Applications of Carboxylesterase Activity in Environmental Monitoring and Toxicity Identification Evaluations (TIEs)

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1 Introduction

The purpose of this review is to examine uses of carboxylesterase activity in environmental monitoring with a specific emphasis on pyrethroid insecticides. The chapter begins with an overview of the enzyme class, including general structure, function, catalytic mechanism, and substrate specificity. This section serves to introduce carboxylesterases, their biological significance, and their role in metabolism and detoxification reactions. Following this section, an in-depth analysis of different reports of applications of carboxylesterase activity in environmental monitoring is presented on an organism-specific basis. From an environmental standpoint, one of the most important carboxylesterase-mediated reactions is the hydrolysis and subsequent detoxification of pyrethroid insecticides. This reaction is one of the main detoxification pathways for pyrethroids in numerous organisms ranging from worms to fish to humans and is also an important pathway for the development of insect resistance to pyrethroid-associated toxicity. Accordingly, this class of insecticide is reviewed in more detail, with emphasis on toxicity and physical properties. The high hydrophobicity of pyrethroids is specifically addressed with a discussion of the effects of surface adsorption upon the observed toxicity in aquatic testing systems. A particular point is that changing agricultural practices combined with new legislation are causing a shift in insecticide usage patterns from organophosphates (OPs) and carbamates to pyrethroids. The effects of this shift are complex and potentially far reaching, especially the environmental consequences. In particular, the extreme toxicity of pyrethroids to many aquatic organisms, combined with their hydrophobicity, has resulted in concern regarding their potential environmental effects. This concern is exacerbated by the fact that current Toxicity Identification Evaluation (TIE) protocols devised for the identification of insecticides (and other environmental contaminants) in aqueous and sediment samples do not identify pyrethroid-associated toxicity with complete certainty. To address this shortfall, the use of carboxylesterase activity to hydrolyze pyrethroids in aquatic toxicity testing has been proposed as a simple, mechanistically based method to selectively identify pyrethroid-associated toxicity. This chapter reviews TIE protocols and the role of carboxylesterase activity in the development of TIE methods. A series of case studies are presented in which carboxylesterase activity was employed to identify pyrethroid-associated toxicity. Additional methods for the selective detection of pyrethroid-associated toxicity are also examined, including the use of temperature differentials and piperonyl butoxide (PBO). The strengths and weaknesses of the carboxylesterase-addition technique are also analyzed, with a number of distinct recommendations made for future development. Taken together, this review provides a detailed analysis of multiple applications of carboxylesterase to environmental monitoring and strongly advocates for further work on this enzyme system.

2 Carboxylesterases

Carboxylesterases are enzymes in the α/β-hydrolase fold family that catalyze the hydrolysis of carboxyl esters via the addition of water, as shown in Fig.1 (Junge and Krisch 1975; Myers et al. 1988; Ollis et al. 1992; Aldridge 1993; Cygler et al. 1993; Heikinheimo et al. 1999; Oakeshott et al. 1999; Satoh and Hosokawa 2006; Hosokawa et al. 2007). The α/β-hydrolase fold is a superfamily of enzymes that also includes cholinesterases (Quinn 1987, 1997, 1999), epoxide hydrolases (Morisseau and Hammock 2005; Newman et al. 2005), and phosphotriesterases (such as paraoxonase) (Sogorb et al. 2004) as well as other enzymes (Ollis et al. 1992; Hotelier et al. 2004). In standard esterase nomenclature, carboxylesterases are termed B-esterases in that they are inhibited by OPs, as opposed to A-esterases, which are defined as hydrolyzing uncharged esters that are not inhibited by OPs or other acylating inhibitors (Aldridge 1953a,b, 1993). Carboxylesterases are found in many tissues including liver, lung, small intestine, heart, kidney, muscle, brain, testis, adipose tissue, nasal and respiratory tissues, leukocytes, and the blood (see Satoh and Hosokawa 1998, and references therein). However, carboxylesterase expression and activity are tissue- and organism dependent, with levels and activities varying widely (Imai 2006). Carboxylesterases consist of multiple isozymes that vary with both the tissue and organism (Hosokawa et al. 1995; Satoh and Hosokawa 1995; Imai 2006), making individual nomenclature complicated. These

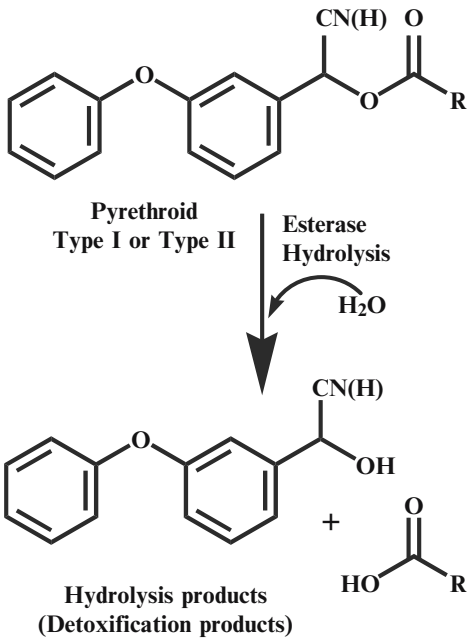


Fig. 1 Esterase-mediated hydrolysis of pyrethroids. Esterases hydrolyze an ester via the addition of water to form the corresponding alcohol and acid, which are generally detoxification products

enzymes play a significant role in the metabolism and subsequent detoxification of many agrochemicals and pharmaceuticals (representative structures are shown in Fig. 2) (Redinbo and Potter 2005; Potter and Wadkins 2006). In particular, carboxylesterases hydrolyze pyrethroids (Abernathy and Casida 1973; Stok et al. 2004a; Wheelock et al. 2004) and bind stoichiometrically to carbamates (Gupta and Dettbarn 1993; Sogorb and Vilanova 2002) and organophosphates (Kao et al. 1985; Casida and Quistad 2004). Carboxylesterases are also important in the metabolism of a number of therapeutics (Williams 1985), including the cholesterol-lowering drug lovastatin (Tang and Kalow 1995), the antiinfluenza drug oseltamivir (Tamiflu) (Shi et al. 2006), the narcotic analgesic meperidine (Demerol) (Zhang et al. 1999), and cocaine and heroin (Pindel et al. 1997). Carboxylesterase activity is also used extensively in soft- and pro-drug design (Bodor and Buchwald 2000, 2003, 2004), as demonstrated by activation of the cancer therapeutic pro-drug CPT-11 through its conversion to SN-38 (Potter et al. 1998; Wadkins et al. 2001). Given the importance of this enzyme class in metabolizing this suite of compounds, interest in the study of their function, distribution, and selectivity is greatly increasing. It should also be

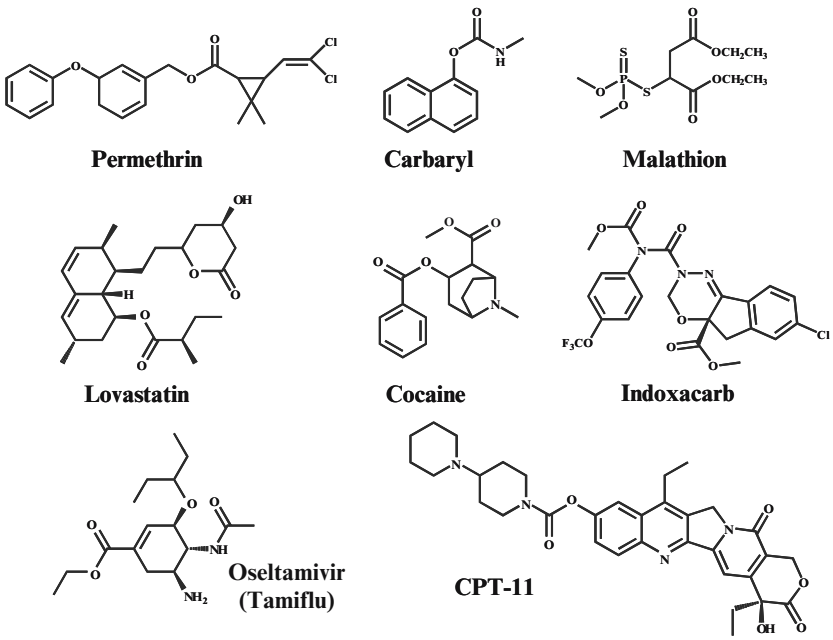


Fig. 2 Structures of common agrochemicals and pharmaceuticals that interact with esterases. Permethrin is a pyrethroid, carbaryl is a carbamate, malathion is an organophosphate (OP), indoxacarb is an oxadiazine, lovastatin is a cholesterol-lowering drug used in the treatment of cardiovascular disease, cocaine is a tropane alkaloid, oseltamivir or Tamiflu is an antiviral drug used in the treatment of influenza, and CPT-11 or Irinotecan is a chemotherapy agent that is a topoisomerase 1 inhibitor used mainly in the treatment of colon cancer. All compounds, with the exception of carbaryl, are hydrolyzed by carboxylesterases

mentioned that there are enzymes in other protein families that are not technically carboxylesterases but do hydrolyze esters; however, this review focuses solely on carboxylesterase-mediated ester hydrolysis.

2.1 Esterase Hydrolysis: Mechanism and Inhibition

The mechanism by which esterases hydrolyze their substrates has been examined by many research groups using both biochemical and structural means. The detailed mechanism of hydrolysis has been reviewed elsewhere (Satoh and Hosokawa 1995, 1998; Quinn 1997, 1999; Satoh et al. 2002; Sogorb and Vilanova 2002; Redinbo et al. 2003) and is only briefly presented here. Interested readers are referred to the immediately foregoing references for a more extended presentation of the hydrolysis mechanism. The publication of crystal structures of mammalian carboxylesterases (Fig. 3) has greatly contributed to our understanding of the enzyme mechanism

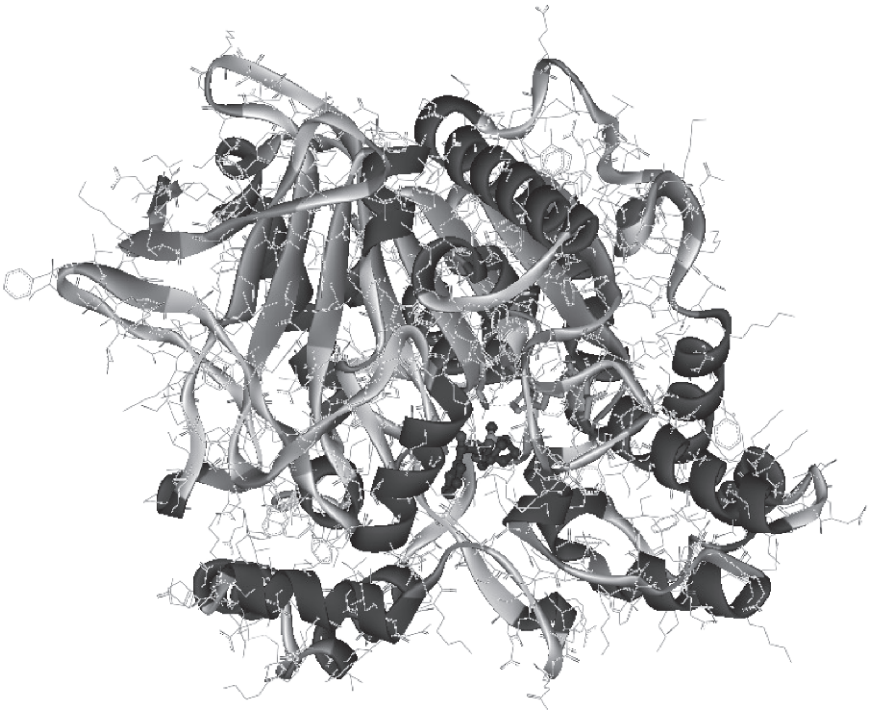


Fig. 3 Solid ribbon structure of human liver carboxylesterase 1 (hCE1) complexed with homatropine, shown as a darker ball-and-stick structure in the *lower right part* of the figure. The figure was generated from the crystal structure of Bencharit et al. (Bencharit et al. 2003a,b), from the RCSB protein data bank (http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1MX5). [The image was created with DS ViewerPro 5.0 (Accelrys, San Diego, CA) and is reproduced from Wheelock et al. (2005c) with kind permission from the *Journal of Pesticide Science*.]

(Bencharit et al. 2002, 2003a,b, 2006; Fleming et al. 2005, 2007). The general mechanism involves a catalytic triad centered on the GXSXG active serine motif for serine esterases (Ollis et al. 1992). For carboxylesterases, this motif consists of a Ser, His, and either a Glu or Asp residue (Ser221, His468, and Glu354 for human carboxylesterase 1, hCE1) (Bencharit et al. 2003a,b); however, recent work has illustrated a potential fourth catalytic residue, which is also a serine amino acid (Stok et al. 2004b). Carboxylesterases cleave esters via a two-step process that involves the formation and degradation of an acyl-enzyme intermediate. The catalytic or nucleophilic serine is first activated to generate the oxygen nucleophile, which then attacks the carbonyl carbon of the ester substrate, leading to formation of the acyl-enzyme intermediate. The alcohol hydrolysis product is then released to undergo nucleophilic attack by water, leading to the release of the carboxylic acid and return of the catalytic amino acids to their original state.

