The Use of Cholinesterases in Ecotoxicology

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1 Introduction

The need for reproducible and accurate biomarkers in Environmental Toxicology has led researchers to implement methods to evaluate the physiological effects caused by contaminants. Such methods are of particular biological importance and ecological interest if they allow the measurement of direct impairment of key endpoints in the test organisms or nontarget species. Neurotransmission impairment via cholinesterase

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ChE inhibition is the target of two important classes of modern pesticides, the organophosphates (OPs), and the carbamates (CBs). Because of their extensive use in modern agriculture, these two classes of compounds are widely employed. Metcalfe et al. (2002) estimated for the California Department of Food and Agriculture that the ban from current agricultural use of such compounds would cause the loss of 209,000 jobs and would result in a national economic loss of $17 billion. Therefore, it is not difficult to conclude that these chemical agents will continue to be used, despite the fact that humans and many nontarget organisms are exposed to them (spray drift from crop application, run off from agricultural fields that contaminate adjacent water bodies, residues in food, etc.) (Vermeire et al. 2001).

Protecting agroecosystems from pesticide effects commonly entails measuring the levels of pesticides that occur in manifold environmental media. The inhibition of cholinesterases (ChEs) is poised to assume a leading role as a tool for monitoring the presence of those classes of pesticides or other agents that are ChE inhibitors, and their use may assist in determining the effects of such compounds on nontarget organisms. Denoyelle et al. (2007) have already provided evidence for what is possible in using such methods, by assessing the effects in apple orchards of certain pesticides on earthworms (*Allolobophora chlorotica*).

The prospective overall importance of ChEs to modern ecotoxicity assessment is high, particularly acetylcholinesterase (AChE). AChE acts to terminate the nervous impulse at the synaptic cleft and is directly involved in the intoxications caused both by the OP and CB pesticides. The toxic effect elicited by both types of pesticides has assured this biomarker a leading role in future environmental analyses. Although the OP pesticides may be hazardous, they are still extensively used in agriculture, and they exhibit certain favorable characteristics; in addition to successfully controlling many economic pests, and the OPs have low environmental persistence (when compared with organochlorine insecticides), low bioaccumulation (Sánchez-Hernandez 2001). These are the main reasons that justify their wide and continuing use, despite the fact that they also have high, nonspecific acute toxicity that can lead to frequent intoxication of nontarget organisms.

ChEs are extremely complex; namely, they display differential forms, expression, biologic functions, location, and catalytic activity. In addition to their involvement in neurodegenerative disorders, ChEs have other biologic functions (Small et al. 1996). These authors enumerated some of the functions that may be attributed to ChEs, including regulation of neurite growth, cell proliferation, tumorigenic processes, cell adhesion molecules, and megakaryocytopoiesis. Mammalian brain AChE (an enzyme that is released by presynaptic neurons during the neuronal communication process), for example, is mainly an enzyme located in postsynaptic neurons, is anchored to the cellular membrane by a single glycosylated protein, and is specialized in the hydrolysis of the neurotransmitter acetylcholine (Boschetti and Brodbeck 1996). AChE is of fundamental importance in terminating the nervous impulse; consequently, the regulation of its synthesis depends on, through a mechanism of feedback, the activation of nerve cells by propagation of an action potential. Schweitzer (1993) showed that AChE, which is mainly located in the postsynaptic cell, is constitutively expressed when nerve cells are resting; however, activation (by depolarization) of these cells leads to the release of AChE molecules to the extracellular space. Furthermore, this author found that the

secretion and release of AChE from nerve cells could be of two types: (1) a constitutive, and (2) a regulated pathway that can be activated following neuronal activity. These two pathways differ not only in the amount of AChE released but also in the preferential (asymmetric and globular) forms that are released. The expression of human AChE is controlled via a number of distinct pathways, one involving cAMP (Wan et al. 2000; Tsim 1998), which can be regulated by a calcitonine gene-related peptide (Tsim 1998).

The physiological role attributed to AChE is not limited only to the regulation of neurotransmission. The involvement of nerve growth factor in the synthesis of AChE was first demonstrated by Greene and Rukenstein (1981), indicating that the activity of this enzyme could also affect the neuronal differentiation processes. More recently, other research has produced evidence of this association. The role of AChE in neurodifferentiation was shown by Deschênes-Furry et al. (2003) and Choi et al. (1998). Yang et al. (2002) demonstrated the potential activity of AChE at the onset of apoptosis in nerve cells, with its potential implication in the neurodegenerative pathophysiology of certain diseases, such as Alzheimer's Disease. The increased AChE activity observed in neurodegeneration (a characteristic of Alzheimer's disease) is associated with the presence of high concentrations of reactive oxygen and nitrogen species (ROS and RNS, respectively). These results were published by Melo et al. (2003), whose work established a direct linkage between oxidative stress and the enzymatic activity of AChE of the human brain.

Butyrylcholinesterase (BChE), the other ChE present in the majority of vertebrates, is found mainly in the plasma and has an unclear function (Jbilo et al. 1994). However, BChE is thought to be involved in regulation of cell proliferation and the early stages of neuronal differentiation (Mack and Robitzki 2000). In addition to its presence in body fluids, BChE can exist in hematopoietic cells, liver, lung, heart, at cholinergic synapses, in the central nervous system, in tumors and in developing embryonic tissues (Mack and Robitzki 2000).

The use of ChE inhibition has thus become increasingly common among the batteries of biomarkers used in ecotoxicological assessment. Its use is favored because of several attributes, such as sensitivity to a large number of significant environmental contaminants, low cost, easy performance of quantification assays, adaptability to a vast number of species from distinct ecosystems, good reproducibility, and biological/ecological relevance. This latter factor is, indeed, of major importance. Padilla (1995) showed that the assessment of ChE in body fluids of organisms exposed to OP compounds correlated well with levels of ChE inhibition in target organs (namely, the central nervous system), and was closely accompanied by clinical symptoms and behavioral modifications. However, the time course of the intoxication must be assessed frequently, when ChE is employed as a biomarker. The possibility of establishing a direct relationship between ChE inhibition, and the behavioral/biochemical/physiological changes that occur in exposed organisms, is an important factor to consider in environmental analysis. The reason is that altered behavior may have severe population implications that result from potential impairment of reproduction, migration, and predator avoidance.

Inhibition of cholinesterasic activity is also relevant ecologically, because mortality of exposed organisms is a potential outcome. Fulton and Key (2001) highlighted the fact that cholinesterasic inhibition was closely accompanied by a rise in mortality;

much research demonstrates that the survival of aquatic organisms is impaired when inhibition from anti-cholinesterase compound exposure approached 70%. The potential impact of anticholinesterasic agents on animal behavior was shown by Chebbi and David (2009), following exposure of *Cyprinus carpio* to quinalphos. These authors observed that subsequent to a significant reduction of AChE activity, a major modification occurred in the swimming pattern of the mentioned fish species, such as erratic, darting, and burst-swimming. Pronounced impairment of behavior may constitute an adverse outcome that has evident ecological repercussions. Similarly, data obtained by Sandahl et al. (2005), after they exposed Coho salmon (*Oncorhynchus kisutch*) to chlorpyrifos, showed that a good correlation was obtained by plotting AChE inhibition against spontaneous swimming and feeding behaviors. Furthermore, this team found a remarkable result: ecologically relevant concentrations of chlorpyrifos could cause significant AChE inhibition and, simultaneously, cause clearly evident behavioral changes. The behavioral changes that result from AChE inhibition are not always obvious (Bain et al. 2004). These authors indicate that the anticholinesterasic effects caused by fenitrothion on the lizard species *Pogona vitticeps* were not followed by any significant modification in prey-capture ability.

The effects that occur concurrently with ChE inhibition may be important at the population level (Duquesne 2006). This author showed that after exposure of the crustacean *Daphnia magna* to paraoxon-methyl, a transient phase occurred during which ChE inhibition was noticeable, and simultaneously, several individual life traits were affected. These traits included survival, reduction in reproductive performance, and a decrease in body size. These population effects were a direct consequence of the toxic effects, and resulted from a reduced rate of population growth.

The neurotoxicity of xenobiotics may also produce effects at the cellular level. After exposure, unicellular organisms may display altered cell morphology and functionality, with consequences at the population level (lower number of cells). Falugi et al. (2002) showed that exposure of the protozoan species to basudin, a neurotoxicant, could result in cholinesterasic inhibition, followed closely by effects such as altered cell morphology, lower cell density, and impairment in the formation of aggregates.

