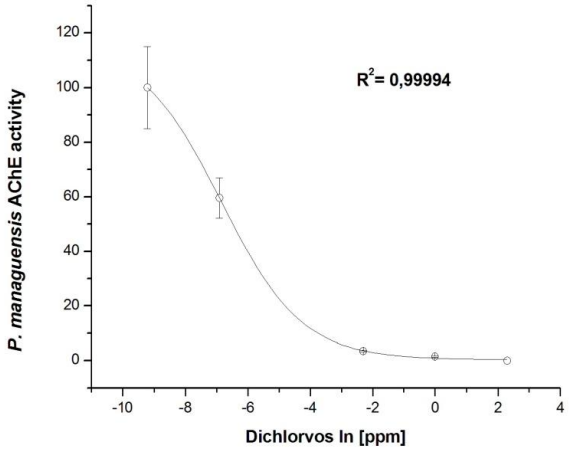

Figure 4 - Effect of one organophosphate and two carbamate pesticides on the activity of brain ChEs of *P. managuensis.* (A) Carbaryl, (B) Carbofuran, (C) Dichlorvos.

Table 4. Values of IC_{20}, IC_{50} and K_i for ChE activity of *P. managuensis* in presence of organophosphate and carbamate pesticides.

Pesticide	**IC_{20}(µM)**	**IC_{50} (µM)**	**K_i (µM)**
Dichlorvos	0.28	1.68	1.58×10^{-2}
Carbaryl	0.77	4.35	4.08×10^{-2}
Carbofuran	0.14	0.28	2.63×10^{-3}

4. DISCUSSION

The use of specific substrate and inhibitors evidenced the presence of AChE and BChE-like activities in the brain of *P. managuensis*. PChE-like activity (or ChE activity of an active site whose size is intermediary between AChE and BChE) was observed in the brain of *C. macropomum* and such activity was strongly inhibited when exposed to the specific BChE inhibitor Iso-OMPA and the specific AChE inhibitor BW284c51 (Assis et al., 2014). The absence of BChE activity was reported in the brain of several fish species (Pezzementi and Chatonnet, 2010; Silva et al., 2013). However, this enzyme is predominant in liver and plasma (Çokugras, 2003).

Although it was evidenced the presence of BChE-like activity in the brain of *P. managuensis*, the substrate acetylthiocholine was hydrolyzed more efficiently than butyrylthiocholine. This fact can be observed by the K_m values found here that were 0.587 ± 0.95 mM and 4.06 ± 0.82 mM for acetyl and butyrylthiocholine, respectively. These values are in accordance with those present in previous works for other fish species showed in Table 1. The analyzes of V_{max} ratio and K_m ratio in the BChE-like activity pointed to predominance of AChE or absence of BChE in the brain of *P. managuensis*. According to Pezzementi et al. (1991) the expected values for such ratios are: a) low V_{max} ratio (< 1) and a K_m ratio ≥ 1 for AChE due to its inhibition by excess substrate; b) V_{max} ratio ≥ 1 and km ratio < 1. Thus, the values found here indicate that bands should be considered as AChE since the value of V_{max} ratio was low (< 1), while km ratio was > 1 and they are consistent with those related to AChE activity present in literature (Rodríguez-Fuentes and Gold-Bouchot, 2004; Assis et al., 2014).

The maximum activity for AChE was observed at pH 8.0 and temperature 35°C. This result is similar to that found *Pleuronectes platessa*, 8.5 and 33°C (Bocquené et al., 1990), as well as for *Cichla ocellaris* (Silva et al., 2013) and *Colossoma macropomum* (Assis et al., 2010) which yelded higher activity at pH 8.0 and 45°C. *Cichla ocellaris* and *C. macropomum* presented optimum temperature slightly higher than that of *P. managuensis* probably due to their evolution have been occurred in a warmer environment such as the Amazonian basin. This fact could also justify their higher thermostability. Optimum temperatures of fish AChE range from 20 to 45°C and pH vary between 6.5 and 8.5 (Assis et al., 2011). However, the characterization of AChE physicochemical properties are required since, in general, tissues present non-specific esterases that may contribute for the activity under study and lead to misinterpretations, mainly in ecotoxicology studies (Ferreira et al., 2010; Howcroft et al., 2011).