Several different types of carboxylesterase inhibitors have been reported in the literature. The main structural motifs include trifluoromethyl ketone (TFK)-containing inhibitors (Székács et al. 1992; Wheelock et al. 2001), OP derivatives (Casida and Quistad 2004), carbamates (Gupta and Dettbarn 1993; Sogorb and Vilanova 2002), diones (Hyatt et al. 2005, 2007; Wadkins et al. 2005; Hicks et al. 2007), and sulfonamides (Wadkins et al. 2004). Each of these compound classes has been used to study carboxylesterase biochemistry and function. TFK-containing inhibitors are based upon the inclusion of an electron-deficient carbonyl moiety in the molecule, which covalently binds to the enzyme (Brodbeck et al. 1979; Wheelock et al. 2002). These compounds are transition-state analogue inhibitors that exhibit slow tight-binding kinetics; however, the covalent bonding is reversible, and the enzyme is reactivated over days to weeks (Abdel-Aal and Hammock 1986). TFK-containing inhibitors are useful tools for studying esterase-mediated biological processes (Wheelock et al. 2002, in press) and have been proposed as potential biocontrol agents (Rosa et al. 2006). For example, work by Wheelock et al. (2006) demonstrated that the TFK-based compound 1,1,1-trifluoro-3-octylthiol-propan-2-one (OTFP) was more efficient at inhibiting carboxylesterase activity than chlorpyrifos-oxon. Inhibition of porcine esterase by chlorpyrifos-oxon was essentially 100% (as determined by enzyme activity assay); however, 72 hr later, ~72% of activity had been recovered, whereas the OTFP-inhibited esterase remained fully inhibited. Accordingly, caution should be taken when using OP-based inhibitors in esterase studies. However, the oxon-forms of OP insecticides, such as paraoxon (*O*,*O*-diethyl *p*-nitrophenyl phosphate), are generally potent carboxylesterase inhibitors ($k_i = 1.4 \times 10^6$ M^{-1} min^{-1} for rat serum carboxylesterase; Maxwell and Brecht 2001). The phosphorylated enzyme can either release the OP substrate (by undergoing hydrolysis similar to an acyl group, but at a much slower rate), or it can undergo aging, where the enzyme is essentially catalytically dead (i.e., the OP acts as a suicide substrate) (Maxwell 1992a). A mechanistic description of these processes is shown in Fig. 4. A similar reaction can occur with carbamates; however, the methylcarbamoylated enzyme is less stable than the phosphorylated enzyme, thus accounting for the decreased toxicity of some carbamates relative to OPs (Casida and Quistad 2004). A number of papers have reviewed the

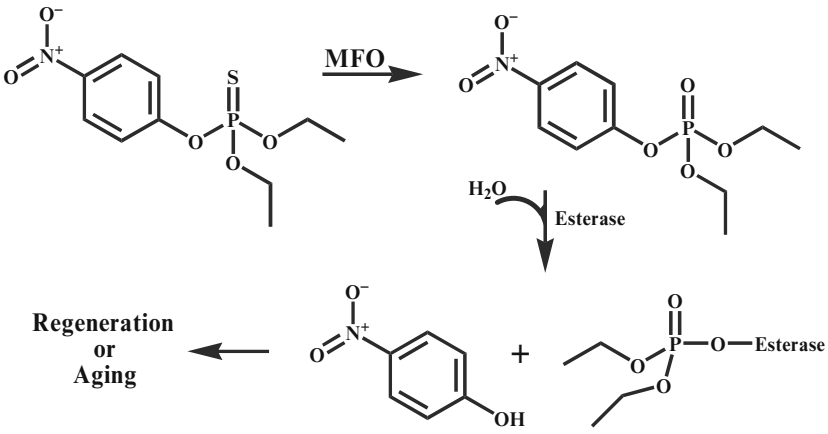


Fig. 4 Carboxylesterase inhibition mechanism for the organophosphate insecticide parathion. Parathion is first activated via mixed-function oxidases (*MFO*) to the "active" oxon form (paraoxon), which is the inhibitory structure of the compound. Paraoxon then binds to the esterase and is hydrolyzed by the addition of water, releasing *p*-nitrophenol. The phosphorylated esterase can then either release the phosphate group and regain catalytic activity, or become "aged" where the phosphate remains permanently bound and the enzyme loses catalytic activity. [Figure 4 is reproduced from Wheelock et al. (2005c), with kind permission from the *Journal of Pesticide Science*.]

interactions of carbamates and OPs with carboxylesterases (Fukuto 1990; Gupta and Dettbarn 1993; Sogorb and Vilanova 2002; Casida and Quistad 2004, 2005).

2.2 Role of Carboxylesterases in Agriculture

Carboxylesterases play an important role in agrochemical efficacy and detoxification (Ahmad and Forgash 1976a,b; Casida and Quistad 1998, 2004; Sogorb and Vilanova 2002; Wheelock et al. 2005c) by interacting with three major classes of agrochemicals: OPs (Kao et al. 1985; Satoh and Hosokawa 2000), carbamates (Gupta and Dettbarn 1993), and pyrethroids (Casida et al. 1983). Major pathways for agrochemical metabolism have been reviewed elsewhere, and involve a number of different enzyme systems that are beyond the scope of this review, including P450 monooxygenases (P450 MOs) (Kulkarni and Hodgson 1984), glutathione *S*-transferases (GSTs) (Fournier et al. 1992; Enayati et al. 2005), phosphotriesterases (Sogorb et al. 2004), as well as carboxylesterases (Wheelock et al. 2005c). It is well known that variability in carboxylesterase levels and relative isozyme abundance contribute to the selective toxicity of ester-containing insecticides in a range of organisms from fish to insects and mammals (Brooks 1986; Chiang and Sun 1996; Li and Fan 1997; Wheelock et al. 2003, 2005a; Huang and Ottea 2004; Stok et al. 2004a).

OPs and carbamates exhibit their toxicity by inhibiting acetylcholinesterase (Maxwell 1992b; Gupta and Dettbarn 1993) and causing widespread disruption of the nervous system as a result of buildup of the neurotransmitter acetylcholine (Quinn 1987). OPs require activation by P450s to the corresponding oxon form (Fig. 5) before they effectively inhibit acetylcholinesterase, whereas carbamates do not require metabolic activation. Carbamates and OPs bind stoichiometrically to carboxylesterases, leading several researchers to postulate that carboxylesterases act as an agrochemical sink to protect acetylcholinesterase from OP/carbamate-mediated toxicity (Gupta and Kadel 1990; Maxwell et al. 1994; Yang and Dettbarn 1998; Dettbarn et al. 1999; Sweeney and Maxwell 1999; Kuster 2005). Cleavage of pyrethroids by carboxylesterases (see Fig. 1) is one of the main detoxification pathways for this group of pesticides in both mammals (Casida 1980) and insects (Davies 1985). Accordingly, organism exposure to OPs and/or carbamates, followed by pyrethroids, can cause synergistic toxicity (Gaughan et al. 1980; Martin et al. 2003), and these effects have been demonstrated in multiple organisms (Abernathy and Casida 1973; Casida et al. 1983; Denton et al. 2003; Choi et al. 2004). For example, one of the principal metabolic pathways for malathion is via carboxylesterase-mediated hydrolysis, and the toxicity of malathion correlates with carboxylesterase levels in humans, rats, and mice (Talcott 1979; Talcott et al. 1979a,b,c). Li and Fan (1996) showed that, in five different species of freshwater fish, carboxylesterase inhibition increased malathion toxicity. Accordingly, the

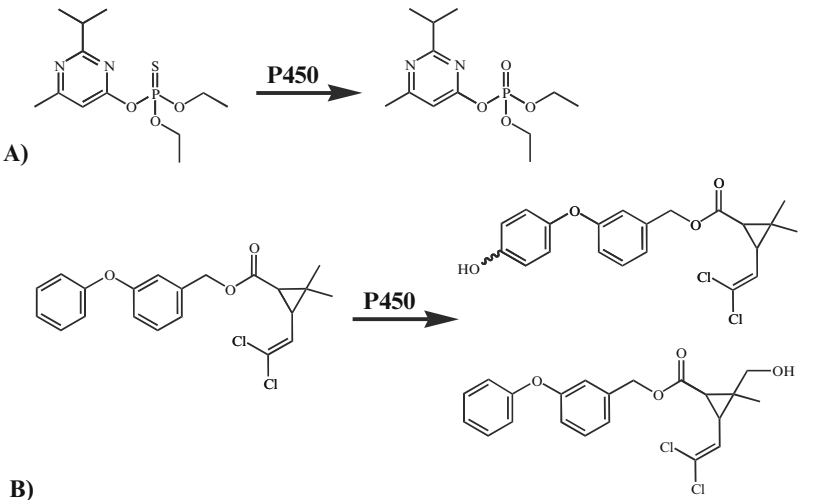


Fig. 5 Cytochrome P450-mediated metabolism of (A) diazinon and (B) permethrin. The addition of piperonyl butoxide (PBO) inhibits P450s and has divergent effects upon the observed toxicity to reactions *A* and *B*. P450s convert diazinon (and OPs in general) to the active oxon form, which inhibits acetylcholinesterase, thus increasing toxicity. Conversely, P450-mediated metabolism of permethrin (and pyrethroids in general) is a detoxification process

concept of agrochemical interactions is well established, and the importance of examining the toxicity of chemical mixtures has been emphasized in recent literature (Lydy et al. 2004).

A major concern in agrochemical use is the development of insect resistance (Casida and Quistad 1998), which can lead to increased pesticide use, cost increases, and detrimental environmental effects. A prominent pathway of resistance development to agrochemicals involves increased levels of carboxylesterases (Newcomb et al. 1997; Hemingway and Karunaratne 1998; Byrne et al. 2000; Harold and Ottea 2000; Oakeshott et al. 2005; Cui et al. 2007). This pathway has been demonstrated in multiple species of insects with OPs and pyrethroids (Immaraju et al. 1990; Elzen et al. 1992; Solomon and Fitzgerald 1993; Cahill et al. 1995; Mazzarri and Georghiou 1995; Zhao et al. 1996; Ahmad et al. 2002; McAbee et al. 2004). The study of carboxylesterases and their interactions with OPs and pyrethroids is therefore economically important. One particular area of interest is the development of isozyme-selective inhibitors of insect pyrethroid-hydrolyzing esterases. This type of inhibitor could be coapplied with pyrethroids as a synergist, in a similar fashion as PBO, to control esterase-mediated resistance. Other areas of active research include the synthesis of selective pyrethroid surrogate substrates to measure carboxylesterase activity to identify resistance mechanisms (McAbee et al. 2004; Devonshire et al. 2007). Carboxylesterase-mediated agrochemical resistance can also have ramifications for human health. Pyrethroids and OPs are extensively used for vector-borne disease control and are particularly important in the fight against malaria (Curtis and Mnzava 2000; Greenwood et al. 2005; Coleman and Hemingway 2007).

2.3 Use of Carboxylesterase Activity in Environmental Monitoring

Increasingly, there is need to monitor the environment for the presence of agrochemicals. Bioassays are commonly used in environmental monitoring and depend upon well-established biological endpoints that correlate with agrochemical exposure. A classic example is the use of acetylcholinesterase activity as an index of OP exposure (Fulton and Key 2001). Because exposure to OPs results in inhibition of acetylcholinesterase activity, it can be used to provide a measure of organism exposure (Rickwood and Galloway 2004). This approach has a number of advantages: (1) it is a mechanistically based biological effect that can be directly correlated with compound exposure, (2) it does not require expensive analytical instrumentation, and (3) it can be performed in the field. Positive hits can be validated, as necessary, with analytical data [i.e., gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS) data]. Although well established in the literature (Sturm et al. 2000; Fulton and Key 2001; Galloway et al. 2002; Rickwood and Galloway 2004), recent reports have questioned the use of acetylcholinesterase as a sole biomarker of OP and/or carbamate exposure (Galloway et al.

2004b; Rickwood and Galloway 2004; Wheelock et al. 2005a). Studies have shown that OPs and potentially carbamates have increased affinity for carboxylesterase over acetylcholinesterase, suggesting that carboxylesterase activity will provide a more sensitive endpoint (Gupta and Dettbarn 1993; Escartin and Porte 1997; Wogram et al. 2001; O'Neill et al. 2004; Wheelock et al. 2005a).

Because an organism's sensitivity to pyrethroid, OP, or carbamate exposure may be influenced by its endogenous carboxylesterase activity, that activity may be useful in predicting the effects of agrochemical exposure upon ecosystem health (Barata et al. 2004). However, there are currently insufficient data available in the literature to fully validate this concept. Emerging research involves the use of carboxylesterase activity as a biomarker of organism exposure or susceptibility to agrochemicals (Huang et al. 1997; Sanchez-Hernandez et al. 1998; Galloway et al. 2004b). Of the limited work performed in this area, most has focused on esterase levels in fish (James 1986; Wogram et al. 2001). Early work demonstrated that carboxylesterase levels in rainbow trout have different expression levels throughout development (Kingsbury and Masters 1972), suggesting that different life stages may vary in susceptibility to esterase inhibitors or ability to hydrolyze substrates. Further work showed that rainbow trout have lower levels of carboxylesterase activity than mammals (Glickman and Lech 1981, 1982; Glickman and Casida 1982; Glickman et al. 1982). Additionally, it has been found that activity varies among fish species (Barron et al. 1999; Wheelock et al. 2005a) and can account for differences in susceptibility to pyrethroid toxicity (Glickman et al. 1979). However the extreme susceptibility of many fish species to pyrethroid toxicity may be more a function of sensitivity of their sodium channels then low carboxylesterase activity (Glickman and Casida 1982; Glickman and Lech 1982), although carboxylesterase activity may play some role in toxicity.