The aim of the present review article is to address practical issues associated with the use of cholinesterase inhibition as a tool to determine the effects that anthropogenic contaminants have on nontarget wild species. In this article, we will also address the following points vis-a-vis ChEs: the need for previous characterization of cholinesterasic forms, the validity of use for field vs. laboratory quantification, main drawbacks as a biomarker, the future potential for use, and relevance for use with several classes of contaminants.

2 Types of Cholinesterases

The nature of the ChEs that exist in vertebrates and invertebrates are quite different. Vertebrates usually possess several distinct molecular forms of the enzyme, whereas invertebrates typically exhibit only one form (Massoulié and Bon 1982).

Fournier et al. (1988) showed that the native AChE form present in the insect *Drosophila melanogaster* was characterized by noncovalent association of two dimeric subunits, resulting from proteolysis of a precursor encoded for by the *Ace* locus. In contrast, Talesa et al. (1997) demonstrated the presence of two acetylcholinesterasic forms in the annelid species *Spirographis spallanzanii*; these differed in several characteristics, such as relative amounts present, the way in which they anchored to the membrane and pharmacological properties (namely, different sensitivities to edrophonium or procainamide). Both are amphiphilic globular forms, and the main distinguishing feature between the two enzymes was their different manner of attachment to the cellular membrane. Whereas the predominant form established strong electrostatic interactions with the membrane, the other one was anchored through a phosphatidylinositol linkage. Unlike the forms reported by Fournier et al. (1988), the acetylcholinesterasic forms found by Talesa et al. (1997) were more likely to result from expression of different genes. The acetylcholinesterasic forms found in tissues of *Octopus vulgaris* by Talesa et al. (1995a) were more complex. These authors found that AChE from this species could be divided into amphiphilic, dimeric, and hydrophilic tetrameric forms that share a common pharmacological relationship with heparin. This tendency was similar for both forms that existed in *O. vulgaris*, and pointed to the existence of a single gene underlying the expression of AChE.

The ChE forms found in the mollusc species *Mytilus edulis*, *M. galloprovincialis,* and *Corbicula fluminea* were investigated by Mora et al. (1999). These authors reported that the cholinesterasic forms identified in *M. edulis* and *M. galloprovincialis* were almost identical in terms of mass (180 kDa), whereas the form found in the other mollusc species, a clam, had a higher mass (240 kDa). However, all forms shared a similar membrane anchorage in that they were connected by a glycosyl inositol phosphate residue. More recently, representatives of one of these same mussel species (*M. galloprovincialis*), collected in the Adriatic Sea, was analyzed by Talesa et al. (2001), who found three forms of ChE in its tissues. They found two hydrophilic, spontaneously soluble forms (accounting for approximately 80% of all hydrolytic activity) in the hemolymph that had dimeric and globular tetrameric structures. The third form was mainly an amphiphilic globular dimer attached to the cellular membrane by a phosphatidylinositol tail insertion. This form promptly interacted with detergent (such as Triton X-100 and Brij 96) and was likely to undergo self-aggregation.

The existence of highly variable polymorphism of AChE forms in vertebrates was illustrated by Rocío Marcos et al. (1998). The authors isolated, identified, and characterized the AChE present in sheep platelets, and showed the presence of several forms that could be differentiated according to their relative solubility in low- and high-level saline solutions and in detergents. Furthermore, the authors observed that the physical–chemical characteristics of the different forms could account for the differential solubility: the AChE of sheep platelets could be extremely hydrophilic (soluble in aqueous salt solutions) or could be amphiphilic. The amphiphilic, globular, membrane-bound form is thought to be the initial stage in which AChE is found in sheep platelets; however, it can be cleaved as a consequence of the activity of endogenous proteases and phospholipases, or by endogenous chemical reduction.

The cholinesterases present in the medicinal leach, *Hirudo medicinalis*, were also characterized by Talesa et al. (1995b). They found two main types of ChE: a low salt-soluble hydrophilic monomer and a detergent-soluble amphiphilic glycolipid-anchored dimer that differed markedly in terms of substrate preference and inhibitor efficacy.

3 Characterization of Cholinesterases

Different forms of ChEs can produce distinct types of hydrolytic capacity. Vertebrates show two main types of enzymes with cholinesterasic activity: AChE and BChE (Massoulié et al. 2008). Both cholinesterasic forms also exist in humans and are simultaneously present in the human brain. These two distinct forms are differentiated on genetic, structural, and kinetic bases. Age also affects the relationship between AChE and BChE, because their relative content varies with progressing age (Giacobini 2004). Other forms of variability also exist. Different AChE forms may result from the alternative splicing of a single gene (Massoulié et al. 2008). Aquatic organisms are also likely to produce different cholinesterasic forms, whose existence may be manifested through their selective affinity for specific inhibitors and/or substrates (Kozlovskaya et al. 1993).

Because of the inherent variability of ChE morphology and hydrolytic activity, one of the mandatory pre-requisites for use of cholinesterase inhibition in environmental analysis is the full characterization of the cholinesterasic form that is present in the tissue of the test organism(s) selected for use. If the most prominent form of a ChE present is not identified, it is impossible to positively identify the cholinesterasic forms for which hydrolytic activity is being assayed. In general, cholinesterase characterization involves using specific inhibitors to establish an inhibition profile of the enzymatic form under study; concurrently, it also involves the study of the substrates that the enzyme prefers.

ChEs belong to the family of enzymes designated as esterases that retain the capability of hydrolyzing carboxylic esters. ChEs can be distinguished from other esterases in that they exhibit a preference for hydrolyzing choline esters rather than other carboxylic esters; ChEs are generally inhibited by physostigmine (eserine) at concentrations in the range of 10^{-5} M (Nunes et al. 2005; Eto 1984). Eserine can also inhibit other esterases (e.g., carboxylesterases) that may be present in the same tissue, and this inhibitory effect is dependent on the substrate used in the enzymatic determinations (Laguerre et al. 2009), as has been reported in the snail species *Xeropicta derbentina*.

ChEs can be classified according to their preference for specific substrates: AChEs have a strong preference for acetylcholine; BChEs prefer butyrylcholine; propionylcholinesterases are better at degrading propionylcholine. Accordingly, numerous studies have been conducted to define the most common types of ChEs that occur in living organisms. In spite of being present in virtually all organisms, marked differences in ChEs have been observed in terms of substrate preference and hydrolytic

activity. Drawing from the points made above, vertebrates and invertebrates possess different types of cholinesterases that must be fully characterized prior to their use as tools in environmental assessment.

3.1 Unicellular Organisms

Intermediate hydrolytic characteristics were found in the protozoan *Dictyostelium discoideum* (Falugi et al. 2002). These authors revealed that this protozoan had an enzymatic form with cholinesterasic activity, which preferred acetylcholine and propionylcholine as substrates, whereas butyrylcholine was not metabolized.

3.2 Earthworms

ChEs of different natures and structures may exhibit different properties, and the inhibitory effects of specific ones may differ from species to species. One must keep in mind the variability that exists among these enzymes when establishing ecotoxicological assessments, particularly when assessing the toxic effects of pesticides in distinct organisms. The first efforts to characterize ChEs were made during the late 1970s and early 1980s. Andersen et al. (1978) refers to the presence of a cholinesterasic form in tissues of *Eisenia foetida* that showed a marked preference for propionylthiocholine (PSCh) as a substrate; consequently, this enzyme could neither be classified as an AChE nor as a BChE. However, this enzyme was inhibited by the presence of pesticides (both OPs and CBs), a finding that allowed researchers to conclude that it was a B-esterase. Principato et al. (1978) identified a cholinesterasic form in *Allolobophora caliginosa* that was inhibited by eserine, and it showed higher catalytic activity when in the presence of the substrate acetylthiocholine (ASCh). These findings led to the conclusion that tissues of this species were likely to have a predominance of AChE, rather than any other form. However, Principato et al. (1989) also characterized the other form as a propionylcholinesterase that could also exist in the same species. Stenersen (1980) found that the tissues of the earthworm *E. foetida* were rich in two cholinesterasic forms; the predominant form was a propionylcholinesterase, whereas the other form displayed the properties of a nonspecific ChE.