Four (Cd^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}) out of ten analyzed ions presented inhibitory effect at 1 mM, a concentration considered elevated for environment samples not associated to mining or industrial areas (Assis et al, 2011). However, AChE presented relatively low sensitivity to the ions Cd^{2+} and Zn^{2+} (inhibition < 50%) and sharp sensitivity to Cu^{2+} and Hg^{2+} (inhibition ≥ 50% at 1mM). Several reports in literature described inhibition of AChE activity by Cu^{2+} such as: 23% for pirarucu, *Arapaima gigas*; 35% for tambaqui, *Colossoma macropomum*; 23% for cobia, *Rachycentron canadum*; 78% for electric eel, *Electrophorus electricus*; 53% for tucunaré, *Cichla ocellaris* (Assis, 2011; Oliveira et al., 2012; Silva et al., 2013).

The most expressive inhibition was observed for Hg^{2+} which completely inactivated AChE at 1mM. This same level of inhibition was found for AChE of *C. ocellaris* (Silva et al, 2013). In this study, 50% of the AChE activity of *P. managuensis* was inhibited in the concentration of 0.01 mM. Therefore, this enzyme can be highly inhibited at a very low concentration of Hg^{2+} which corroborate the potential of *P. managuensis* AChE as a promising tool as biomarker of this ion.

Among the pesticides under study the carbamate carbofuran presented the most inhibitory effect on the activity of brain AChE of *P. managuensis* as well as *C. ocellaris* brain AChE and in contrast to the enzyme of *C. macropomum* which showed to be more sensitive to dichlorvos (Table 5).

Table 5. Values of IC_{20}, IC_{50} and K_i estimated for *P. managuensis*, *C. ocellaris* and *C. macropomum* brain AChE in the presence of organophosphate and carbamate pesticides.

Pesticide	**IC_{20} (µM)**	**IC_{50} (µM)**	**K_i (µM)**	**Reference**
Dichlorvos				
P. managuensis	0.28	1.68	1.58×10^{-2}	Present work
C. ocellaris	4.02	5.52	6.76×10^{-2}	Silva et al., 2013
C. macropomum	-	0.04	1.37×10^{-4}	Assis et al., 2010
Carbaryl				
P. managuensis	0.77	4.35	4.08×10^{-2}	Present work
C. ocellaris	1.18	4.41	5.4×10^{-2}	Silva et al., 2013
C. macropomum	-	33.8	1.16×10^{-1}	Assis et al., 2010
Carbofuran				
P. managuensis	0.14	0.28	2.63×10^{-3}	Present work
C. ocellaris	0.082	0.21	2.57×10^{-3}	Silva et al., 2013
C. macropomum	-	0.92	3.15×10^{-3}	Assis et al., 2010

(-) not determined

Carbamate insecticides are direct inhibitors of AChE by carbamilation of the active site not requiring biotransformation so that they can induce toxic effects faster than most of organophosphorus compounds (Tham et al., 2009). The IC_{20} and IC_{50} values found for the pesticides under study in the present work using *P. managuensis* are lower than the maximum concentration levels recommended by national and international regulations. The *Resolution*

nº 20/1986 of CONAMA (Brazilian Environmental Council) recommends a maximum concentration level for organophosphates of 10 µg/L (0.45 µM dichlorvos) for class 1 and 2 waters (waters for domestic supply after simple treatment, for primary contact sports, for irrigation of vegetable for fresh consumption and for aquaculture) and 100 µg/L (4.5 µM dichlorvos) for class 3 (waters for domestic supply after conventional treatment, for livestock and for irrigation forage). The *USEPA National Primary Drinking Water Standards* (1984) recommend a maximum limit of 0.04 mg/L (aproximately 1.8 µM) for the carbamate carbofuran. Here, the results showed that *P. managuensis* could undergo significant deleterious effects due to the strong inhibition of AChE activity in concentrations lower than those provided by current regulations. It is noteworthy that the sublethal contamination could significantly alter several biochemical, physiological and morphological processes when absorbed by the fish organs.

5. CONCLUSION

Acetylcholinesterase was identified in the brain of *P. managuensis* and all the pesticides under study showed inhibitory effect on its activity, mainly the carbamate carbofuran in concentrations lower than the values established by current national and international regulations related to the maximum levels of these pesticides in natural water bodies. Most of the heavy metals did not showed potential to influence the activity of AChE in the concentration of 1 mM. However, the enzyme from this species showed a great potential as a biomarker for the mercury ion (Hg^{2+}). The analyzes of Vmax ratio and Km ratio in the BChE-like activity pointed to predominance of AChE or absence of BChE in the brain of *P. managuensis*. This is the first work in the literature reporting biochemical properties of the brain AChE of *Parachromis managuensis* pointing to the possibility of a routine and effective monitoring of anticholinesterasic agents.