Carboxylesterase activity in relation to agrochemical exposure has been examined in additional species and may therefore be useful for ecosystem-wide environmental monitoring projects (Wilson and Henderson 1992; Chevre et al. 2003; Galloway et al. 2004a,b). A significant advantage of carboxylesterase activity-based biomarkers is that assays can be nondestructive in contrast to use of brain acetylcholinesterase activity. Plasma carboxylesterase activity is generally low in mammals, but it is higher in fish, amphibian and birds (Thompson et al. 1991a). Thompson et al. (1991b) reported that serum carboxylesterases were generally more sensitive to OP exposure then brain acetylcholinesterase. A number of different species have been examined for carboxylesterase activity in response to agrochemical exposure. Of the many studies performed, only a few are discussed here to illustrate the prospects for application to ecosystem-wide monitoring.

2.3.1 Applications in Fish

Work by Wogram et al. (2001) compared the sensitivity of acetylcholinesterase, butyrylcholinesterase, and carboxylesterase in the three-spined stickleback (*Gasterosteus aculeatus*) following exposure to parathion. Results were mixed,

with exposure to 0.01 and 0.1 µg/L not affecting activity of any of the three enzymes, although exposures at 1.0 µg/L decreased butyrylcholinesterase activity in the liver (~60%), axial muscle (30%), and gills (30%). No effects were observed on acetylcholinesterase or carboxylesterase activity. Carboxylesterase activity has been reported to be an appropriate biomarker in the fathead minnow (*Pimephales promelas*) (Denton et al. 2003), the spotted gar (*Lepisosteus oculatus*) (Huang et al. 1997), gilthead seabream (*Sparus aurata*) larvae, and the Nile tilapia (*Oreochromis niloticus*) (Pathiratne and George 1998). Ferrari et al. (2007) demonstrated that carboxylesterase inhibition is a good biomarker of exposure to azinphos-methyl and carbaryl in juvenile rainbow trout (*Oncorhynchus mykiss*), with significant carboxylesterase inhibition observed after 24 hr exposure to 2.5 µg/L and 1 mg/L azinphos-methyl and carbaryl, respectively. Work with channel catfish (*Ictalurus punctatus*) indicated that carboxylesterases were inherently more sensitive to inhibition by OP compounds, including chlorpyrifos-oxon, paraoxon, and DEF (*S*,*S*,*S*-tributylphosphorotrithioate), but those data did not confirm that carboxylesterases protected against acetylcholinesterase inhibition (Straus and Chambers 1995). However, data from two populations of mosquitofish (*Gamusia affinis*) showed that carboxylesterases exhibited a much higher affinity for OPs than acetylcholinesterases, suggesting a protective role (Chambers 1976). Küster (2005) examined the use of zebrafish (*Danio rerio*) embryos in assessing insecticide exposure using methyl-paraoxon. Data showed that carboxylesterase activity was ~40 times higher then acetylcholinesterase activity at a very early stage of development (6-somite stage), suggesting that carboxylesterases could serve as a stoichiometric buffer system. However, at later stages of development (48 hr postfertilization), the enzyme activities were approximately equal and data showed similar inhibition of the two enzymes following methyl-paraoxon exposure. Therefore, results were inconclusive as to whether carboxylesterase would be superior to acetylcholinesterase for environmental monitoring in this system. Follow-up studies by Küster and Altenburger (2006) concluded that carboxylesterase activity was not preferentially inhibited relative to acetylcholinesterase following methyl-paraoxon exposure. However, the authors made the point that their choice of substrate for carboxylesterase activity assays (*S*-phenylthioacetate) may have confounded the results as this substrate can also be hydrolyzed by paraoxonases (PONs) and arylesteraes (A-esterases) (Küster and Altenburger 2006). *S*-Phenylthioacetate has been used for measuring both carboxylesterase and acetylcholinesterase activity by a number of authors (Bonacci et al. 2004; Corsi et al. 2004; Arufe et al. 2007), which underscores the importance of substrate selection in ensuring an accurate measurement of the desired endpoint.

It has also been reported that both the total carboxylesterase specific activity as well as individual response to OP inhibition are organism specific. A comparison of topmouth gudgeon (*Pseudorasbora parva*), goldfish (*Carassius auratus*), Nile tilapia (*Tilapia nilotica*), mosquitofish (*Cambsua affinis*), and rainbow trout (*Oncorhynchus mykiss*) found that overall activity in the liver was species dependent (Li and Fan 1996, 1997). In addition, the tissue distribution of carboxylesterase activity was species dependent. However, Baron et al. (1999) reported that the carboxylesterase activity of whole-fish homogenates from juvenile rainbow trout

(*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), fathead minnows (*Pimephales promelas*), and bluegill (*Lepomis macrochirus*) did not vary significantly. Tissue-specific variations were observed, with greater activity in the liver than gills. Results from Wheelock et al. (2005a) supported these findings with assays from three teleost species, Chinook salmon (*Oncorhynchus tshawytscha*), medaka (*Oryzias latipes*), and Sacramento splittail (*Pogonichthys macrolepidotus*), showing that whole-body homogenates demonstrated essentially equivalent carboxylesterase activity. Wheelock et al. (2005a) also examined the dose–response relationship in Chinook salmon following exposure to an OP and pyrethroid insecticide. Exposure to chlorpyrifos at a high dose (7.3 µg/L), but not a low dose (1.2 µg/L), significantly inhibited acetylcholinesterase activity in both brain and muscle tissue (85% and 92% inhibition, respectively), whereas esfenvalerate exposure had no effect in this species. In contrast, liver carboxylesterase activity was significantly inhibited at both the low and high chlorpyrifos doses (56% and 79% inhibition, respectively), while esfenvalerate exposure still had little effect. The inhibition of carboxylesterase activity at levels of chlorpyrifos that did not affect acetylcholinesterase activity suggests that some salmon carboxylesterase isozymes may be more sensitive than acetylcholinesterase to inhibition by OPs.

Carboxylesterase activity has also been used as a biomarker of exposure to non-acetylcholinesterase-inhibiting compounds. For example, the white-spotted rabbitfish (*Siganus canaliculatus*) has been suggested as a biomarker of organotin exposure (Al-Ghais et al. 2000). Taken together, these studies provide a solid base demonstrating that carboxylesterase may be useful for development as a biomarker of exposure to OP and/or carbamate insecticides, as well as other contaminants. However, the inconsistent nature of study results makes direct comparisons difficult, and a great deal of additional data is required before strong conclusions can be drawn.

2.3.2 Applications in Bivalves

Bivalve mussels have been used extensively for environmental monitoring (O'Connor 2002; Sarkar et al. 2006). It has been demonstrated in these organisms that carboxylesterase activity has greater sensitivity then does cholinesterase to inhibition by OPs or carbamates (Ozretic and Krajnovic-Ozretic 1992; Escartin and Porte 1997; Basack et al. 1998; Galloway et al. 2002). Specifically, studies with the mussel *Mytilus galloprovincialis* examined the sensitivity of acetylcholinesterase, butyrylcholinesterase, and carboxylesterase to fenitrothion, fenitrooxon, and carbofuran exposure (Escartin and Porte 1997). Results showed that carboxylesterase activity in the digestive glands and gills was significantly higher than was acetylcholinesterase (~40 fold and 3 fold, respectively) or butyrylcholinesterase activity (~100 fold and 70 fold, respectively). In addition, carboxylesterase activity was much more sensitive to both fenitrothion and fenitrooxon exposure than was acetylcholinesterase activity, although both enzymes were equally sensitive to carbofuran exposure. Similar work in the mussel *Mytilus edulis* indicated that

carboxylesterase activity was slightly more sensitive than was acetylcholinesterase to paraoxon and chlorpyrifos exposure (Galloway et al. 2002).

2.3.3 Applications in Crustaceans

Crayfish (*Procambarus clarkia*) are reported to be a useful model species for biomarker studies (Vioque-Fernandez et al. 2007b), but results in field studies did not exhibit direct correlations between pesticide concentrations and esterase activity, suggesting that other factors could affect esterase activity (Vioque-Fernandez et al. 2007a). Field studies were performed with the freshwater crustacean *Asellus aquaticus* (L.) (an isopod) in sites above and below a sewage treatment facility above the Mersey estuary in England (O'Neill et al. 2004). Results showed that both acetylcholinesterase and carboxylesterase activity declined at sites downstream of the sewage effluent discharge; however, carboxylesterase activity was more severely inhibited (27% of control values) and continued to decline at greater distances from the treatment facility.

Studies with the cladoceran *Daphnia magna* have evaluated the use of carboxylesterase activity as a marker of OP exposure. Results showed that carboxylesterase activity was more sensitive to malathion and chlorpyrifos exposure than to acetylcholinesterase, whereas the two enzymes demonstrated equivalent sensitivity to the carbamate carbofuran (Barata et al. 2004). Follow-up field studies in the Delta del Ebro in northeast Spain demonstrated that both acetylcholinesterase and carboxylesterase activity strongly correlated with fenitrothion levels (Barata et al. 2007). Laboratory studies demonstrated that carboxylesterase activity in *Daphnia magna* is sensitive to malathion exposure, with a complete loss in activity following a 15- or 30-min exposure to 0.2 ppm malathion (Bond and Bradley 1997).

2.3.4 Applications in Algae

A number of studies have evaluated esterase activity in algae for use in environmental monitoring. However, the mechanisms by which esterase activity is reduced in algae are unclear. All algae studies cited examined responses in esterase activity following exposure to non-OP or carbamate insecticides. Early work by Blaise and Ménard (1998) demonstrated that esterase activity could be an appropriate indicator of sediment toxicity. This concept was expanded by Regel et al. (2002), who examined esterase activity in two species of algae (*Microcystis aeruginosa* and *Selenastrum capricornutum*) following exposure to acid mine drainage in a South Australian stream. Exposure to acid mine drainage for 1 hr resulted in 30%–70% reduction in esterase activity, which was maintained for 24 hr. Similar studies were performed with seven benthic marine algae, with the diatom *Entomoneis* cf. *punctulata* being the most suitable for assay development. A whole-sediment and water-only toxicity test was developed for algae based upon inhibition of esterase activity, with results showing sensitivity to copper and sediment particles (copper tailings) (Adams and Stauber 2004). Follow-up studies in which *Entomoneis* cf *punctulata* was exposed to hydrocarbon-contaminated sediments showed this assay to have efficacy (Simpson

et al. 2007). These authors recommended the use of algal esterase activity as a whole-sediment TIE method to determine the contribution of hydrocarbon contamination to sediment toxicity.

2.3.5 Applications in Terrestrial Organisms

Studies on carboxylesterase activity following exposure to insecticides are not limited to aquatic organisms. Several studies have been performed in terrestrial organisms including birds, lizards, and earthworms. The use of earthworms in environmental monitoring was proposed because earthworms ingest large amounts of soil and are continuously exposed to contaminants through their alimentary surfaces. These applications have recently been reviewed and may be of interest to readers (Sanchez-Hernandez 2006; Castellanos and Sanchez-Hernandez, 2007).

Extensive studies have been performed in avian systems (Thompson 1993). A study comparing plasma cholinesterase and carboxylesterase activity in pigeons (*Columba livia*), American kestrels (*Falco sparverius*), red-tailed hawks (*Buteo jamaicensis*), and Swainson's hawks (*Buteo lineatus*) reported generally lower inhibition of carboxylesterase activity than cholinesterase activity following OP exposure (Bartkowiak and Wilson 1995). Studies aimed at developing a nondestructive biomarker of OP exposure examined blood esterase levels in Japanese quail (*Coturnix coturnix japonica*) and swallows (*Hirundo rustica*) following azamethiphos exposure (Fossi et al. 1992, 1994; Lari et al. 1994). Data showed that carboxylesterase activity was consistently less inhibited then acetylcholinesterase activity, potentially indicating that carboxylesterase activity was a more sensitive endpoint. Carboxylesterase activity in the nestling European starling (*Sturnus vulgaris*) was found to be more sensitive to diazinon exposure then to acetylcholinesterase, but methyl-paraoxon and aldicarb exhibited higher affinities for plasma acetylcholinesterase (Parker and Goldstein 2000). Additional studies in starlings observed a dose–response relationship in serum cholinesterase and carboxylesterase activities following exposure to demeton-*S*-methyl and triazophos (Thompson et al. 1991b). Field studies were conducted with nestling and adult great tits (*Parus major*) following spray drift exposure to pirimicarb and dimethoate (Cordi et al. 1997). Adults demonstrated significant reductions in butyrylcholinesterase activity 24 hr after dimethoate and pirimicarb exposure (51% and 67% of preexposure values, respectively). However, no significant inhibition was observed for serum carboxylesterase activity. Studies with nestlings showed significant decreases in both butyrylcholinesterase and carboxylesterase activity following exposure to dimethoate (66% and 77% of preexposure values, respectively). Interestingly, significant inhibition (27%) was observed in dead nestlings from hedges treated with pirimicarb, whereas no significant reductions in butyrylcholinesterase activity were observed. Interspecies differences in esterases were evaluated in seven species of wild birds to investigate their different susceptibilities to OPs, with results evidencing an inverse correlation between brain acetylcholinesterase and plasma carboxylesterase activity in relationship to body size (Fossi et al. 1996). Carboxylesterase levels were examined in a series of European raptors, with data

suggesting that diet affected enzyme activity (Roy et al. 2005). It has been speculated that the wide range of esterases present in the pheasant (*Phasianus colchicus*) may protect it against anticholinergenic pesticides and thereby contribute to its success in regions of the United States where other avian species are adversely affected by pesticides (Baker et al. 1966).