Stenersen et al. (1992) reported that ChEs of three species of earthworms (*Eisenia fetida*, *E. veneta,* and *E. andrei*) could be distinguished by their tendency to be inhibited by the carbamate pesticide carbaryl. *E. andrei* and *E. fetida* had distinct cholinesterasic forms, and one of these could be completely inhibited by carbaryl. The remaining forms were extremely resistant to this pesticide, which may be accounted for by the lack of responsiveness of these species to carbamate exposure. Aamodt et al. (2007) reported the dual role of the ChEs in *E. fetida*. The authors distinguished two cholinesterasic forms, both of which were inhibited by carbaryl. However, one of the forms was promptly regenerated, whereas the second had not recovered, even after 21 days. Such findings are noteworthy, since toxicological

monitoring of anticholinesterasic effects in the wild account for such biological variations, which in turn may explain increased survivals of a particular species.

Modern soil ecotoxicologists may also assess pollution through the use of cholinesterasic inhibition. When doing so, they must provide a full characterization of the cholinesterasic forms present in the test organism that is to serve as the model for toxicological interaction. One example was described by Caselli et al. (2006), when attempting to characterize the cholinesterasic forms present in the earthworm *Eisenia andrei*. These authors discovered the hydrolytic preference of the ChE present was for ASCh and PSCh; simultaneous inhibition of this enzymatic form was more effective after exposure to BW284C51, a compound that strongly reduced ASCh and PSCh hydrolysis. This chemical, however, only caused a slight inhibitory effect when the substrate used was BSCh. Exposure to tetra(monoisopropyl) pyrophosphortetramide (iso-OMPA) did not elicit any significant inhibitory effect, for all tested substrates. These results suggest the presence of an intermediate cholinesterasic form that can, to a similar extent, simultaneously hydrolyze ASCh and PSCh, without affecting butyrylthiocholine (BSCh). A similar procedure was adopted by Rault et al. (2007), for characterizing the ChEs of several species of earthworms (namely, *Lumbricus terrestris*, *L. castaneus*, *Aporrectodea nocturna*, *A. caliginosa*, *Allolobophora chlorotica*, and *Aporrectodea rosea*). Some observations were extremely interesting: the enzymatic activities were stable during the sampling campaign (12 month), favoring the use of ChE inhibition as an effect criterion during all four seasons. All tested species, with the exception of *A. chlorotica*, had AChE as the predominant form. This is an extremely important finding, because the quantification of biomarkers, including cholinesterasic activity, is a promising tool in soil ecotoxicity assessment (Scott-Fordsmand and Weeks 2000). However, the use of ChE inhibition as an effect criterion, when assessed in earthworms, is novel and must be further validated under field conditions (Rodríguez-Castellanos and Sanchez-Hernandez 2007a). Earthworms respond to anticholinesterasic compounds, but their response is somewhat unpredictable; issues such as high inter-individual variation must be taken into account and clarified for the successful use of cholinesterasic inhibition with earthworms.

3.3 Crustaceans

Varó et al. (2002) showed that the main cholinesterasic forms present in the crustacean species *Artemia salina* and *A. parthenogenetica* were different, despite the phylogenetic proximity of the two species. In fact, the cholinesterasic forms present in these two invertebrates were classifiable neither as AChEs, nor as BChEs. Indeed, these two enzymes retained intermediate characteristics, since their maximum hydrolytic activity was recorded with PSCh.

Forget et al. (2002) studied the hydrolytic profile of the ChEs found in the estuarine copepod species *Eurytemora affinis*. The authors found that AChE was the predominant form among all ChEs, because it preferred ASCh as a substrate. Although it degraded PSCh and butyrylthiocholine, the hydrolysis rates were much slower.

Furthermore, a distinct profile existed for this enzyme form that exempted it from being a pseudocholinesterase (PsChE); it lacked responsiveness toward the inhibitor iso-OMPA, but was susceptible to significant inhibition by eserine. Similar results were obtained for the marine copepod *Tigriopus brevicornis* (Forget and Bocquené 1999). These results underscore the importance of using autochthonous species that were previously characterized for cholinesterasic activity in environmental studies conducted in marine and estuarine environments. Stefano et al. (2008) indicated that the most important cholinesterasic form in gill and muscle tissues of the marine scallop *Pecten jacobaeus* was AChE, and this form shared common characteristics with mammalian AChE. The study results showed that the substrate preferred ASCh, and that the hydrolytic activity was almost fully inhibited by BW284c51, which is a specific AChE inhibitor.

The muscle tissue of the crayfish species *Procambarus clarkii* was shown to be rich in both AChE and BChE (Escartín and Porte 1996) but had higher AChE activity.

Key and Fulton (2002) showed that the grass shrimp *Palaemonetes pugio*, possessed AChE as the predominant ChE form, demonstrated by a higher preference for ASCh as a substrate. Furthermore, AChE activity in this species was inhibited by eserine, BW284c51, but not by iso-OMPA, showing that AChE was predominant over other ChEs.

Frasco et al. (2006) identified the main cholinesterasic form present in the eye tissues of the prawn *Palaemon serratus* as AChE. Similarly, the results obtained by Xuereb et al. (2007) showed that the most important ChE in the crustacean *Gammarus pulex* was AChE, because it preferred ASCh as a substrate and was inhibited by BW284c51.

3.4 Molluscs

Talesa et al. (2001) characterized the ChEs of *M. galloprovincialis.* These authors found that all forms of ChEs in the tissues of this mussel were likely to prefer ASCh as a substrate, and they found one form that did not hydrolyze butyrylthiocholine. The ChEs found were barely inhibited by compounds such as eserine and paraoxon, and no inhibition was observed for propoxur and diisopropylfluorophosphate. However, these ChEs were especially sensitive to BW284c51, which is a specific inhibitor of AChEs. This finding points to the intermediate hydrolytic characteristics of these ChEs.

An extreme case has been reported by Valbonesi et al. (2003), who observed no cholinesterasic activity for the bivalve species *Tapes philippinarum*. However, the same work showed that AChE was the predominant cholinesterasic form in the tissues of *M. galloprovincialis* and *Ostrea edulis*. These results were an important part of an effort to define an adequate battery of indicator organisms for use in the assessment of agricultural chemical residues (that were rich in anticholinesterasic agents) in the area of the Mediterranean. The authors suggested that both bivalves, *M. galloprovincialis* and *O. edulis*, can be used as suitable test organisms for this

purpose; in contrast, *T. philippinarum* is definitely not an adequate species for such biomonitoring of marine contamination by OP or CB pesticides.

AChE was found by Brown et al. (2004) in the gill homogenates of *M. edulis*. Despite being an invertebrate species, *M. edulis* gill ChE activity was significantly inhibited by exposure to the AChE-specific inhibitor compound BW284c51.

3.5 Fish

The concurrent presence of distinct forms of ChEs in the same organisms also occurs. Varó et al. (2003) showed that the main cholinesterasic form in the brain tissue of the fish *Dicentrarchus labrax* was AChE, whereas muscle tissue simultaneously possessed AChE and BChE. Similar results were obtained by Solé et al. (2008), when assessing the esterasic activity of muscle tissue from the marine fish *Lipophrys pholis*. The authors concluded that the predominant cholinesterasic form present was AChE. A comparable conclusion was obtained by Rodríguez-Fuentes and Gold-Bouchot (2004) for the most predominant ChE form present in brain of the freshwater fish tilapia (*Oreochromus niloticus*). AChE was more abundant in nervous tissue, while atypical forms were more evident in liver and muscle tissues.

Similarly, the study conducted by Arufe et al. (2007) showed that the predominant cholinesterasic form in the gilthead seabream (*Sparus aurata*) larvae was AChE. The authors determined the substrate preference of whole-body cholinesterasic activity of yolk sac seabream larvae and observed a noticeable preference for AChE, followed by propyonylthiocholine, and finally BChE. Additionally, the enzymatic activity of homogenized tissues was almost fully inhibited by BW284c51, indicating the presence of AChE. Jung et al. (2007) also discovered that a considerable portion of the cholinesterasic activity in the muscle tissue of the sole species *Limanda yokohamae* was attributed to BChE. However, the most important form identified in the nervous tissue was AChE.

Sturm et al. (1999a) reported the presence of AChE as the dominant cholinesterasic form in brain tissue of several marine fish species, such as *L. limanda*, *Platichthys flesus*, and *Serranus cabrilla*. This was an indisputable finding because AChE is predominant among all cholinesterasic forms that exist in vertebrate species; however, muscle tissue is somewhat different, in that the simultaneous presence of butyryl- and acetylcholinesterase was observed. One of the major findings described in this paper for this tissue was the responsiveness of the characterized butyrylcholinesterase. This enzymatic form was much more sensitive toward OP pesticides than was AChE; this observation may be important in future marine risk assessment.