6. ACKNOWLEDGMENTS

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7. REFERENCES

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 (10), 2243–2248.

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

Assis, C.R.D., Linhares, A.G., Oliveira, V.M., França, R.C.P., Santos, J.F., Marcuschi, M., Maciel Carvalho, E.V.M., Bezerra, R.S., Carvalho Jr, L.B., 2014. Characterization of catalytic efficiency parameters of brain cholinesterases in tropical fish. Fish Physiol. Biochem. 40 (6), 1659-1668.

Barbosa, J.M., Leitão, S.S., 2003. *Parachromis managuensis*: um ciclídeo introduzido no Brasil. In: XII Conbep, 2003, Porto Seguro. Anais do XII CONBEP. Porto Seguro: FAEP-BR, 2003. v.1. p.33-34.

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 (2), 75–89.

Botté, E.S., Jerry, D.R., Codi King, S., Smith–Keune, C., Negri, A.P., 2012. Effects of chlorpyrifos on cholinesterase activity and stress markers in the tropical reef fish *Acanthochromis polyacanthus*. Mar. Pollut. Bull. 65 (4-9), 384-393.

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), 3099–3108.

Çokugras, A.N., 2003. Butyrylcholinesterase: structure and physiological importance. Turk. J. Biochem. 28 (2), 54–61.

Coimbra, R.S.C.; Santos, C.R.S.; Saraiva, V.B.; Oliveira, M.M. 2013. Biomarcadores como ferramentas na avaliação da qualidade do pescado contaminado com metais traço. Boletim do

Observatório Ambiental Alberto Ribeiro Lamego, Campos dos Goytacazes/RJ. 7 (1), 153-172.

FAO, Food and Agriculture Organization, 2007. Pesticides in Food Report 2007. FAO Plant Production and Protection Paper 191. Food and Agriculture Organization, Rome, Italy.

Ferreira, N.G.C., Santos. M.J.G., Domingues, L., Calhôa, C.F., Monteiro, M., Amorin, M.J., 2010. Basal levels of Enzimatic biomarkers and energy reserves in *Porcellionides pruinosus*. Soil Biol. Biochem. 42 (12), 2128 -2136.

Ghazala, Mahboob, S., Ahmad, L., Sultana, S., AlGhanim, K., Al-Misned, F., Ahmad, Z., 2014. Fish cholinesterases as biomarkers of sublethal effects of organophosphorus and carbamates in tissues of *Labeo Rohita.* J Biochem. Mol. Toxicol. 28 (3), 137-142.

Hart, K.A., Pimentel, D. Environmental and economic costs of pesticide use. In: Pimentel D, editor. Encyclopedia of pest management. New York: Marcel Dekker; 2002. p. 237–239.

Howcroft, C.F., Gravato, C., Amorim, M.J.B., Soares, A.M.V.M., Guilhermino, L., 2001. Biochemical characterization of cholinesterase in *Enchytraeus albidus* and assesment of *in vivo* and *in vitro* effects of different soil properties, copper and phenmedipham. Ecotoxicology 20 (1), 119-130.

Hughes, R.J., Bennett, J., 1985. Effect of metal ions on the activity of acetylcholinesterase. Biochem. Soc. Trans. 13 (1), 219-220.

Jonsson, C.M.; Aoyama, H., 2010. Alteration of enzymatic activity in aquatic organisms by agricultural pollutants: a general approach and the susceptibility of the acid phosphatase. Química Nova. 33 (4), 920-928.

Marques, P.R.B.O.; Yamanaka, H., 2008. Biosensors based on the enzymatic inhibition process. Química Nova. 31 (7), 1791-1799.

Marques, S.M., Antunes, S.C., Nunes, B., Gonçalves, F., Pereira, R., 2011. Antioxidant response and metal accumulation in tissues of Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine. Ecotoxicology. 20 (6), 1315–1327.

Nigam, A.K., Srivastava, N., Rai, A.K., Kumari, U., Mittal, A.K., Mittal, S., 2012. The fist evidence of cholinesterase in skin mucus of carps and its applicability as biomarker of organophosphate exposure. Environ. Toxicol. 29 (7), 788-796.