Field studies with the lizard *Gallotia galloti* found a 50% reduction in serum carboxylesterase activity following spray application with parathion (Sanchez et al. 1997a). In addition, laboratory studies with *Gallotia galloti* found that carboxylesterase activity was inhibited for a longer duration than was brain acetylcholinesterase activity following parathion exposure, suggesting that carboxylesterase activity was a superior biomarker (Sanchez et al. 1997b). However, field studies with *Gallotia galloti* in the Canary Islands found that carboxylesterase and acetylcholinesterase activity were essentially equally inhibited following exposure to the OP trichlorphon, whereas butyrylcholinesterase activity was only slightly affected (Fossi et al. 1995). The toad *Chaunus schneideri* was assessed for its ability to serve as an indicator organism for agrochemical exposure (Attademo et al. 2007). Toads were collected in rice fields and surrounding environments and in a reference (control) pristine forest. Carboxylesterase activity in plasma of toads collected from agricultural areas was depressed relative to control values, suggesting that plasma carboxylesterase activity could serve as a nondestructive test for exposure to pesticides.

2.3.6 Miscellaneous Applications

Hamers et al. (2000) reported the development of a small-volume bioassay for quantifying the inhibition potency of OPs and carbamates in rainwater. They compared the use of purified acetylcholinesterase from the electric eel (*Electrophorus electricus*) and carboxylesterase activity from a homogenate of honeybee heads (*Apix mellifera*). Results showed that carboxylesterase activity demonstrated greater sensitivity than acetylcholinesterase activity, with an assay detection limit of "esterase inhibiting potency in rainwater" of 2 ng dichlorvos equivalents per liter. Based upon this assay, they were able to quantify esterase inhibition potential in four rainwater samples, measuring dichlorvos equivalents of 12–125 ng/L. Follow-up studies employed the assay to determine esterase inhibition potency of rainwater collected over 26 consecutive periods, and reported that dichlorvos equivalents exceeded permissible levels in The Netherlands (Hamers et al. 2003). Studies conducted with the lugworm (*Arenicola marina*) found that carboxylesterase levels were significantly lower than those of cholinesterase suggesting to the authors that cholinesterase would be a potentially useful biomarker for this species (Hannam et al. 2007). Studies in the marine worm *Nereis (Hediste) diversicolor* following exposure to temephos showed significant acetylcholinesterase and carboxylesterase inhibition, with carboxylesterase displaying the greatest sensitivity (Fourcy et al. 2002). Carboxylesterase activity has been used in a multiple biomarker study designed to measure the effects of dimethoate exposure in spiders (Babczynska et al. 2006).

Carboxylesterase activity has also been investigated for applications in the selective bioactivation of herbicides in weeds (Gershater et al. 2006). Proteins from a range of important commodity crops and economically important weeds were assayed for carboxylesterase activity. The crops included maize, rice, sorghum, soybean, flax, and lucerne, and the weeds were *Abutilon theophrasti*, *Echinochloa crusgalli*, *Phalaris canariensis*, *Setaria faberii*, *Setaria virdis*, *Sorghum halepense*, and the model plant *Arabidopsis thaliana*. Hydrolysis activity was measured using a range of herbicidal esters including 2,4-D methyl ester, clodinafop propargyl, fenthioprop ethyl, fenoxaprop ethyl, bromoxynil octanoate, and cloquintocet mexyl as well as the insecticide permethrin. Significant hydrolysis of the majority of herbicides was observed, with very few exceptions. Consequently, the applications of carboxylesterase activity in environmental monitoring can potentially be extended to plants and their ability to metabolize ester-containing pesticides. Ileperuma and coworkers (2007) crystallized a carboxylesterase from a kiwifruit species (*Actinidia eriantha*) and showed that it was significantly inhibited by paraoxon, demonstrating that a plant carboxylesterase had a similar inhibitor-binding mechanism as mammalian orthologues. An additional area not covered in this review involves microbial esterases (Bornscheuer 2002), which have important roles in the degradation of agrochemicals (Karpouzas and Singh 2006; Singh and Walker 2006). It is very possible that microbial enzymes could be employed in biomonitoring and bioremediation approaches (Sutherland et al. 2002, 2004), with the particular benefit that it is possible to engineer microbial enzymes with the desired activity and substrate specificity (Yang et al. 2003). Further work should examine the potential to exploit this system.

2.3.7 Future Applications

Taken together, these studies demonstrate a concerted effort to evaluate use of carboxylesterase activity as an indicator of agrochemical exposure. Although results are mixed, existing data suggest that the concept has merit and justifies continued development of this endpoint for use in environmental monitoring studies. However, the use of carboxylesterase activity as a biomarker of exposure will be challenging. Little work has been done to characterize the constitutive levels of esterases in most species, and it is difficult to correlate measured levels of enzyme activity with observed effects. Reduced enzyme activity may either be an indication of enzyme inhibition following exposure to OPs or carbamates or an indication of low esterase expression resulting from other environmental factors. Moreover, very little is known about inducers of carboxylesterase levels. It is possible that some environmental contaminants cause increases in constitutive carboxylesterase levels. It is therefore necessary to develop a method to quantify absolute levels of carboxylesterase. One potential approach is the development of species-specific antibodies, allowing for quantification of the level of carboxylesterase present and subsequent determination of inhibition levels. Because enzyme purification and antibody generation are time intensive, this approach will be expensive. Another potential method is use of reactivation protocols as a biomarker of pesticide exposure to differentiate dilution-

reversible inhibitions (carbamate exposure) from dilution-irreversible effects (OP exposure) (Sanchez-Hernandez 2006; Vioque-Fernandez et al. 2007b).

It is also possible that esterase activity could be used as a biomarker of susceptibility, similar to work done with activation of OPs by cytochrome P450s (Keizer et al. 1995). By examining a large numbers of individuals, it could be possible to establish a range of constitutive enzyme levels for endogenous carboxylesterase titers. These levels could then be scored on their ability to hydrolyze pyrethroids, or bind OPs and/or carbamates, with lower scores rated as more sensitive to pyrethroid and/or OP/carbamate toxicity. This process would essentially create an index of susceptibility based upon carboxylesterase activity. One limitation to this concept is illustrated by the fact that rainbow trout sodium channels are more sensitive to pyrethroid toxicity than mouse sodium channels (Glickman and Lech 1982). Therefore, caution must be exercised in interpreting susceptibility based upon constitutive carboxylesterase activity. However, a biomarker of susceptibility to agrochemicals would be useful for examining ecosystem effects and could eventually be employed in risk assessments. Further research should focus on characterizing esterase hydrolytic profiles in a range of species with a goal to examine the relationship to agrochemical toxicity.

An important point in the development of standardized carboxylesterase monitoring protocols will be the selection of the substrate employed for activity measurements (Wheelock et al. 2005a,c). The choice of substrate can profoundly influence observed enzyme activity. The most common substrates currently used include acetyl esters of *p*-nitrophenol (*p*-nitrophenyl acetate, PNPA) or naphthol (α- and β-naphthyl acetate). These substrates are employed because assay endpoints can be measured easily and inexpensively with spectrophotometers. However, the biological significance of these compounds is not known. Some authors have attempted to employ more environmentally realistic substrates, such as pyrethroid surrogate substrates (Riddles et al. 1983; Butte and Kemper 1999; Wheelock et al. 2003; Stok et al. 2004a; Huang et al. 2005, 2006; Devonshire et al. 2007). Although more appropriate measurements of pyrethroid hydrolysis activity, these substrates are not necessarily appropriate for estimating the extent of hydrolysis (i.e., detoxification) of other compounds. In addition, the rates of hydrolysis of pyrethroid surrogate substrates are often so low that they are impractical for many organisms (Wheelock et al. 2005a). It is therefore not appropriate to use a single substrate to characterize esterase activity of crude tissue homogenate. Because multiple esterase isoforms are usually present in the preparation, it is optimal to have a battery of substrates for full characterization of enzyme activity. For example, analyses performed on PNPA and pyrethroid hydrolysis activity in human liver microsomes showed very little correlation between the hydrolytic profiles ($r^2 = 0.29$ for a fenvalerate surrogate), suggesting that different enzymes are involved in the hydrolysis of the two substrates (Wheelock et al. 2003). Therefore, monitoring of PNPA activity, or that of other general substrates, may not provide an accurate account of pyrethroid hydrolysis. Similarly, Stok et al. (2004a) reported that the portion of pyrethroid hydrolysis activity in mouse liver microsomes was only 0.5% that of total esterase activity (as measured by PNPA). Ultimately, it is best to use a suite of substrates for environ-

mental monitoring purposes. The employment of standard substrates with facile esters would enable a theoretical maximum measure of hydrolysis activity as well as comparisons with literature values. However, if such data were combined with more-specific substrates, then a broader measure of the effects of exposure to insecticides and/or other esterase-inhibiting compounds could be performed.

If one is looking at esterase activity in a high-throughput system to evaluate its ability to degrade environmental chemicals, having surrogate substrates is very important because they can lead to inexpensive and quantitative assays that can be routinely performed. For example, an alternative made increasingly attractive by the use of 96- and 384-well plate readers and sophisticated robotics is the use of an array of esterases with varying sensitivity, with substrates such as OPs and carbamates, as an environmental screen for inhibitors of these enzymes (Wortberg et al. 1996). Robotic systems and computer control allow enzymes with different substrates to be used in a high-throughput manner, enabling the rapid screening of multiple surrogate substrates. There are mathematical approaches that can be used to integrate the output of such studies for the tentative identification of environmental contaminants. Such arrays offer a broader range of sensitivity to xenobiotics than any one enzyme. For example, the pattern of inhibition may suggest the composition of mixtures or the identity of a specific inhibitor. In addition, surrogate substrates can be used to drive the purification of esterases from environmental organisms, to guide selection of recombinant esterases in artificial evolution and protein engineering, and for quality control of esterase batches in TIE procedures discussed later. However, it must be remembered that despite their many advantages surrogates are not the substrates of interest. The hypothesis that the substrate used is an appropriate surrogate for the environmental chemical targeted must be tested repeatedly. Also, with the advances in LC-MS and other analytical technologies, it is increasingly attractive to use the actual environmental contaminant of interest rather than or in addition to surrogate substrates.

It is clear that there is interest and need for an increased battery of biomarkers and that a number of researchers are working avidly on the problem (Thompson 1999; Hyne and Maher 2003; Galloway 2006; Galloway et al. 2006; Sarkar et al. 2006). It is likely that carboxylesterase activity will be a valuable addition to methods for determining organism exposure and susceptibility to environmental contaminants. However, it is important to standardize activity assays, ensure that appropriate substrates are used, and make certain that data are interpreted correctly.

3 Pyrethroids

3.1 Introduction

Agrochemical usage practices are currently shifting, with a general movement away from OPs toward pyrethroid insecticides (Casida and Quistad 1998). These trends have been strengthened by passage of The Food Quality Protection Act

(FQPA) in 1996 (Wagner 1997; Glade 1998). The FQPA mandated that EPA consider the "*available information concerning the cumulative effects of such residues and other substances that have a common mechanism of toxicity … in establishing, modifying, leaving in effect, or revoking a tolerance for a pesticide chemical residue*" (Mileson et al. 1998). Accordingly, the use of OP insecticides is decreasing in California, with a subsequent increase in pyrethroid usage (Epstein et al. 2000). Pyrethroids now account for more than 18% of the world insecticide market (Pap 2003). The ecological implications of this large-scale shift in pesticide use are unknown. Pyrethroids generally have low mammalian toxicity (Abernathy and Casida 1973; Casida et al. 1983; Casida and Quistad 1995), especially when compared to many OP pesticides. However, there have been several reports regarding the sensitivity of aquatic invertebrates and some fish species to pyrethroids (Bradbury and Coats 1989b; Werner et al. 2002; Denton et al. 2003). There is growing concern that the ecological consequences of increased pyrethroid use on aquatic ecosystems could be far reaching.