Sturm et al. (1999b) characterized the cholinesterasic forms present in the tissues of the freshwater fish species *Gasterosteus aculeatus*. They observed that this enzyme form's capacity to hydrolyze ASCh, and its near complete inhibition by eserine, indicated that this fish had a ChE in its muscle tissue. However, this

cholinesterasic form was intermediate in terms of substrate preference, since it could degrade both ASCh and BSCh. The authors postulated that *G. aculeatus* muscle ChE was an atypical PsChE that had intermediate sensitivity to the inhibitory compound BW284C51 (complete inhibitor of AChE in mammals).

Garcia et al. (2000) reported similar results when studying the ChE content of the freshwater fish species *Poecilia reticulata*. These authors found that the main cholinesterasic form present in head tissue of the mentioned species was AChE. In agreement with these results, Nunes et al. (2005) found that the most active cholinesterasic form that existed in nervous tissue of *Gambusia holbrooki* was AChE.

The characterization of the predominant ChE forms present in tissues of *Pomatoschistus microps* was performed by Monteiro et al. (2005). They reported that the ChEs present in the homogenized tissues from the head of the organism had a clear preference for ASCh as a substrate and the reaction was inhibited by BW284C51. The results obtained in the entire head homogenate with iso-OMPA (a common BChE inhibitor) showed that a significant inhibitory effect was possible. These two results are consistent with the possibility that an atypical form of ChE exists and seemed to indicate that there was an intermediate behavior of the ChE present in *P. microps* tissues.

ChEs may be present in tissues other than that of the nervous system and muscles. Wogram et al. (2001) showed that the responsiveness of the butyrylcholinesterase found in tissues (liver and axial muscle) of the freshwater fish species three-spined stickleback (*G. aculeatus*) was clearly higher than was AChE, following exposure to parathion. The importance of the significant inhibition of BChE in liver tissue is high: parathion requires a previous metabolic activation (with consequent formation of paraoxon, the active metabolite), namely, through cytochrome P450 oxidative activity to exert its anticholinesterasic activity. Because liver tissue is rich in cytochrome P450, it is natural that the hepatic BChE form becomes immediately inhibited after parathion metabolism. In general terms, hepatic BChE was 1,000-fold more sensitive than was AChE. This finding is important in terms of use of this species in environmental assessment, since extremely short periods of exposure can be better assessed by the quantification of BChE, rather than AChE activity.

The assessment of the anthropogenic impact on reef ecosystems was also the main concern that drove Leticia and Gerardo (2008) to characterize the ChEs of the fish species *Haemulon plumieri*. These authors reported higher enzymatic activities, specifically measured as AChE, in brain and liver tissues.

3.6 Reptiles

Bain et al. (2004) characterized the plasma cholinesterasic forms that existed in the lizard species *P. vitticeps*. The authors observed that the predominant form present was butyrylcholinesterase. A similar result was obtained by Sanchez-Hernandez and Sanchez (2002) in plasma of the reptile species *Gallotia galloti*. Despite the presence of butyrylcholinesterase in serum, a residual amount of AChE was also present.

The total amount of detected serum BChE constituted 74% of total cholinesterasic activity (Sanchez-Hernandez 2003). However, in this same study it was also shown that brain ChEs were exclusively composed of AChE.

An extensive study was conducted by Schmidt (2003) to characterize the ChE activity of reptiles, which included several species such as the spotted turtle (*Clemmys guttata*), river cooter (*Pseudemys concinna*), logerhead sea turtle (*Caretta caretta*), Texas hornet lizard (*Phrynosoma cornutum*), desert hornet lizard (*P. platyrhinos*), round-tailed hornet lizard (*P. modestum*), eastern cottonmouth rattlesnake (*Agkistrodon piscivorus*), western diamondback rattlesnake (*Crotalus atrox*), American alligator (*Alligator mississippiensis*), and Morelet's crocodile (*Crocodylus moreletii*). For all tested species, brain ChE was predominantly composed of AChE, while plasma was particularly rich in butyrylcholinesterase.

4 Usefulness of Cholinesterase Inhibition in Environmental Monitoring

4.1 Classic Use for Assessing the Environmental Effects of Anticholinesterasic Agents

The classic role attributed to AChE inhibition in environmental analysis has been related to the assessment of effects caused by exposure to the OP and CB pesticides. These classes of compounds share a common mechanism of toxicity, i.e., both classes exert their toxic action directly on the active catalytic site of AChE, thus preventing the in vivo physiological hydrolysis of the neurotransmitter acetylcholine that is needed to terminate the nerve impulse. Some OPs are initially inactive and require in vivo metabolic activation; such activation may dramatically increase toxicity, as observed by Jokanovic (2001). OPs are thus considered to be irreversible AChE inhibitors, requiring a full de novo synthesis of the inhibited enzyme, whereas CBs may be hydrolyzed and consequently removed from the active binding site (Zinkl et al. 1991). The most prominent consequences of exposure to these agents are neurotransmission/neuromuscular impairment. These agents are widely employed to control the unwanted presence of insect pests, and both classes of pesticides are characterized by their propensity to exert deleterious effects on nontarget species. The effects they induce have been extensively analyzed by quantifying the actions of AChE in exposed organisms. Such effects (enzymatic inhibition) explain the significance of the classic role attributed to AChE inhibition as an effect criterion in Ecotoxicology. The need to define a sensitive marker for anticholinesterasic agents led Magnotti et al. (1994) to study the ChE activity of 28 fish species. Such ChE activity in fish may act as a sentinel for the presence of OP compounds in environmental assessment studies. This research team found that, among all tested species, sea bass and flatfish possessed the highest levels of ChEs, and these two species are thus construed to represent prime candidates for environmental assessment of neurotoxic compounds. In this study, the authors proposed the use of the

two mentioned fish species as sentinels to monitor for the presence of anthropogenic agents, such as OP pesticides, hypochlorite-activated organothiophosphates and CB pesticides. Based on the high responsiveness of ChEs from these species, the presence of such compounds can be identified and monitored.

An extensive survey was performed by Chuiko (2000) on the effects of dimethyl 2,2-dichlorovinyl phosphate (DDVP) on ChEs in 11 fish species, (*C. carpio*, *Abramis brama*, *A. ballerus*, *Blicca bjoerkna*, *Rutilus rutilus*, *Alburnus alburnus*, *Leuciscus idus*, *Perca fluviatilis*, *Stizostedion lucioperca*, *Esox lucius,* and *Coregonus albula*). The results of this study indicated that the inhibitory effects caused by DDVP on AChE and BChE activities in brain and serum were rapidly manifested in these species, and brain and serum AChE was promptly inhibited by the insecticide in a manner similar to that already observed for mammals. However, serum BChE was extremely sensitive to DDVP and was more sensitive than were mammalian ChEs.

ChE inhibition has been a widely applied endpoint to assess exposure of OP and related compounds. In addition to the utility of this biomarker in ecotoxicity, human toxicology investigators have quantified red blood cell ChE inhibition to diagnose OP exposure in humans. MacGregor et al. (2005) showed that human sensitivity to the insecticide dichlorvos was similar to that of other test organisms, such as rodents, primates, and dogs. This is important, because it shows that the physiologic role attributed to ChEs is well preserved among vertebrates and is an accurate and reliable marker of neurotoxicity.

Comparing the sensitivity of anticholinesterasic compounds in invertebrates has been a topic of interest to Sánchez-Hernandez (2007). In his review, several invertebrate organisms were defined as possessing high levels of carboxylesterases (CbE), and the sensitivity of these enzymes to OP pesticides is much higher than that of the AChE present in the central nervous system. Therefore, the levels of CbE must be considered when characterizing the toxic response of such organisms to OP pesticides. Therefore, quantifying CbE, rather than AChE inhibition, after OP pesticide exposure (Sánchez-Hernandez 2007) may be equally or more useful.

There are numerous research articles in the field of Ecotoxicology that address the use of AChE as a biomarker. Sancho et al. (2000) exposed eels (*Anguilla anguilla*) to the CB thiobencarb, and monitored the AChE activity in the eyes of this organism for a period of 1 week after exposure. The authors concluded that AChE inhibition could function as a sensitive and satisfactory biomarker for verifying the exposure of this organism to the tested carbamate. Moreover, the ocular tissues showed that sensory organs are also prone to the inhibitory effects of carbamates.