Nunes, B., 2011. The use of cholinesterases in ecotoxicology. Reviews of Environmental Contamination and toxicology. Springer, New York. pp. 29-59, vol.212.

Nunes, B., Barbosa, A.R., Antunes, S.C., Gonçalvez, F., 2014. Combination effects of anticholinesterasics in acetylcholinesterase of a fish species: effects of a metallic compound, an organophosphate pesticide, and a pharmaceutical drug. Environ. Sci. Pollut. Res. 21 (9), 6258–6262.

Oliveira, V.M.; Assis, C.R.D.; Silva, R.P.F.; Bezerra, R.S., 2012. Efeitos do $FeCl_2$ e $CuCl_2$ na atividade da acetilcolinesterase cerebral de *Oreochromis niloticus.* Natural Resources. 2 (2), 27-36.

Olson, D.L., Christensen, G.M., 1980. Effects of water pollutants and other chemicals on fish acetylcholinesterase (*in vitro*). Environ. Res. 21 (2), 327-335.

Pezzementi, L., Sanders, M., Jenkins, T., Holliman, D., Toutant, J.P., Bradley, R.J., 1991. Structure and function of cholinesterase from amphioxus. In: Massoulié, J., Bacou, F., Barnard, E., Chatonnet, A., Doctor, B., Quinn, D. (eds) Cholinesterases: structure, function, mechanism, genetics and cell biology. American Chemical Society, Washington, DC, pp 24–31.

Pezzementi, L., Chatonnet, A., 2010. Evolution of cholinesterases in the animal kingdom. Chemico-Biol. Interact. 187 (1-3), 27–33.

Quinn. D.M., 1987. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. Chem. Rev. 87 (5), 955–979.

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. Ecotoxicol. Environ. Saf. 69 (3), 466-471.

Rodrigues, S.R., Caldeira, C., Castro, B.B., Gonçalves, F., Nunes, B., Antunes, S.C., 2011. Cholinesterase (ChE) inhibition in pumpkinseed (*Lepomis gibbosus*) as environmental

biomarker: ChE characterization and potential neurotoxic effects of xenobiotics. Pestic. Biochem. Physiol. 99 (2), 181–188.

Sedmak, J.J., Grossberg, S.E., 1977. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. 79 (1-2), 544–552.

Silva, K.C.C., Assis, C.R.D., Oliveira, V.M., Carvalho JR, L.B., Bezerra, R.S., 2013. Kinetic and physicochemical properties of brain acetylcholinesterase from the peacock bass (*Cichla ocellaris*) and in vitro effect of pesticides and metal ions. Aquat. Toxicol. 126, 191-197.

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 (1), 135–138.

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

Valbonesi, P., Brunelli, F., Marttioli, M., Rossi, T., Fabbri, E., 2011. Cholinesterase activities and sensitivity to pesticides in different tissues of silver European eel, *Anguilla anguill.* Comp. Biochem. Physiol., Part C. 154 (4), 353 -359.

Tomlinson, G., Mutus, B., McLennan, I., 1980. Modulation of acetylcholinesterase activity by peripheral site ligands. Mol. Pharmacol. 18 (1), 33-39.

Wang. Y., Chen, C., Zhao, X., Wang. Q., Qian. Y., 2015. Assessing joint toxicity of four organophosphate and carbamate insecticides in common carp (*Cyprinus carpio*) using acetylcholinesterase activity as an endpoint. Pestic. Biochem. Physiol. 122, 81–85.

WHO/IPCS/INCHEM. 1986a. Organophosphorus insecticides: a general introduction. Environ Health Crit 63. Geneva, CH: World Health Organization.

WHO/IPCS/INCHEM. 1986b. Carbamate insecticides: a general introduction. Environ Health Crit 64. Geneva, CH: World Health Organization.

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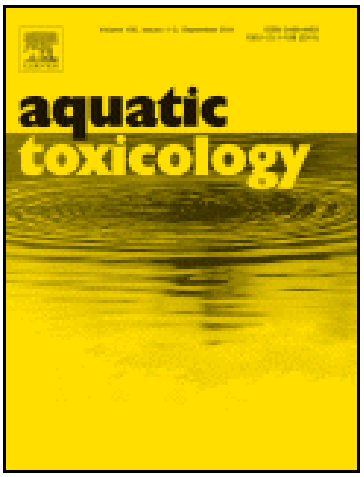

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