3.2 Chemistry

3.2.1 Background

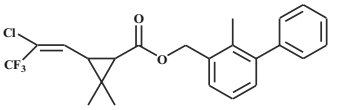
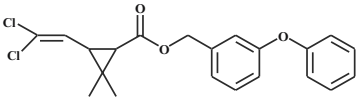
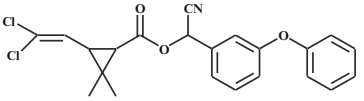
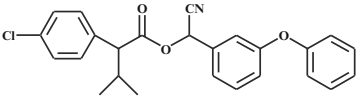
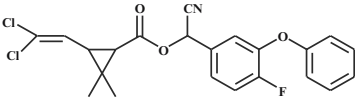
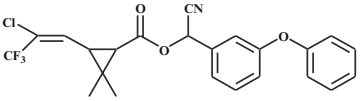
The pyrethroid insecticides are synthetic analogs of the naturally occurring pyrethrum flowers (Casida 1973; Elliott 1976; Davies 1985). Pyrethrum flowers are of the genus *Chrysanthemum*, of which there are two species, those with red and those with white flowers. Only those with white flowers, *Chrysanthemum cinerariaefolium* Vis, contain the insecticidal active components (Katsuda 1999). The original home of *Chrysanthemum* is the Dalmatian region of the former Yugoslavia, on the Mediterranean coast of the Adriatic Sea (Katsuda 1999). Fujitani (1909) first separated the insecticidal active mixture from pyrethrum flowers and termed the ester component "pyrethron." The structure of "pyrethron acid" was further probed by Yamamoto (1923, 1925) using elegant natural product chemistry, confirming the presence of the cyclopropane ring. Full structures for pyrethrins-I and -II were proposed by Staudinger and Ruzicka (1924), with LaForge and Barthel (1945) reporting that natural pyrethins consisted of four homologues (pyrethrins-I and -II, and cinerins-I and -II). The first pyrethroid, allethrin, was developed under the pressure of World War II by Schechter et al. (1949), first as a mixture of eight isomers from three chiral centers and finally as *S*-bioallethrin (the most active isomer, 1*R*, 3*R*, 4'*S*). The extract of pyrethrum flowers has long been used as an insect control agent but was superseded by the more effective and simpler chlorinated hydrocarbon and OP insecticides after World War II (Casida 1980). However, organochlorines and OPs have subsequently either been eliminated or curtailed because of a panoply of adverse characteristics. Their disappearance has made way for a new generation of pyrethrum derivatives that have improved photostability and selective toxicity, rendering these compounds appropriate for agricultural application. In the

1960s and 1970s, Michael Elliott and coworkers, as well as researchers at Sumitomo Chemical Co., developed a series of synthetic pyrethroids, several of which are still important insecticides, which were the precursors of current pyrethroids (Elliott et al. 1965, 1973a,b, 1974; Ohno et al. 1976). Pyrethroid structures are best described in terms of their distinct acid and alcohol moieties, with synthesis involving condensing these distinct groups to form a connecting ester moiety (essentially the reverse of the hydrolysis reaction shown in Fig. 1). Synthetic efforts focusing on the distinct acid and alcohol moieties were made in an attempt to improve the photostability, insecticidal properties, selective toxicity, and physical characteristics of this class of compounds as well as reduce their overall cost (Casida et al. 1983). The acid portion of the structure was standardized as chrysanthemic acid (Elliott et al. 1965), and then a range of alcohol moieties were tested for insecticidal activity. The instability to sunlight of the 5-benzyl-3-furylmethyl alcohol moiety (resmethrin) (Elliott et al. 1967) led to the inclusion of 3-phenoxybenzyl alcohol in pyrethroid structures (Elliott et al. 1973b). A number of earlier pyrethroids were developed with the original chrysanthemic acid moiety and varying alcohol moieties, including pyrethrin I, allethrin, and resmethrin (Casida 1980). To increase environmental stability, structural modifications in chrysanthemic acid were made including the substitution of the dichlorovinyl acid analogue with chlorine in place of methyl in the isobutenyl side chain (Elliott et al. 1973a) (permethrin; or with dibromovinyl acid to form deltamethrin) (Elliott et al. 1974). Another significant discovery was that the cyclopropane carboxylate moiety could be replaced with the corresponding α-isopropyl 4-chlorophenylacetate (fenvalerate) (Ohno et al. 1976). A major change in the alcohol moiety was also made involving the use of 3-phenozybenzaldehyde cyanohydrin instead of 3-phenoxybenzyl alcohol, to give an α-cyano group-substituted ester (Elliott 1976). This substitution is the distinguishing feature between type I and type II pyrethroids (i.e., permethrin vs. cypermethrin). This change had the effect of converting the ester linkage from a primary to a secondary ester, thereby increasing the chemical stability of the compounds. Additional changes in the alcohol moiety to affect the selectivity and toxicity of the compounds were achieved by adding fluorine substituents to the 3-phenoxybenzylalcohol moiety (cyfluthrin). Pyrethroids contain varying chiral centers and as such can have a variety of optical isomers, often with varying biological activity (Pap 2003). For example, there are eight different isomers of cypermethrin. The *S,S*-isomer of fenvalerate has substantial efficacy relative to the *R,R*-isomer or the racemic mixture, leading to the selective manufacture and sale of the *S,S*-isomer (esfenvalerate vs. fenvalerate). Accordingly, it is important that chirality be taken into account when analyzing different pyrethroids (Soderlund and Casida 1977).

3.2.2 Hydrophobicity

Pyrethroids exhibit a high degree of hydrophobicity, as shown in Table 1. The logs of the octanol–water partition coefficients (log K_{OW} or log P) are generally on the order of 5 or greater. This physical characteristic has a number of environmental

Table 1 Structure and physical constants of common pyrethroids [a]

Structure	Name[b] (type)[c]	Water solubility[d]	Vapor pressure[e]	Henry's constant[f]	Log P[g]
	Bifenthrin (I)	0.014	2.4×10^{-2}	7.2×10^{-3}	6.40
	Permethrin (I)	5.5	2.5×10^{-3}	1.4×10^{-6}	6.10
	Cypermethrin (II)	4.0	2.0×10^{-4}	3.4×10^{-7}	6.54
	Fenvalerate (II)	6.0	1.92×10^{-2}	1.4×10^{-7}	5.62
	Cyfluthrin (II)	2.3	<1	3.7×10^{-6}	5.97
	λ-Cyhalothrin (II)	5.0	1.0×10^{-3}	1.9×10^{-7}	7.0

[a]Data are from Laskowski (2002).

[b]Chemical names of each pesticide are as follows: permethrin ((3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylate, CAS 52645-53-1), bifenthrin ((2-methyl[1,1-biphenyl]-3-yl)methyl (1*R*,3*R*)-3-[(1*Z*)-2-chloro-3,3,3-trifluoro-1-propenyl]-2,2-dimethylcyclopropanecarboxylate, CAS 82657-04-3), cypermethrin ((+/−) α-cyano(3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate, CAS 52315-07-8), fenvalerate ((*R/S*)-α-cyano(3-phenoxyphenyl)methyl (α*R/S*)-4-chloro-(1-methylethyl) benzeneacetate, CAS 51630-58-1), cyfluthrin (α-cyano(4-fluoro-3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate, CAS 68359-37-5), λ-cyhalothrin ((*S/R*)-α-cyano(3-phenoxyphenyl)methyl (1*S*,3*S*)-3-[(1*Z*)-2-chloro-3,3,3-trifluoro-1-propenyl]-2,2-dimethylcyclopropanecarboxylate, CAS 91465-08-6).

[c]Type II pyrethroids contain an α-cyano group on the benzylic carbon (see Fig. 1).

[d]Units are μg/L.

[e]Units are mPa.

[f]Units are atm m^3 mol^{-1}.

[g]Values are the log of the partitioning constant between octanol and water (K_{OW}).

ramifications. It was initially thought that the low aqueous solubility of these compounds would prevent their runoff from sites of agricultural application. This issue can be of particular importance in California during dormant spraying, which often coincides with winter storm events (Werner et al. 2002, 2004; Teh et al. 2005). Migration of pyrethroids from soils following application has been observed in association with particulate material. It appears that pyrethroids

adsorb to sediments, which can then be washed offsite, through agricultural drainage ditches and eventually into larger waterways (Gan et al. 2005). There is still concern for the environmental impact of pyrethroids that leach from sites of application into aquatic ecosystems (Gan et al. 2005).

The growth in pyrethroid usage has resulted in a need to monitor environmental samples for the presence and potential toxic effects of pyrethroids. However, the hydrophobicity of pyrethroids can make it challenging to perform toxicity assays with aquatic samples (Lee S. et al. 2002; Wheelock et al. 2005b). These compounds readily adsorb to test containers, resulting in many studies reporting aqueous levels as "nominal concentrations." This fact can affect the outcome of toxicity testing results in that organisms may be exposed to lower pyrethroid concentrations than those targeted. In addition, field-collected samples can lose a large proportion of their pyrethroid (and other hydrophobic compounds) residues through adsorption to sampling and testing containers, resulting in underreporting of observed pyrethroid toxicity.

The effects of such pyrethroid loss during toxicity testing, as well as the magnitude of loss to sampling containers, was tested by Wheelock et al. (2005b). Results with different container types showed that pyrethroids adsorb to the container surface in a time-dependent manner. In addition, toxicity studies demonstrated that the effect of pyrethroid adsorption to the testing container was assay condition dependent. Toxicity studies were designed to examine the effect of sample incubation on toxicity. Spiked pyrethroid solutions were prepared and then added to the sample testing container and allowed to stand for the time intervals indicated in Table 2. Two test species were used. Toxicity testing with *Ceriodaphnia dubia* showed very distinct time-dependent adsorption effects upon observed toxicity with an approximate 50% reduction in toxicity between the 30-min and 4-hr incubation, whereas *Hyalella azteca* did not exhibit a significant change. Of particular importance is the observation that vortexing of the *C. dubia* samples after a 4-hr incubation resulted in almost complete recovery of pyrethroid-associated toxicity. These data suggest that the time-dependent loss of toxicity is in fact an adsorption phenomenon.

To further examine the time-dependent effects upon pyrethroid toxicity, similar studies were performed with fathead minnows (*Pimephales promelas*). These studies showed a slight effect of adsorption upon the observed toxicity between the 30-min incubation and subsequent incubations. An important observation from these studies is the effect of sample stirring upon pyrethroid toxicity. The data show that a 24-hr incubation of the pyrethroid-spiked sample before organism addition resulted in only 30% mortality relative to the 30-min incubation. However, simple stirring of the sample with a glass rod for 2 min was sufficient to restore the observed mortality to 100%. These data are important in that sample handling can potentially have profound effects upon the outcome of toxicity assays. Unfortunately, no studies to date have identified a testing method that can prevent systemic pyrethroid loss during sample collection and handling. It is therefore necessary, at a minimum, that methods rigorously describe how samples were manipulated. Sample shaking and stirring

Table 2 Effect of time to test initiation and sample handling on acute permethrin toxicity

Time (hr)[a]	Permethrin concentration (ng/L)[b]				LC_{50}[c]	95% CI[d]
Hyalella azteca[e]						
	0	25	50	75	LC_{50}	95% CI
0.25	13 ± 12	13 ± 12	87 ± 12	87 ± 12	40	34–46
0.5	6 ± 10	27 ± 23	73 ± 23	87 ± 12	35	28–45
1	7 ± 12	13 ± 12	93 ± 12	100 ± 0	35	33–38
2	13 ± 12	33 ± 23	73 ± 12	93 ± 12	38	29–48
4[f]	13 ± 12	0 ± 0	53 ± 12	93 ± 12	48	41–55
Ceriodaphnia dubia[g]						
	0	125	250	375	LC_{50}	95% CI
0.25	0±0	90 ± 12	100 ± 0	100 ± 0	66	61–78
0.5	5±10	85 ± 19	100 ± 0	100 ± 0	74	55–106
1	0±0	80 ± 16	100 ± 0	100 ± 0	78	58–107
2	0±0	70 ± 26	100 ± 0	100 ± 0	89	58–146
4[h]	0±0	40 ± 23	100 ± 0	100 ± 0	140	106–168
Pimephales promelas[i]						
	0	2000	4000	8000	LC_{50}	95% CI
0.5	0	0	5	100	5900	5400–6300
1	0	0	0	60	7300	—[k]
2	0	0	0	100	6000	6000–6000
4	0	0	0	75	6700	—
4/stirring[j]	0	0	0	100	6200	5300–7300
8	0	0	0	90	6200	5300–7300
8/stirring	0	0	0	90	6200	5300–7300
24	0	0	0	30	>8000	—
24/stirring	0	0	0	100	6000	6000–6000

[a]Test solutions were prepared at given permethrin concentrations and placed into test containers followed by organism addition at the time intervals indicated.

[b]Nominal water concentration.

[c]LC_{50} (concentration to cause 50% lethality) values are reported in ng/L. Values were calculated using Spearman–Karber analysis.

[d]95% confidence interval (CI) of the LC_{50} results.

[e]Three replicates of five *Hyalella azteca* used per treatment. Data are given as 96-hr % mortality. Results are from Wheelock et al. (2005b).

[f]Vortexing of the 4-hr sample did not affect the observed toxicity (data not shown).

[g]Four replicates of five neonate *Ceriodaphnia dubia* per treatment. Data are given as 48-hr % mortality. Results are from Wheelock et al. (2005b).

[h]Vortexing of the 4-hr sample served to increase the toxicity to nearly initial levels with 48-hr mortality being 85% ± 15% (data not shown).