Varó et al. (2002) showed that AChE inhibition occurred after exposure of two species of *Artemia* (namely *A. salina* and *A. parthenogenetica*) to the insecticides chlorpyrifos and dichlorvos. However, AChE inhibition in both of these crustaceans was not indicative of major toxicological implications AChE activity inhibition was not directly correlated with mortality, since death of exposed organisms only occurred at inhibitions exceeding ~80% of the initial enzymatic activity. These results indicate that this enzyme is extremely sensitive as a potential biomarker for pesticides exposure.

The use of OP and CB pesticides in common agricultural processes may have direct consequences on non-target species (Ferrari et al. 2007). These authors studied the inhibitory effects of the OP insecticide azinphos-methyl (AzMe) and the CB

pesticide carbaryl on juveniles of rainbow trout. They concluded that normal patterns of use of both compounds can exert significant real effects on cholinesterasic forms of both head and muscle tissues in fish.

The effects of chlorpyrifos and carbaryl on AChE of hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) were studied by Somnuek et al. (2007). In this work, test organisms having different body sizes were exposed to sublethal concentrations of the test compounds for a period of 4 days. The endpoint was AChE inhibition and was measured in several tissues: brain, liver, muscle, and gills. The authors observed a marked inhibition of enzyme activity in all tissues. Nevertheless and not surprisingly, AChE inhibition was more prominent in brain tissues. The animals were subjected to a recovery period that was too short to permit a total recovery of the inhibitory effects. These results indicate that deleterious effects caused on non-target species may persist for long periods and may be permanent.

Marine environments are also prone to toxic damage by anti-cholinesterasic agents. Accordingly, Brown et al. (2004) showed that the main cholinesterasic form present in *M. edulis* gill homogenates was responsive to the insecticide azamethiphos (calculated IC_{50} of 100 mM). However, when *M. edulis* was used as a sentinel species in marine environmental assessment, the AChE present in *its* tissues was less susceptible to significant inhibition and was less responsive than in other marine species. The AChE present in eye tissues of *P. serratus* exhibited extreme sensitivity toward OP and CB pesticide exposure (Frasco et al. 2006). The authors tested exposure to the CB carbofuran and the OP chlorpyrifos-oxon, and concluded that this crustacean could be useful for assessing pesticide contamination in saltwater.

The issue of sensitivity of ChEs was also assessed by Jung et al. (2007). They performed a study to quantify the inhibitory effect caused by the insecticide iprobenfos on both acetyl- and butyryl-cholinesterase of *L. yokohamae*. The study results showed that sensitivity may not only relate to the type of ChE involved in the toxic response, but may also derive from a tissue-specific trend. Despite being extremely sensitive when present in muscle tissue of the test organisms, AChE from the nervous system was rather insensitive to the inhibitory effects caused by iprobenfos. Muscle butyrylcholinesterase activity was also sensitive to inhibition by the tested insecticide. These findings suggest that the selection of the most appropriate tissue is mandatory when assessing environmental effects caused by specific pesticidal agents.

The discussion concerning the sensitivity of cholinesterasic forms to be used in quantifying deleterious biological effects was also addressed by Rodríguez-Fuentes et al. (2008). In their study, these authors refer to the presence of atypical cholinesterasic forms in two species of marine fish, namely *Pleuronectes vetulus* and *Pleuronichthys verticalis*. The authors discovered that *P. vetulus* had a predominance of AChE over BChE in muscle tissue, and that the total length of the fish was a critical experimental variable, in that total cholinesterasic activity was negatively correlated with total body length. Furthermore, no significant effects resulted from gender differences or sampling sites. This later observation is extremely important, because, in this study, two quite different priority sampling sites were defined: (1) a sampling site near the wastewater release point coming from a sewage treatment plant and (2) an off-coast location, far from any apparent human influence. The situation

with *P. verticalis* was different, because the majority of the cholinesterasic activity in muscle derived from the presence of butyrylcholinesterase. Furthermore, the sensitivity of ChE to pesticide inhibition was higher in males than was the sensitivity registered for females.

ChEs found in *M. galloprovincialis*, *M. edulis,* and *C. fluminea* exhibited a similar pharmacological behavior, which was characterized as extremely refractory to OP compound exposure (Mora et al. 1999). This finding was significant, because the authors suggested that this species only be used when particularly heavy contamination of OP pesticides existed, rather than being employed in low-level contaminant (chronic) surveys. A different result was obtained by Dauberschmidt et al. (1997) after exposing zebra mussel (*Dreissena polymorpha*) to anti-cholinesterasic agents, such as thiometon, disulfoton, and demeton-S-methyl. Despite having identified the presence of ChEs by radiolabeling of the serine residue in the active site, the authors observed an absolute lack of response of ChE to the tested compounds. Furthermore, the calculated ChE activity was extremely low when compared with cholinesterasic activities already reported for other mollusc species. The range of tested concentrations did not elicit any significant effect in terms of cholinesterasic inhibition, even after the death of exposed organisms. These observations allow one to conclude that interspecific differences must always be taken into account, since similar organisms may retain large differences in the nature and levels of enzymes present.

The effectiveness of OP compounds as anticholinesterasic agents was also assessed in reptiles. Sánchez-Hernandez and Walker (2000) observed the inhibitory effects of the insecticides trichlorphon and parathion on serum and brain ChEs of the lizard *G. galloti*. Besides evaluating the potential inhibitory effects by these OP compounds, the authors also observed that the lizards were less sensitive than were certain other organisms (such as birds or mammals) to the inhibitory effects of the OPs. This same species was again used by Sanchez-Hernandez (2003) to assess the potential impact of agrochemicals on serum BChE activity. The inhibition levels of BChE in animals collected from highly impacted agricultural landscapes were studied in comparison with the results from organisms collected at pesticide-free sampling sites. Results were that the quantification of serum cholinesterase (BChE) in *G. galloti* was a reliable tool to assess the contamination by anticholinesterasic compounds under field conditions. Similarly, Bain et al. (2004) tested the anticholinesterasic effects of fenitrothion on the Australian lizard species *P. vitticeps*. This organism was responsive, in terms of ChE inhibition, to the presence of the insecticide. Other than a significant and dose-dependent ChE inhibition, no other evident symptoms (e.g., changes in diurnal body temperature or alterations in standard metabolic rate) were correlated with exposure to this insecticide.

Effects on ChEs, following accidental exposures of non-target organisms in the wild, may not always be transient. Even if the effects derived from that exposure do not culminate in death, medium to long-term effects are likely to occur and are often not benign. This was shown by Sancho et al. (1998), in a study in which Europeans eels (*A. anguilla*) were acutely exposed to the insecticide fenitrothion. After exposure, a batch of organisms immediately analyzed for plasma AChE activity showed a marked inhibition. A second batch of exposed animals was allowed to

recover; however, recovery was only permitted for a limited period (1 week), after which quantification disclosed AChE activity to be lower than for non-exposed organisms. These results provided evidence that physiological impairment may derive from exposure to anthropogenic chemicals and may be sustained for long periods. These authors suggested that the type of inhibition they observed was not transient, and that a de novo synthesis of the impaired enzyme is required for re-establishing normal levels of activity.

Serum BChE and CbE inhibition were used as effect criteria in a field survey conducted by Sanchez et al. (1997). The authors quantified the levels of these enzymes in plasma of the reptile species *G. galloti*. According to the results obtained, these enzymes could be effectively used as biomarkers to assess pesticidal contamination in reptiles. The authors of this study described the significant inhibitory effects caused by parathion on serum BChE and CbE, for a period of 23 days after the initial spraying and exposure. Consequently, the authors sustained the view that these markers can serve as long-term indicators of chemical contamination if assessed in the serum of the mentioned lizard species.

AChE inhibition was also the criterion adopted by Gao and Zhu (2002), when studying the resistance to OP pesticides exhibited by the insect species *Schizaphis graminum*. The authors concluded that the efficacy of the insecticides (or their active metabolites) chlorpyrifos oxon, paraoxon, methyl paraoxon, malaoxon, demeton-S-methyl, and omethoate was similar in all organisms, but the ones that showed higher resistance had higher levels of AChE. In fact, the authors concluded, resistance came from the increased expression of a ChE gene.