[i]Fathead minnows obtained from Aquatox, Inc. (Hot Springs, AK) were maintained in EPA moderately hard (EPAMH) water until tested when 7 d old. Each test sample was tested using two replicates of 10 fish each in 400-mL glass beakers containing 250 mL test solution. Permethrin exposures were conducted at 2000, 4000, and 8000 ng/L in EPAMH water. Permethrin was obtained from Accustandard (New Haven, CT), and working standards (100 µg/L) were prepared in high-performance liquid chromatography-grade methanol. Methanol concentration in all test solutions was less than 0.1%. Fish were added to the test solutions 0.5, 1, 2, 4, 8 or 24 hr after permethrin addition. An aliquot of the 4-, 8-, and 24-hr solutions was vigorously stirred for 2 min before fish addition. Test duration was 96 hr, and test solutions were renewed at 48 hr. Fish were fed *Artemia nauplii* 4 hr before sample renewal at 48 hr. Tests were conducted at 25° ± 1°C with a 16 hr light:8 hr dark photoperiod. Mortality was noted daily. The concentration required to cause 50% mortality

(continued)

Table 2 (continued)

(LC_{50}) in each of the treatments was calculated from the mortality data using a computer program (ToxCalc, Ver. 5.0.23; Tidepool Scientific, McKinnleyville, CA). Standard deviations were not calculated because data were only collected in duplicates.
[j]Test containers were stirred vigorously for 2 min before organism addition.
[k]95% CI values could not be determined from the mortality data.

is of special importance. As demonstrated with the fathead minnow studies, stirring of the sample is sufficient to recover some of the pyrethroid-associated toxicity. It is assumed that the turbulence created during the stirring process is sufficient to create enough force to overcome the hydrophobic associations between pyrethroids and containers. The difficulty will be in developing a reproducible method for resuspending pyrethroids during sample handling. It is therefore extremely important that these effects be considered when performing toxicity assays. It is likely that a number of different toxicity testing systems are affected by the observations presented in Table 2, which could have important ramifications in environmental toxicity testing.

3.3 Toxicology

Pyrethroid toxicity varies greatly with species and pyrethroid type (Bradbury and Coats 1989b). The LD_{50} for pyrethroids are structure-, organism-, and life stage dependent. For example, the oral LD_{50} for deltamethrin in adult rats is 81 mg/kg versus 5.1 mg/kg in weanling rats (Sheets et al. 1994). Pyrethroids are primarily sodium channel toxins that prolong neural excitation, but they exhibit few or no direct cytotoxic effects (Casida et al. 1983). The major site of action of all pyrethroids is the voltage-dependent sodium channel (Narahashi 1996); however, a number of other potential interaction sites exist, such as the voltage-gated chloride channels (Forshaw et al. 1993), GABA-gated chloride channels (Bloomquist et al. 1986), and possibly protein phosphorylation (Enan and Matsumura 1993). The degree of sodium channel excitability is dose related, but the nature of the excitability is structure dependent (Coats 1990). Most pyrethroids exhibit much greater toxicity to insects than to mammals because insects have increased sodium channel sensitivity, lower body temperature, a lipophilic cuticle, and smaller body size (Bradberry et al. 2005). In addition, mammals are protected by poor dermal absorption of pyrethroids and rapid metabolism to nontoxic metabolites (Bradberry et al. 2005).

3.3.1 Human Toxicity

Pyrethroids generally exhibit low mammalian toxicity (Vijverberg and van den Bercken 1990; Ray and Forshaw 2000). Despite their extensive worldwide use, there are relatively few reports of human pyrethroid poisoning (Ray and Forshaw

2000). Fewer than 10 deaths have been reported from ingestion or following occupational exposure (Bradberry et al. 2005). In adults, 10 mg/kg oral doses have been reported to cause seizures (Tippe 1993), and some pyrethroids have been reported to cause systemic occupational poisoning in China (He et al. 1989). However, pyrethroids are rapidly hydrolyzed in the liver, thereby preventing the nervous system effects that are lethal to insects (Aldridge 1990). Many pyrethroids are less toxic to mammals than are the very safe natural pyrethrins. However. some of the more stable pyrethroids can give symptoms following occupational exposure. Pyrethrum formulations extracted from chrysanthemum flowers can also contain sesquiterpene lactones, which can cause allergic rhinitis and contact dermatitis (O'Malley 1997). However, processes to remove these components were developed many years ago, and synthetic pyrethroids do not contain these natural by-products.

The main routes of pyrethroid metabolism are through the action of esterases (see Fig. 1) and cytochrome P450s (see Fig. 5). As shown in Fig. 5, P450s can hydroxylate pyrethroids in a number of positions, thereby increasing their water solubility and providing chemical moieties for further conjugation and Phase II metabolic processes (Casida and Quistad 1995). Esterases hydrolyze pyrethroids to the corresponding alcohol and acid, and esterase activity correlates with pyrethroid-associated toxicity (Abernathy and Casida 1973). Further detail on metabolism of pyrethroids is beyond the scope of this review, but readers interested in this topic are referred to a number of available reviews (Bradbury and Coats 1989b; Aldridge 1990; Coats 1990; O'Malley 1997; Ray and Forshaw 2000; Bradberry et al. 2005).

3.3.2 Environmental Toxicity

Pyrethroids exhibit much greater toxicity to aquatic organisms than to mammals (Coats et al. 1989). Laboratory tests have shown that pyrethroids are extremely toxic to fish such as fathead minnow, rainbow trout, brook trout, bluegill, and sheepshead minnow (TDC 2003; Bradbury and Coats 1989a). Pyrethroids have negative temperature coefficients of toxicity, which means that their toxicity increases in colder water (Ware and Whitacre 2004). Pyrethroids are acutely toxic to aquatic insects and crustaceans, with most median lethal concentrations (LC_{50}s) well below 1 µg/L (TDC 2003). Anderson et al. (2006a) reported freshwater bifenthrin and permethrin LC_{50}s below 1 µg/L for the amphipod *H. azteca* (9.3 and 21.1 ng/L) and the mayfly genus *Procloeon* (84 and 90 ng/L); however, the LC_{50}s for the dipteran *Chironomus dilutus* were 6,000 and 10,000 ng/L, respectively. Wheelock et al. (2004) reported *C. dubia* LC_{50}s for five pyrethroids that ranged from 140 to 680 ng/L. Because pyrethroids quickly adsorb to laboratory exposure chambers, the reported thresholds probably underestimate the actual sensitivity of water column organisms (Wheelock et al. 2005b). It should also be stressed that the majority of laboratory-based toxicity studies are aqueous-only exposures that do not take into account the effects of organic material on observed toxicity. It is distinctly possible that the presence of

organic material in the testing system will significantly reduce the observed acute toxicity (Bondarenko et al. 2006; Yang et al. 2006a,b,c).

As previously stated, pyrethroids are hydrophobic chemicals that have a tendency to associate with sediment or humic particles. It is therefore more likely to detect pyrethroids in suspended and bedded sediments, and there is greater potential for environmental impacts in this compartment. Maund et al. (2002) reported that the toxicity of cypermethrin-containing sediments was dependent on sediment organic carbon content, with 10-d LC_{50}s as low as 3.6 mg/kg for the freshwater amphipod *H. azteca* and 13 mg/kg for the freshwater dipteran *Chironomus tentans*. Amweg et al. (2005) reported *H. azteca* LC_{50}s for several pyrethroids based on toxicity tests with spiked sediments containing various amounts of organic carbon that ranged from 6.6 ng/g for bifenthrin to 249 ng/g for permethrin. Anderson et al. (2007a) reported several pyrethroid LC_{50}s for two marine amphipod species. The LC_{50}s for bifenthrin, cypermethrin, and permethrin were 0.008, 0.011, and 0.140 mg/kg, respectively, for *Eohaustorius estuarius*, and 0.95, 0.47, and 8.9 mg/kg, respectively, for *Ampelisca abdita*.

Several sublethal effects have also been reported. Denton (2001) reported impacts on fish, including behavioral changes such as rapid gill movement, erratic swimming, altered schooling activity, and swimming at the water surface. Concentrations of pyrethroids as low as 10 ng/L reduced daphnid reproduction and lowered feeding filtration rates (Day 1989). Cypermethrin exposures below 0.004 µg/L significantly impaired salmonid olfactory responses, which could disrupt reproductive functions (Moore and Waring 2001). Several pyrethroids and their breakdown products were found to have endocrine activity (Tyler et al. 2000). For a comprehensive review of the effects of pyrethroids in field studies, see Oros and Werner (2005).

Fish demonstrate extreme sensitivity to pyrethroids (Bradbury and Coats 1989a), which is thought to be partly the consequence of their slow metabolism of the parent compound (Denton et al. 2003). The sensitivity of many fish to pyrethroid application has led to the development of so-called "fish-safe" derivatives including cycloprothrin, etofenprox, flufenprox, and silafluofen (Pap 2003). These compounds exhibit significantly decreased toxicity toward rainbow trout; for example, the LC_{50} for silafluofen is $>100{,}000$ µg/L. However, of the few studies that have reported pyrethroid metabolism in piscine species, esterase measurements were generally made only on tissue pools, rather than on individual fish (Glickman and Lech 1981; Glickman et al. 1982). Although useful, these data only provide information on the average enzyme activity in a population or species and do not indicate the activity range among individuals. Data derived from measurements in individuals are important to determine if some individuals metabolize pesticides slower than others, which could potentially correlate with increased sensitivity to pyrethroid or OP exposure. It has been hypothesized that fish with lower levels of esterase activity are more sensitive to pyrethroid and OP toxicity (Wheelock et al. 2005a). Understanding the range of esterase activity in environmentally sensitive species will be useful for interpreting the impact of increased pyrethroid usage upon aquatic ecosystems.

3.4 Presence in Ambient Samples

Because of their hydrophobicity, pyrethroids do not remain in surface water, but quickly partition onto particulate matter in the environment (Liu et al. 2004). Although the bioavailable concentration of pyrethroid pesticides in the water column is reduced by adsorption, toxic concentrations are sometimes present (Spurlock et al. 2005). A number of California Department of Pesticide Regulation (DPR) studies have detected pyrethroids in California surface waters (Bacey et al. 2003, 2005; Kelley and Starner 2004), but there are few studies of pyrethroids in surface waters outside of California (Schulz 2004). Of the studies conducted other than in California, Schulz (2004) reviewed 15 studies that detected a variety of pyrethroids in surface waters. Five of these studies were conducted in the southern United States and 10 were conducted in Europe and South Africa.

In the DPR studies, total pyrethroid concentrations were measured, but Liu et al. (2004) estimated that dissolved concentrations of pyrethroids in stream waters would be less than 1% of the total. Although the dissolved concentrations in stream water were quite low, the authors demonstrated that the dissolved concentrations of pyrethroids in runoff from a nursery operation were as high as 27% of total residues. Spurlock et al. (2005) tested a probabilistic screening model for dissolved pyrethroid concentrations and determined that although pyrethroids in the water column have reduced bioavailability in the presence of suspended sediment, toxic concentrations could still be present.

Toxic concentrations of pyrethroids are more readily found in bedded sediments. Weston et al. (2004, 2005) detected pyrethroids in sediments associated with agricultural drainages and suburban creeks in central California. Amweg et al. (2006) detected pyrethroids in urban creeks in the San Francisco Bay area of California. Using the toxic unit approach, these authors implicated pyrethroid pesticides as the cause of observed sediment toxicity in their studies. Studies conducted by the University of California Davis have also detected pyrethroids in the sediments of agriculturally dominated creeks and urban drainages and have identified pyrethroids as the cause of observed toxicity through TIE methods (Anderson et al. 2006b, in press; Phillips et al. 2006).

Pyrethroid pesticides have not been routinely measured in large-scale environmental monitoring programs. The United States Geological Survey National Water Quality Assessment Program is the largest urban monitoring program and only screens for permethrin (www.usgs.gov). Some regional studies have measured pyrethroids in water and sediment at concentrations that are toxic to resident organisms. The Surface Water Ambient Monitoring Program (SWAMP) now includes routine analyses of pyrethroids in California watersheds and has detected pyrethroids in surface water and sediment (http://www.waterboards.ca.gov/swamp/index.html). Recent advances in LC-MS technology should reduce the development time and cost of analyzing for multiple pyrethroids in ecosystems. Also, immunoassays that are compound and pyrethroid class selective have been developed for

multiple pyrethroids and their key metabolites (Wengatz et al. 1998; Shan et al. 1999, 2000; Watanabe et al. 2001; Lee et al. 2002, 2004; Mak et al. 2005). These low-cost assays should facilitate environmental monitoring.

4 Toxicity Identification Evaluations (TIEs)

4.1 Overview

In recent years, there has been increased emphasis on the use of routine aquatic organism toxicity tests to measure ambient water quality (Bailey et al. 1995, 1996, 2000; de Vlaming et al. 2000; Werner et al. 2000). Studies have been conducted to assess receiving water impacts of discharged storm water (Bailey et al. 1997; Werner et al. 2002), municipal and industrial effluent (Bailey et al. 1995), and agricultural runoff (de Vlaming et al. 2000; Werner et al. 2000; Anderson et al. 2002). Studies have also been conducted to determine the environmental impact of contaminated sediments (Anderson et al. 2001; Hunt et al. 2001). Concomitantly, increased attention has focused on methods for identifying the chemical(s) that are responsible for the toxicity so that appropriate control measures can be taken. The United States Environmental Protection Agency (USEPA) has published a series of TIE methods designed to identify the causes of observed toxicity in aqueous samples using chemical characterization, identification and confirmation procedures (USEPA 1991, 1993a,b, 1996). Additionally, sediment TIE procedures will soon be published (Anderson et al., 2007b; in press; 2007 USEPA).