Rahman et al. (2000) showed that OP compounds are unlikely to exert a simple inhibitory effect on the ChEs of exposed organisms. The novel phosphorothionate (2-butenoic acid)-3-(diethoxy phosphinothioyl)-methyl ester was shown to be effective on the AChE activity of rodents but could also affect the activity of several ATPases of the exposed animals. In addition, the authors obtained interesting data that pointed to a gender-selective toxicity (larger susceptibility of females) and a greater sensitivity of cholinesterasic forms to the tested compound. Inhibitory profiles of the effects on different enzymes were created by the authors, but it was clear that recovery of enzymatic activities had not occurred after a period of 28 days. These findings underline the importance of ChEs as putative markers for intoxication by OP compounds.

Hai et al. (1997) showed that the freshwater fish species *C. carpio* was sensitive to the inhibitory effects of the insecticide dichlorvos, with substantial AChE inhibition occurring following exposure. However, this compound not only elicited the common and expected effects related to AChE inhibition but also caused major redox impairment, such as effects on superoxide dismutase, catalase, and lipid peroxidation. It was interesting to note that it was possible to establish a relationship between the two endpoints (neurotoxicity vs. oxidative stress). From these results, it is possible to observe that anthropogenic compounds may exert deleterious effects on biota by various pathways. It is absolutely mandatory to establish comprehensive batteries of biomarkers to fully understand the array of toxic responses that may occur in the wild. Risk assessment studies, based on biomarkers (such as quantification of cholinesterasic activity), may be influenced in unpredictable ways by various confounding factors in the real world.

The use of ChE inhibition as an effect criterion was proposed by Sturm et al. (1999b) for assessing the presence of anti-cholinesterasic agents in streams. This research team selected muscle tissue of the freshwater fish species *G. aculeatus* to assess the combined effects of OP pesticide residues present in the area streams. Their results showed that it was possible to successfully use this biomarker to monitor freshwater environments that are potentially contaminated by OP compounds. The authors reported that the ChE activities measured at several sampling sites varied during the year according to a gradient that corresponded to amounts of organophosphates present. It was concluded that ChE inhibition is a sensitive tool for environmental analysis. The use of fish in biomonitoring programs requires knowledge of the effect of environmental variables on the studied response. For example, cholinesterasic activity in sensitive fish may vary with fluctuations in salinity (Wang et al. 2001). The authors also showed that the neurotoxicity of the CB insecticide aldicarb (evaluated in terms of ChE inhibition) was enhanced in rainbow trout (*O. mykiss*) but not in hybrid striped bass (*Morone saxatilis*×*chrysops*). This finding is important, because during the selection of test organisms for use in biomonitoring, some species possess distinct sensitivities to OPs, and these sensitivities may be affected by the abiotic conditions that exist during toxicant exposure.

The use of living organisms as sentinels of chemical contamination can also be confounded by intrinsic biologic characteristics, such as the age of the test individuals. To study the effects of age on the toxicity of anthropogenic contaminants, Sánchez-Fortún and Barahona (2001) exposed groups of *Artemia salina* of different ages to the insecticide carbophenothion (ChE inhibitor). Increasing age was responsible for enhanced toxicity. Furthermore, the protective role that a specified compound (e.g., ChE-reactivating oxime 2-pyridine aldoxime methochloride; 2-PAM) exerted on carbophenothion toxicity was addressed in the same study. The results showed that by enhancing the regeneration rate of ChEs, 2-PAM significantly reduced the toxicity of this pesticide. The protection offered by compounds such as 2-PAM can also be used as an indirect biomarker of exposure to anticholinesterasic agents. McInnes et al. (1996) observed that wild birds captured in the vicinity of agricultural fields in which the OP insecticide chlorpyrifos was used had lower levels of ChE, AChE, and BChE, when compared with animals from non-contaminated areas. Furthermore, the tested wildfowl required higher amounts of 2-PAM to reactivate AChE activity, which had an unequivocal sign that the birds suffered OP poisoning. A similar study was conducted by Parsons et al. (1999) to assess the presence of anticholinesterasic pesticide residues that could affect bird colonies located mainly in northeast US estuaries. Evaluation of anticholinesterasic effects was again performed through the use of the reactivating oxime, 2-PAM, which gave positive results of the sort mentioned before: birds from agricultural areas were more likely to suffer symptoms of OP poisoning and to require 2-PAM for regenerating serum ChE levels.

Total AChE inhibition and reactivation were the toxicological parameters selected by Maul and Farris (2005) to show the potential impact of anticholinesterasic agrochemicals on *Cardinalis cardinalis*. The authors found that, despite large fluctuations among the groups of tested individuals, the majority of birds captured in OP-use areas had total ChE activities below the diagnostic threshold. Moreover, reactivation rates were also higher, indicating the usefulness of this parameter as an

indicator of OP or CB use. Birds were also the subject of a study undertaken by Iko et al. (2003). These researchers hypothesized that the population decline observed for the mountain plover (*Charadrius montanus*) was possibly associated with a toxic effect caused by agricultural use of OPs. To verify this hypothesis, the work team assessed the serum levels of ChE and found no recent contamination by OPs, as measured by ChE depression; furthermore, potential contamination of wild birds was not confirmed, since no significant differences were observed from animals that came from different areas.

The reactivation of plasma BChE by 2-PAM in *G. galloti* was the best criterion to diagnose exposure to OP and CB pesticides, as shown by Sánchez-Hernández et al. (2004). These authors monitored several populations of this species that existed in specific geographical locations. The organisms captured near agricultural fields had lower serum BChE activities and required higher amounts of 2-PAM.

The Antarctic Ocean is considered to be one of the last pristine environments, and the need to assess anthropogenic pollutant effects of species in it were undertaken by Stefano et al. (2008). They studied the inhibitory effects caused by the pesticides azamethiphos and diisopropylfluorophosphate on the ChEs of *P. jacobaeus*. These authors found that azamethiphos was highly effective in inhibiting cholinesterasic activity, but diisopropylfluorophosphate did not have this effect.

Arufe et al. (2007) studied the effects of azinphosmethyl on the mortality in relation to AChE in *S. aurata* larvae. The study results demonstrated that this insecticide could exert lethal effects in exposed larvae, and the effects were accompanied by a significant AChE inhibition. The relevance of the results obtained by Xuereb et al. (2007) was discussed after they assessed the effects of the OP insecticide chlorpyrifos on ChEs of *G. pulex*. They concluded that the mortality of individuals of this crustacean species occurred at AChE inhibitions of approximately 50% of the initial activity. This means that lethal results can be produced at ecologically relevant concentrations of chlorpyrifos.

The toxic effects of commercial formulations (mixtures), rather than only the technical compounds are important in environmental toxicology. Gambi et al. (2007) showed the differences between the pesticide carbaryl and its commercial formulation on the enzyme kinetics of the earthworm *Eisenia andrei*. The effects of the single compounds and formulated pesticides were somewhat similar in inhibiting AChE. However, when the inhibition was time- and dose-correlated, differences existed in the way the exposure occurred. Notwithstanding, the authors emphasized the importance of AChE inhibition as an effect criterion for pesticide exposure in earthworms. Earthworms are often the unintended targets of pesticides, and when they interact it often culminates in lethal effects. To evaluate the impact of pesticides on earthworms, Panda and Sahu (2004) quantified the extent of AChE inhibition that occurred in tissues of the oligochaete *Drawida willsi*, after exposure to three common insecticides (butachlor, malathion, and carbofuran). These authors observed a significant decrease of AChE activity after exposure to the last two compounds, and this effect persisted for periods ranging from 45 to 75 days. This result indicates the potential deleterious effect that may be derived from pesticide use, even when the use is at levels that give ecologically relevant dosages.

The environmental toxicity of OP pesticides is not limited to immediate or acute effects, as illustrated in a study of the interaction of OPs and ChEs to assess the ecotoxicological impact of pesticides in earthworms (*E. fetida* and *L. terrestris*) (Rodríguez-Castellano and Sanchez-Hernandez 2007b). These authors observed the phenomenon of ChE "aging," which depends upon the release of an alkyl group from the OP–ChE complex, following the inhibition of ChE activity by OPs. This release has a direct consequence: the enzyme can no longer be reactivated, neither spontaneously, nor by the activity of reactivating chemicals, such as pralidoximes. This "aging" of ChEs in earthworms is highly dependent on the chemical structure of the OP pesticide tested, and may possibly be reverted through the use of pralidoxime; consequently, the amount of the antidote used to reactivate inhibited ChEs in earthworms can serve as a contamination index to evaluate OP pesticidal contamination.