TIE methods have been broadly applied to identify the causes of toxicity in water and sediment. TIE testing has routinely identified OP insecticides, including diazinon and chlorpyrifos, as causes of toxicity in municipal effluents, surface waters, and sediments in Northern California (Bailey et al. 2000; de Vlaming et al. 2000; Werner et al. 2000; Anderson et al. 2003; Hunt et al. 2003; Phillips et al. 2004). As discussed previously, pyrethroids are difficult to identify using standard TIE methods because of their physicochemical properties (Sharom and Solomon 1981; Casida et al. 1983; Wheelock et al. 2005b) and the lack of inexpensive, sensitive, and selective analytical methods capable of detecting these insecticides at biologically relevant concentrations (Leng et al. 1999; Lee et al. 2002). In addition to the standard USEPA TIE treatments, there are several TIE treatments that can provide specific lines of evidence for pyrethroid toxicity: carboxylesterase addition (Wheelock et al. 2004, 2006), addition of piperonyl butoxide (USEPA 1991, 1993b; Amweg and Weston 2007), and temperature reduction (Weston 2006). These procedures satisfy the criteria for widespread applicability in that they are rapid, relatively inexpensive, and do not require a high level of expertise or expensive equipment. Although PBO addition and temperature reduction help to characterize pyrethroid-associated toxicity, reduction of toxicity with the addition of carboxylesterase provides a stronger line of evidence when identifying pyrethroids as the cause of toxicity.

4.2 TIE Procedures

The generic TIE protocols are performed in three phases: toxicity characterization (Phase I), toxicant identification (Phase II), and toxicant confirmation (Phase III). Table 3 provides a basic list of standard and emerging Phase I and II TIE methods for water and sediment. Each of these TIE treatments are applied to the test sample and comparison of the level of baseline toxicity with the TIE treatments identifies the physicochemical characteristics of the toxicants. It is essential that proper controls and blanks be used with each TIE treatment, and that a high level of QA/QC is maintained throughout the TIE process. If the standard suite of Phase I treatments are ineffective in identifying cause(s) of toxicity, other techniques can be used, including anion- and cation-ion exchange resins and activated charcoal molecular sieves (Burgess et al. 1997). Application of the TIE process has demonstrated its applicability to virtually every test species and in a variety of test matrices.

The EPA Phase I TIE procedures (USEPA 1991, 1992, 1996) describe a process in which the sample is split into aliquots, each of which is subjected to a single TIE manipulation concurrently with the other treatments ("parallel" treatment approach). However, EPA points out that the Phase I TIE characterization procedures are relatively broad and can indicate more than one class of toxicity. Additional tests or an altered approach may be needed to delineate/confirm the role of a particular chemical class in the effluent toxicity, especially when multiple toxicants are present (USEPA 1993a,b). For example, when the primary toxicant is present in high concentrations, it may mask the other potential toxicant(s) in the sample; ammonia is a common example. In these cases, sequential treatments ("stacked" treatment approach) can be used to evaluate the role of secondary toxicants, e.g., removal of ammonia by Zeolite followed by removal of nonpolar organics by solid-phase extraction (SPE) treatment in cases where multiple toxicants are present at toxic concentrations.

Results of Phase I can be compared with pretreatment program data and chemical-specific data to identify potential toxicants. However, chemical analyses conducted in the absence of Phase I TIE information, e.g., chemical class of toxicant(s), to guide the type of analysis are usually wasted expenditures. For this reason, EPA cautions that chemical-specific tracking should be conducted after the toxicant(s) is(are) identified and confirmed in Phase II and Phase III TIEs, respectively (USEPA 1993a,b).

Phase I characterization provides information on the chemical classes responsible for toxicity and is applicable to both acute and chronic endpoints. Following is a brief description of the aquatic treatments that can be used for water column and interstitial water samples (see Table 3 for a summary). Ammonia toxicity can be assessed by Zeolite removal or the graduated pH test. Toxicity resulting from the presence of divalent cation metals can be detected via the addition of ethylenediaminetetraacetic acid (EDTA), and sodium thiosulfate (STS) is added to reduce toxicity from oxidants and specific metals. Contaminants associated with particles are removed by filtration or centrifugation (2500 *g* at 4°C). Volatile constituents

Table 3 Brief description of some standard and emerging phase I and II Toxicity Identification Evaluation (TIE) methods for water and sediment

Sediment treatment	Description	Citations
Zeolite	Addition of 10%–20% zeolite to sediment reduces interstitial and overlying water ammonia	Besser et al. 1998; Burgess et al. 2003
Chelating resin (SIR-300)	Addition of 10% SIR-300 chelates heavy metal ions and reduces metal bioavailability	Burgess et al. 2000
Coconut charcoal (PCC)	Addition of 10%–15% PCC to sediment reduces toxicity caused by organic contaminants	Lebo et al. 1999, 2003; Ho et al. 2002, 2004
Ambersorb 563	Addition of 10% Ambersorb to sediment reduces toxicity caused by organic contaminants	Kosian et al. 1999; Lebo et al. 1999; West et al. 2001
Carboxylesterase	Addition of enzyme to overlying water reduces toxicity caused by pyrethroid pesticides by breaking down compounds into nontoxic forms	Wheelock et al. 2004, 2006; Weston and Amweg, 2007
Piperonyl butoxide (PBO)	Addition of PBO to overlying water can reduce toxicity caused by organophosphate pesticides, and increase toxicity caused by pyrethroid pesticides and DDT	USEPA 1991; Kakko et al. 2000
Temperature reduction	Testing warm water organisms at colder temperatures can increase the toxicity of pyrethroids and DDT	Ware and Whitacre 2004
Water treatment	**Description**	**Citations**
Aeration	The sample is aerated for 1 hr to determine if toxicity is caused by volatile compounds or surfactants	USEPA 1991, 1993b, 1996
Filtration or centrifugation	Reduces toxicity that is particle related; also used as a pretreatment step for the column treatments	USEPA 1991, 1996
EDTA[a]	Organic chelating agent that preferentially binds with divalent metals, such as copper, nickel, lead, zinc, cadmium, mercury, and other transition metals to form nontoxic complexes	USEPA 1991, 1996
Sodium thiosulfate	Reduces toxicity caused by oxidants and some cationic metals	USEPA 1991, 1996
pH adjustment and volatilization	Reduces ammonia in sample by converting it to the unionized fraction through pH adjustment and volatilizing it by stirring	USEPA 1991, 1993b; Burgess et al. 2003

Graduated pH	Determines if pH-dependent toxicants are responsible for the observed toxicity; the toxicity of ammonia, sulfide, and some metals changes with pH	USEPA 1991, 1993b, 1996
Zeolite column solid-phase extraction	Reduces toxicity caused by ammonia	USEPA 1991; Burgess et al. 2004
Cation column solid-phase extraction and elution	Removes metals from the sample; column can be eluted with 1 N hydrochloric acid (HCl) and resulting eluate tested to determine if substances removed by the column were toxic	USEPA 1993b, 1996; Burgess et al. 1997
Nonpolar organic column solid-phase extraction and elution	Removes nonpolar organic compounds; column can be eluted with solvent to determine if substances removed by the column were toxic	USEPA 1991, 1993b, 1996
Carboxylesterase	Addition of enzyme to sample reduces toxicity caused by pyrethroid pesticides by breaking down compounds into nontoxic forms	Wheelock et al. 2004, 2006
Piperonyl butoxide (PBO)	Addition of PBO can reduce toxicity caused by organophosphate pesticides and increase toxicity caused by pyrethroid pesticides and DDT	USEPA 1991; Kakko et al. 2000
Temperature reduction	Testing warm water organisms at colder temperatures can increase the toxicity of pyrethroids and DDT	Ware and Whitacre 2004

[a]Ethylene diaminetetrachloracetic acid.

such as sulfide are oxidized or volatilized by sample aeration. Nonpolar organic compounds are removed/detected by passing the water samples over a C8 or C18 SPE column. The SPE column can then be eluted with methanol or other solvents. A portion of the eluting solvent is then added back to the laboratory control water to determine if the SPE-bound organic compounds cause toxicity. A cation-exchange column is used to remove metal contaminants, and the column is then eluted with 1 N HCl to add back bound metals to clean dilution water. A sequential treatment using the SPE columns is conducted to resolve mixtures of organic and metal contaminants. The presence of metabolically activated compounds such as OPs is examined through the addition of PBO, which should result in a decrease in the toxic signature. An increase in toxicity following PBO addition usually indicates the presence of pyrethroids (Casida 1970; Casida and Quistad 1995; Ameg and Weston 2007).

The Phase II guidance manual (USEPA 1993b) describes procedures for use in identification of specific classes of toxicants, including: ammonia, cationic metals, polar and nonpolar organic chemicals, chlorine, and filterable toxicants. Phase II treatment techniques are similar to Phase I and are applicable to most acute and chronic test methods. Phase II incorporates chemical-specific analytical procedures, including gas chromatography (GC), GC/mass spectrophotometry (GC/MS), high-performance liquid chromatography (HPLC)/MS, atomic absorption (AA), or ion-coupled plasma (ICP)/MS to identify toxicants. Interested readers are referred to the EPA Phase II manual (USEPA 1993b), and to Waller et al. (2005) for a detailed description of Phase II TIE procedures and examples of TIE case studies.

Phase III TIE procedures involve a thorough confirmation of the cause(s) of toxicity and constitute a key part of the TIE process. Suspected toxicant(s) identified in Phase I and Phase II are confirmed through application of one or more Phase III steps, including correlation approach, symptom approach, species sensitivity approach, spiking approach, and mass balance approach (USEPA 1991, 1992, 1993a,b, 1996)

Sediment TIEs are also becoming more common and are more appropriate for pyrethroids because of their hydrophobicity (Anderson et al., in press). The following solid-phase TIE treatments may be used in conjunction with the interstitial water treatments described earlier (Anderson et al., in press). Solid-phase treatments include amending the sediment with substances that reduce toxicity caused by particular classes of chemicals or adding substances to the overlying water in the exposure chamber. As with the aquatic methods, all three phases of the TIE process can be utilized for sediments. Phase I sediment amendments include addition of a carbonaceous resin (e.g., Amberlite XAD4) or of powdered coconut charcoal to reduce the bioavailability of organic chemicals. Cation-chelating resin (e.g., SIR-300) is added to reduce the bioavailability of cationic metals. Zeolite is added to remove unionized ammonia. Overlying water treatments include the addition of carboxylesterase enzyme and bovine serum albumin (BSA) in separate treatments to identify toxicity from pyrethroid pesticides, and the addition of PBO to differentiate between pyrethroid and OP pesticides (see following for descriptions of the carboxylesterase and albumin methods). Phase II treatments

include the separation and elution of the Amberlite and SIR-300 resins. The eluate is added to control water to verify that chemicals sorbed to the resins were eluted at toxic concentrations. Phase III procedures include comparing concentrations of chemicals in sediments or in the solvent eluates to known toxicity thresholds.

4.3 TIE Use in Ambient Waters and Sediments

TIEs on ambient waters and sediments are used to determine the cause of sample toxicity, for example, as part of the development of a Total Maximum Daily Load evaluation (TMDL). When toxicity tests or other biological indicators produce evidence of water quality impairment caused by contaminated sediments, the water bodies in which the sediments occur may be placed on the Clean Water Act §303[d] list of impaired waters. For each water body on the §303[d] list, states are required to develop individual TMDLs capable of identifying the cause of impairment, locating all sources of the causative pollutant, and allocating loadings of the pollutant among the various sources. The goal of the TMDL is to restrict loadings so that ambient pollution concentrations will decrease to levels that no longer contribute to impairment.

Ambient water TIEs proceed in the same manner as effluent TIEs using the USEPA Phase I, II, and III protocols already described (USEPA 1991, 1992, 1993a,b, 1996). Once toxicity is observed in an ambient water sample, the cause can be investigated with the TIE process. In contrast to effluent samples that usually provide a consistent toxicity signal, ambient waters tend to be influenced by non-point sources of toxicity and could have intermittent toxicity caused by pulses of contaminants. Generally, if toxicity is suspected in ambient water, sufficient volume is collected for initial toxicity testing as well as amounts adequate for the entire TIE process.

Sediments act as a sink for many contaminants and are often viewed as integrators of anthropogenic impacts (USEPA 2004). Because many contaminants, particularly hydrophobic chemicals, can persist in sediments, the toxicity signal is generally more persistent. Sediment TIEs are conducted using solid-phase and interstitial water treatments. As with the ambient water samples, interstitial water TIEs follow the USEPA protocols with minor modifications for volume. These modifications, along with a suite of solid-phase treatment methods, are described in an upcoming USEPA sediment TIE guidance document (USEPA, 2007), and in Anderson et al. (in press).

After sediment toxicity is observed, a decision matrix guides the TIE process as described by Anderson et al. (in press). The process begins with an initial assessment of the magnitude of toxicity using solid-phase and interstitial water dilution series tests. Solid-phase dilutions may be necessary when complete mortality is observed in the original sample to allow improved resolution between baseline and TIE treatments. Once the magnitudes of toxicity are determined, a single sediment concentration is selected for the solid-phase TIE, and a dilution series is selected for the interstitial water TIE. In this approach, solid-phase TIEs are generally conducted on a single concentration of sediment for efficiency but can be conducted

on multiple concentrations if resources allow. The solid-phase concentration is chosen to provide the best resolution among the TIE treatments. Interstitial water TIEs are conducted with a dilution series that brackets the LC_{50} from the initial test. In situations where one sediment matrix produces insufficient toxicity to allow resolution of differences between the TIE treatment and baseline, the TIE proceeds using the matrix with the highest magnitude of toxicity.