Intoxication and inhibition of ChEs by OP compounds is usually followed by a period of recovery. The study by Barata et al. (2004) reported such behavior sensitivity and recovery efficacy of AChE and CbE in juveniles of the freshwater crustacean *D. magna*. In general, CbE was most sensitive to the OPs malathion, and chlorpyrifos; however, both enzymes had equal sensitivity to the CB carbofuran. Recovery rates were similar for all three tested compounds and for both enzymes, since a period of 12–96 h was sufficient to mitigate the inhibitory effects. The environmental significance of this research article was subsequently underscored by other observations that were clear in showing that exposure to OPs and CBs have different consequences. The mortality rates of *D. magna* juveniles increased after OP pesticide inhibition of about 50% of the basal AChE activity; in contrast, carbofuran caused a more pronounced response, since mortality was significantly increased after a very slight AChE inhibition. It is assumed that these results have ecological importance, because they demonstrate that mechanistically similar compounds may have distinctly different outcomes in terms of lethality.

The cholinesterases present in muscle tissue of the crayfish *P. clarkii* were responsive to high concentrations of the insecticide fenitrothion (Escartín and Porte 1996). Both AChE and BChE were significantly inhibited after exposure to a concentration of 20 μg/L. The AChE inhibitory response was dose-dependent; death resulted after reaching AChE inhibition rates of between 39 and 42%. The time course of the intoxication showed that the enzyme inhibition was long lasting. According to the mathematical model that described the recovery kinetics of this intoxication, the predicted time of recovery for muscle AChE was 29 days; therefore, the mode of action of OPs implies a definitive, irreversible inhibition of the affected enzyme, requiring de novo synthesis.

4.2 Use for Diffuse Contamination Sources

Malany et al. (1999) showed that AChE hydrolytic activity was highly dependent upon the electrostatic environment in which hydrolysis takes place. The enzyme itself shows a strong electrostatic dipole that is believed to be important for its

hydrolytic capacity. In most AChE forms, the movement of substrates into the catalytic gorge is favored for cationic molecules, such as acetylcholine (Massoulié et al. 2008). It is thus licit to affirm that AChE activity may be theoretically modulated by many ionic interactions with charged substances that may exist in the environment, namely those deriving from anthropogenic activities. In fact, several studies point to the establishment of an allosteric interaction, not in the active site, but in a distinct segment of the protein, that can explain the inhibition observed for some agents (Kitz et al. 1970). The use of AChE inhibition for environmental analysis has been traditionally linked to pesticide contamination of the aquatic and terrestrial compartments. However, the alleged versatility of AChE inhibition, as an effect criterion after exposure to other non-specific classes of contaminants, has led researchers to employ this biomarker in broad studies for the assessment of effects of diffuse sources of contamination. Kopecka et al. (2004) offers a good example of this trend, since these authors suggested that AChE inhibition be used in selected species to evaluate the anthropogenic contamination status in the area of the Gulf of Gdansk (Baltic Sea, Poland). The authors found that, in general terms, AChE inhibition (measured in gill tissue of the mussel species *Mytilus trossulus*, and also in the muscle tissue of the fish *P. flesus*) followed the patterns and gradients of contamination, mainly resulting from the presence of hydrocarbon runoff (from accidental oil spills), port activities, and domestic and industrial sewage discharges.

4.2.1 Metals

Inhibition of ChEs was reported for a significant number of non-OP pesticides; compounds such as metals or detergents have been found to significantly inhibit in vitro AChE activity in sensitive species, such as *P. reticulata* (Garcia et al. 2000). However, these results seem contradictory with the results published by Romani et al. (2003), who showed that chronic exposure of the fish species *S. aurata* to sublethal concentrations of metallic copper could result in an increase of acetylcholinesterasic activity, both in muscle and brain tissue.

Despite the above-mentioned studies, the point concerning significant interference of metals by AChE (Frasco et al. 2005) suggests that this trend may be an artifact. According to Frasco et al. (2005), AChE inhibition by metals may not be an actual inhibition and may not derive from interference with the catalytic activity of the enzyme. Rather, the inhibition may result from an interference with an important particle present in the reactive medium. The most common methodology employed for determining AChE activity was developed by Ellman et al. (1961). Their method is one in which the enzymatic catalytic activity is responsible for cleavage of the artificial substrate ASCh; this leads to the formation of thiocholine, which in turn reacts with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid; DTNB)) to form a colored complex that can be assessed at a wavelength of 412 nm. Metals, as observed by the authors, can react with all particles present in the reaction media, reducing the formation of the colored complex, and, thereby, leading to the erroneous conclusion that AChE is being inhibited. These observations are only

relevant under in vitro conditions, because exposure in living organisms cannot be measured the same way. This may explain the results obtained by Cunha et al. (2007), when they studied the potential effects of metallic species on ChEs of the gastropods *Monodonta lineata* and *Nucella lapillus*. These authors observed that cadmium increased the ChE activity of *N. lapillus*, whereas no effects were observed on the ChE of *M. lineata* following in vivo exposure. However, in vitro copper exposure decreased the ChE activity of both species, but exposure in vivo was incapable of exerting any effects.

Several examples of published studies indicate the lack of in vivo response of ChEs to metals. Corsi et al. (2004) showed that the cholinesterasic forms present in the scallop *Adamussium colbecki* were refractory to zinc metal exposure, compromising its use in biomonitoring programs for assessing metal contamination in Antarctica. Metallic compounds, such as zinc, copper, and cadmium, were incapable of inducing any sensitive modification in ChE activity from the digestive gland of the snail species, *Helix aspersa* (Dahm et al. 2006). Lead was used to study the influence of metals on several parameters in the grasshopper species *Aiolopus thalassinus* (Schmidt and Ibrahim 1994); these authors observed that lead was capable of significantly inducing ChE activity in this insect. Stefano et al. (2008) reported a high refractory behavior of the ChEs of *P. jacobaeus* after in vitro exposure to zinc; however, the same enzymes were dose-dependently inhibited by cadmium. These apparently contradictory results show that metallic inhibition of ChEs is entirely dependent on the chemical species tested.

The discussion on the feasibility of using ChE inhibition to assess metal toxicity is directly connected to the dose needed to exert a deleterious effect. Sánchez-Hernandez (2001) is quite clear about this point. The concentrations of a metallic species needed to cause a significant ChE inhibition in test organisms is incomparably higher than the amounts of pesticide needed to cause a similar effect. It is not plausible that such high concentrations will ever, in fact, be commonly attained under normal environmentally relevant conditions. Hence, wild organisms are not expected to succumb to effects from metal exposure that results from ChE inhibition.

4.2.2 Surfactants, Detergents, and Other Organic Compounds

Guilhermino et al. (2000) reported a significant inhibition of AChE by surfactant agents in the crustacean species *D. magna*. The authors monitored the influence on these daphnids of the detergents dodecyl benzyl sulfonate, sodium dodecylsulphate (SDS), and a commercial domestic formulation and concluded that all agents were capable of inhibiting AChE, following both in vitro and in vivo exposures. However, Nunes et al. (2005) observed that the alleged inhibitory effect initially reported may derive from the specific physical effect of the detergent activity of such compounds. This work showed that after homogenizing the nervous tissue of a test organism, detergents (such as SDS) tend to form micelles that dissolve portions of cellular membranes. These micelles integrate AChE that is lipid-bound to cellular membranes and prevent the enzyme from interacting with the chemicals used in the

Ellman assay. Such micelles can be reverted by modifying the dieletric constant of buffered media by adding ethanol. Ethanol destabilizes the micelles and results in the release of membrane-bound AChE. By adding ethanol the authors were able to reduce the inhibitory effects of SDS. This suggests that the inhibitory effects caused by exposure to detergents may be a protocol artifact. Li (2008) showed that inhibition of ChE of the planarian species *Dugesia japonica* by detergents or surfactants was clearly dependent on the chemical structure of the compound. This author found that ChE inhibition was possible after exposure of test organisms to Hyamine 1622, pentadecafluorooctanoid acid, perfluooctane sulfonate, and four nonylphenol. In contrast, exposure of planarians to Triton X-100 caused a significant increase in cholinesterasic activity. From these results, no clear pattern arose, indicating that any general conclusion of ChE inhibition by these chemicals is uncertain.

Gonçalves et al. (2010) showed that no effects occurred after acute in vivo exposure of *G. holbrooki* to two types of detergents: anionic (sodium dodecyl sulfate) and cationic (benzalkonium chloride) compounds. Despite the different typology of the tested substances, no evidence existed to show AChE impairment. From these results, again no clear pattern emerged, which leaves doubt that these ChEs are actually inhibited by these chemicals.

The inhibition of AChE by nonspecific agents was referred to by Pham et al. (2010), when they were considering the biological and environmental effects caused by ionic liquids. These authors published an extensive review about the putative effects of ionic liquids, and observed that they could impair a large number of physiologic pathways, such as oxidative homeostasis, AMP deaminase, and most importantly, AChE inhibition.

Solé et al. (2008) showed the utility of AChE inhibition in marine Ecotoxicology. The authors observed a significant inhibition of muscle tissue AChE in the fish *L. pholis*, probably from exposure to neurotoxic compounds present in the marine environment that derive from urban and industrial pollution along the Portuguese Atlantic coast. Klumpp et al. (2007) employed ChE inhibition as a tool to assess anthropogenic effects on the marine fish *Plectropomus leopardus* taken from the Great Barrier Reef. They concluded that ChE was inhibited in fish captured at sampling sites located near pontoons, indicating that man-made chemicals are impacting these areas by exerting deleterious effects on wildlife. The fish species *H. plumieri* can be a sensitive species for assessing the effects of man-made chemicals in reef ecosystems (Leticia and Gerardo 2008). The results of this study showed that the AChE present in the tissues of this species was inhibited by chlorpyrifos and benzo(a)pyrene, which were used as model pollutants. This finding affirmed the potential role for this marine organism as a bioindicator species for monitoring organic chemical contamination.

With the aim of using standardized tools in a future biomonitoring programs adapted to the Antarctic region, Bonacci et al. (2009) studied the potential for using ChE activity in gill from the scallop species, *A. colbecki*. This project allowed the researchers to observe significant cholinesterasic inhibition following in vitro exposure of gill tissue to a combination of Aroclor 1260 (a polychlorinated benzene mixture) and to EPA 610 (a mixture of 16 polyaromatic hydrocarbons).

5 Future Perspectives for the Use of Cholinesterases in Environmental Assessment

From the studies reviewed in this article one can clearly conclude that ChE inhibition may be a valid tool for ecotoxicological assessment of anthropogenic and natural chemicals. However, its valid use requires knowledge about the biological function, forms, and especially, the types of inhibitory interactions of CHEs that may derive from the presence of several chemical classes in the environment. A summary of the main points researchers should remember when employing ChE inhibition assays is as follows:

1. Most important for researchers wanting to rely on ChE-based assessment is to characterize the catalytic properties of the enzyme(s) used; the details on the predominant form of ChEs present in a given tissue of a specific species must be known. As observed for a large number of research articles, ChE characterization is an important underpinning for successful use, particularly when using an unknown species in field studies. Without a positive identification of the type and activity of the cholinesterasic form in tissues of a given species, it will be impossible to express results in terms of a specific cholinesterasic form. Moreover, if one does not know, in detail, the predominant cholinesterasic form present in the evaluated organism, then quantifying the basal levels of enzymatic activities for all isozymes will be rendered impossible. After the forms present are known, one can also successfully test for sensitivity in field monitoring surveys, which may enhance the value of any ecotoxicity assessment.
2. Results show that ChEs are not only different in their substrate affinity/inhibition, but can also be distinct in molecular terms; to differentiate ChE forms, electrophoresis analysis can be a useful tool to discriminate the trends in molecular weight of the enzymatic forms present. Electrophoresis may also be useful for understanding the behavior of carboxylesterases and their isozymes, which are also sensitive to the presence of pesticides in the wild (Kristoff et al. 2010).
3. Researchers must define the mechanisms by which ChE inhibition may occur if at all possible. Without knowing the inhibition mechanism one cannot be certain that results may be affected by artifacts. Indeed, it is possible to overestimate the importance of ChE inhibition as an effect criterion in environmental studies if one does not know the molecular mechanisms that underlie inhibition. What has been found to occur with metallic species and detergents are paradigmatic: some metals and tensioactive compounds are able to significantly inhibit ChE activity in several organisms but only when using in vitro approaches. This means that the enzyme, under in vivo conditions is a actual contaminated field, may or may not be inhibited, because in vitro results may or may not predict those that will occur in vivo. Furthermore, the use of standardized assays that are based on the catalytic activity of an enzyme and a colorimetric reaction (e.g., Ellman method), and occur in a water-based buffered medium, are subject to several confounding factors. When in vitro conditions are used to study the mechanistic aspects of the toxic response, it is common to incubate contaminants with cellular suspensions.

Under such conditions, the contaminant that is being tested may react (e.g., metallic compounds) or otherwise make unviable (e.g., detergents), the chemical reactions that underlie the testing protocol.

4. Researchers who employ cholinesterasic inhibition as an effect criterion must consider the different sensitivities that exist from organism to organism. One can find species that are refractory to common anticholinesterasic agents, whereas others are extremely sensitive.
5. The biological implications of enzymatic inhibition must also be analyzed, because they may represent pronounced behavioral changes that possess ecological relevance or may cause serious ecosystem imbalances in a test system.

The manifold data collected in recent years from the profusion of studies on ChE inhibition as an effect criterion is ample proof of the viability of this monitoring tool. Cholinesterasic inhibition has been used in laboratory-based bioassays, in field monitoring of OP and CB pesticide exposure, and as an indirect measure or diagnostic parameter of contamination. This method has also been used as an analytical tool to improve the understanding of the relationship between chemicals and biological structures present in different species. However, the diversity of ChEs (differentiated by their chemical nature, form, type, hydrolytic activity, inhibition profile, and sensitivity to common pesticides) sometimes confounds the understanding of how contaminants can threaten wildlife. To avoid confounding factors, it is mandatory that critical steps be developed to allow the correct and useful employment of ChE assessment in the environmental sciences.

6 Summary

Cholinesterase (ChE) is one of the most employed biomarkers in environmental analysis. Among ChEs, potentially the most significant in environmental terms is acetylcholinesterase (AChE), an enzymatic form that terminates the nerve impulse. Because of its physiological role, AChE has long been considered a highly specific biomarker for organisms exposed to anticholinesterasic agents, primarily agrochemicals (organophosphate and carbamate pesticides). The effects of these pesticides depend upon their selective inhibition of AChE. Because large amounts of such pesticides are employed, it is plausible that they exert neurotoxic effects on some non-target species. Therefore, AChE is among the most valuable of diagnostic tools that can be used to verify exposure to such chemical agents. It is well known that assays are available for use in quantifying AChE in multiple tissues of several test organisms. Enzymes other than AChE (e.g., butyrylcholinesterase and carboxylesterases) have also been used as putative markers for detecting the environmental presence of contaminating compounds. Researchers must use a step-by-step approach to identify the most prominent cholinesterasic form present in a given species, so that this form can be distinguished from others that may interfere with its use. Such fundamental work must be completed prior to using ChEs for any monitoring to assess for anticholinesterasic effects.

Despite massive employment in environmental analysis, using ChE inhibition as an endpoint or effect criterion has been unsettled by the discovery that ChEs may interact in the environment in previously unknown ways. Several chemicals, in addition to anticholinesterasic pesticides, are now known to inhibit ChE activity. Such chemicals include detergents, metals, and certain organic compounds such as hydrocarbons. The situation is made worse, because the literature is contradictory as to the ability of such chemicals and elements to interact with ChEs. Some results indicate that ChE inhibition by metals, detergents, and complex mixtures do not or are unlikely to occur. These problems and contradictions are addressed in this review.

It is our purpose in this review to address the following practical issues related to the ChEs:

1. The situations and organisms in which ChEs have been employed as biomarkers in laboratory trials, and the need to fully characterize these enzymatic forms before they are used for environmental assessment purposes.
2. The ways in which the ChEs have been used in field monitoring, and the potential for use of other complimentary markers to diagnose organophosphate exposure, and how drawbacks (such as the absence of reference values) can be overcome.
3. What requirements must be satisfied prior to implementing the use of ChEs as biomarkers in species not yet studied.
4. How direct linkages have been established between ChE inhibition and effects from inhibition observed at higher levels of integration (e.g., behavioral changes and population effects, or other indices of ecological relevance).
5. The potential for ChE inhibition to be applied as an effective parameter of toxicity to detect for the environmental presence of compounds other than the organophosphate and carbamate pesticides, and the limitations associated therewith.

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