4.4 Identification of Pyrethroid-Associated Toxicity with TIEs

Current TIE methodology can characterize and, in some cases, identify pyrethroid toxicity (Anderson et al., in press). However, these methods are still imprecise, and further work is needed to develop specific methods for pyrethroid identification. Even the most robust of these methods are limited in their ability to quantify toxicity contributed by individual pyrethroids when these pesticides occur in mixtures (e.g., permethrin vs. deltamethrin). Previous research has shown that a weight-of-evidence approach is effective at identifying sediment toxicity caused by pyrethroids. TIE evidence includes solid-phase extraction and elution of interstitial water samples, addition of carbonaceous resin to whole-sediment samples, and solvent elution of resins to recover sorbed pesticides. Lines of evidence for pyrethroid toxicity are provided when results from these procedures are combined with detailed chemical analyses and information from the standard TIE treatments.

4.4.1 Use of Temperature Differential

One potential method for characterizing pyrethroid-associated toxicity involves the use of temperature. Pyrethroids have been reported to exhibit a negative temperature coefficient (toxicity increasing with decreasing ambient temperature) in varying degrees depending upon pyrethroid and species (Pap 2003). This effect is thought to be partly caused by reduced metabolism of the parent pyrethroid to less-toxic metabolites (Ware and Whitacre 2004). In support of this theory, increased toxicity of sediments and/or interstitial waters containing pyrethroids has been observed in *H. azteca* tests conducted at 15°C compared with concurrent tests conducted at 23°C (Anderson et al. 2006b; Weston 2006; Phillips et al. 2007; Anderson et al., in press-b). However, we are unaware of any published reports that utilized reduced temperature toxicity tests as part of TIEs aimed at identifying pyrethroid-caused toxicity in ambient freshwaters. Therefore, we performed studies to determine if reduced temperature toxicity tests would be useful in a TIE context. These studies were aimed at the identification of pyrethroid-caused toxicity in two commonly used freshwater test species, fathead minnows (*Pimephales promelas*) and *C. dubia*, using permethrin, bifenthrin, cypermethrin, esfenvalerate, and cyfluthrin at 15°C and 25°C. The 48-hr acute *C. dubia* and 96-hr acute fathead minnow bioassays were conducted in accordance with the U.S. Environmental Protection Agency (USEPA) 5th edition protocol (USEPA 2002), as described in Tables 4 and 5.

Table 4 Effect of reduced temperature on acute toxicity of pyrethroids to *Ceriodaphnia dubia*[a]

	25°C	15°C	
Pyrethroid (type)	LC_{50}[b] (µg/L) (SD)	LC_{50} (µg/L) (SD)	Toxicity ratio[c]
Permethrin (I)	0.21 (0)	0.078 (0)	2.7
Bifenthrin (I)	0.074 (0.004)	0.045 (0.001)	1.6
Cypermethrin (II)	0.22 (0.005)	0.27 (0.032)	0.8
Esfenvalerate (II)	0.14 (0.022)	0.089 (0.007)	1.6
Cyfluthrin (II)	0.21 (0)	0.12 (0.014)	1.8

[a]*C. dubia* acute toxicity tests were conducted with neonates <24 hr old collected within an 8-hr period from in-house cultures. Pyrethroid analytical standards (≥99% pure) obtained from AccuStandard (New Haven, CT) were diluted in HPLC-grade methanol. Each test consisted of 5–6 concentrations of the test material with 4 replicates of 5 neonates each. Test chambers were 20-mL glass scintillation vials containing 18 mL test solutions. Dilution water was reverse osmosis- and granular carbon-treated well water amended with dry salts to attain USEPA moderately hard specifications (EPAMH). Test duration was 48 hr; test solutions were not renewed and test organisms were not fed during the test. Tests were conducted in environmental chambers at 15° ± 2°C and 25° ± 2°C. Photoperiod was 16 hr light:8 hr dark. Mortality was noted daily. LC_{50} values were calculated using ToxCalc (Tidepool Scientific, McKinleyville, CA).
[b]LC_{50} values are nominal concentrations.
[c]Toxicity ratio = LC_{50} at 25°C/LC_{50} at 15°C.

Table 5 Effect of reduced temperature on acute toxicity of pyrethroids to larval fathead minnows (*Pimephales promelas*)[a]

	25°C	15°C	
Pyrethroid (type)	LC_{50}[b] (µg/L) (SD)	LC_{50} (µg/L) (SD)	Toxicity ratio[c]
Permethrin (I)	0.86 (0.03)	1.97 (0.54)	0.4
Bifenthrin (I)	0.52 (0.03)	0.49 (0.04)	1.1
Cypermethrin (II)	2.52 (0.18)	4.00 (0.08)	0.6
Esfenvalerate (II)	0.49 (0.04)	0.38 (0.04)	1.3
Cyfluthrin (II)	0.79 (0.02)	1.37 (0.23)	0.6

[a]Fathead minnows were obtained from Aquatox, Inc. (Hot Springs, AK), and were maintained in USEPA moderately hard (EPAMH) water until tested at 7 d old. Pyrethroid analytical standards (≥99% pure) obtained from AccuStandard (New Haven, CT) were diluted in HPLC-grade methanol. Each test incorporated 5–6 dilutions of the test material using 2 replicates of 10 fish each in 400-mL glass beakers containing 250 mL test solutions. Dilution water was EPAMH laboratory water. Test duration was 96 hr, and test solutions were renewed at 48 hr. Fish were fed *Artemia nauplii* 4 hr before sample renewal at 24 hr. Tests were conducted in an environmental chamber at 15° ± 2°C and 25° ± 2°C. Photoperiod was 16 hr light:8 hr dark. Mortality was noted daily. LC_{50} values were calculated using ToxCalc (Tidepool Scientific, McKinleyville, CA).
[b]LC_{50} values are nominal.
[c]Toxicity ratio = LC_{50} at 25°C/LC_{50} at 15°C.

Results of the temperature study were mixed. The LC_{50} values for *C. dubia* ranged from 0.074–0.22 µg/L at 25°C to 0.045–0.27 µg/L at 15°C (see Table 4). Bifenthrin was most toxic and cypermethrin the least toxic, regardless of test temperature. The toxicity ratios (TRs; LC_{50} at 25°C/LC_{50} at 15°C) were calculated for each of the pyrethroids. If the reduced temperature resulted in increased toxicity, the TRs would be greater than unity. The value for cypermethrin was less than

1.0 whereas the TR values for the other four pyrethroids ranged from 1.6 to 2.7. Thus, with the exception of cypermethrin, reduced temperature resulted in approximately a two- to threefold increase in toxicity and would therefore be useful for identification of pyrethroid-caused toxicity to *C. dubia*.

The results with fathead minnows were quite different from the *C. dubia* studies. At 25°C, LC_{50} values ranged from 0.49 to 2.52 µg/L, compared to 0.49–4.00 µg/L at 15°C. As with the *C. dubia* tests, bifenthrin was the most toxic and cypermethrin the least toxic regardless of test temperature. Table 5 shows that the TR values ranged from 0.4 to 1.1 for Type I pyrethroids and from 0.6 to 1.3 for Type II pyrethroids. In these tests, none of the pyrethroids tested exhibited significantly increased toxicity at 15°C compared to 25°C and, interestingly, three of the pyrethroids (permethrin, cypermethrin, and cyfluthrin) were noticeably less toxic at the lower test temperature. These data indicate that reduced temperature would not be a useful tool for identification of pyrethroid-caused toxicity to fathead minnows. In contrast, for *C. dubia*, reduced temperature toxicity tests would be a useful TIE procedure, adding to the weight-of-evidence for identification of pyrethroid-caused toxicity in aqueous samples.

4.4.2 Use of Piperonyl Butoxide (PBO)

In addition to the use of SPE columns for water samples and resin for sediment samples to characterize the cause of toxicity as an organic, the addition of PBO can characterize pyrethroid-associated toxicity by synergizing the toxicity signal (Kakko et al. 2000; Ameg and Weston, 2007). Certain compounds (e.g., OPs) must be metabolically activated by the test organism before they can exert their toxic effect (Casida and Quistad 2004). Many of these activation reactions consist of oxidative metabolism by a group of enzymes collectively known as mixed-function oxidases (MFOs), of which the heme-protein cytochrome P450s are a subset (Hodgson 1982) (see Fig. 5). Compounds such as PBO, a synthetic methylenedioxy phenyl compound, bind to and block the catalytic activity of some MFOs, preventing the toxicity of metabolically activated OP insecticides, such as diazinon, chlorpyrifos, malathion, parathion, and fenthion (Hamm et al. 2001). Thus, when a nontoxic level of PBO is added to test samples containing one or more of these OPs, the toxicity is greatly reduced or completely blocked. The use of PBO to identify toxicity caused by metabolically activated OP insecticides has been previously described (Ankley et al. 1991) and incorporated in published USEPA TIE manuals (USEPA 1991, 1993b). Conversely, PBO synergizes the toxicity of pyrethroid insecticides by blocking MFO-mediated metabolism of these chemicals (Casida 1970). Thus, PBO addition to samples containing one or more pyrethroids increases and/or prolongs the toxic effect (Casida and Quistad 1995). This dual action of PBO can lead to a confounding signal in TIE testing of samples that contain both OPs and pyrethroids. Alternatively, the action of PBO can be a useful tool to identify the presence/absence of pyrethroids and metabolically activated OPs in aqueous samples. Accordingly, it is important that the use of PBO in TIE testing is performed with the correct controls to enable interpretation

of the results. Several studies have successfully utilized PBO addition to build a weight-of-evidence for pyrethroid toxicity in ambient water and sediment (Anderson et al. 2006b; Phillips et al. 2006, 2007; Amweg and Weston 2007; Anderson et al., in press).

4.4.3 Use of Carboxylesterase Activity

A novel method for pyrethroid detection involving the addition of carboxylesterase during the TIE process was proposed by Wheelock and coworkers (2004, 2006). As discussed previously, carboxylesterases are very efficient at hydrolyzing pyrethroids to their corresponding acid and alcohol, which generally significantly reduces observed toxicity. Carboxylesterases rapidly degrade both Type I and Type II pyrethroids (see Fig. 1). This class of enzymes has been demonstrated to be effective in reducing pyrethroid-associated toxicity in both mammals and insects (Abernathy and Casida 1973) and is a logical tool for removing the toxicity of ester-containing pyrethroids in TIE samples. Previous work identified this enzyme as a good target for identifying pyrethroid-associated toxicity in aquatic samples (Denton et al. 2003). Carboxylesterase addition to aqueous samples, or water overlying sediment in solid-phase exposures, provides evidence of toxicity somewhere between the Phase I TIE (characterization) and Phase II TIE (identification). If toxicity is reduced after enzyme addition, the probable cause of toxicity is attributed to a pyrethroid. However, this test does not identify the specific pyrethroid. Previous work with carboxylesterase addition to identify/remove pyrethroid-associated toxicity validated the concept with *C. dubia* using several pyrethroids, including permethrin, bifenthrin, cypermethrin, cyfluthrin, lambda-cyhalothrin, and esfenvalerate (Wheelock et al. 2004) (Table 6). Additional studies with *H. azteca* demonstrated that nonpyrethroid toxicants (e.g., DDT, cadmium, and chlorpyrifos) were not significantly affected by carboxylesterase addition (Weston and Amweg 2007).

The carboxylesterase preparation method is thoroughly described in Wheelock et al. (2004, 2006) and only briefly here. Carboxylesterase is commercially available and can be purchased as a liquid (ammonium sulfate suspension, 3.2 M, pH 8.0) or a crude lyophilized powder preparation. Each preparation has its individual advantages and disadvantages, which have been evaluated elsewhere (Wheelock et al. 2006). Some researchers have a preference for the liquid preparation for ease of pipetting, whereas others believe that the powder is simpler to handle. In addition, there are specific toxicity issues associated with each preparation because the ammonia concentration of the liquid preparation is extremely high. The commercial carboxylesterases are available as preparations from porcine or rabbit liver. For reasons of cost, all studies to date have used the porcine preparations. Standardized methods for sediment testing with *H. azteca* use the lyophilized enzyme. Before test initiation, the lyophilized enzyme powder is added to Nanopure water to prepare a stock solution that can be added directly to the sample. The amount of enzyme added depends on sample volume and the tolerance of the test organism. It is important that toxicity of the enzyme preparation to the organism is evaluated

Table 6 Effect of carboxylesterase addition upon pyrethroid- and nonpyrethroid-associated toxicity to *Ceriodaphnia dubia* and *Hyalella azteca*

Treatment	Conc.[a] (ng/L)	Esterase (–)	Esterase (+)
	C. dubia 48-hr % mortality[b]		
Control[c]	0	0	0
Permethrin	600	100	0
Bifenthrin	660	100	0
Cypermethrin	1450	100	0
Esfenvalerate	700	100	0
Cyfluthrin	560	100	0
λ-Cyhalothrin	600	100	0
Diazinon	760	100	100
Chlorpyrifos	160	100	100
	H. azteca 10-d % mortality[d]		
Cadmium	—[e]	17	10
DDT	—	13	7
Chlorpyrifos	—	13	7

[a]Conc. indicates the concentration of insecticide at which the samples were spiked. All values are given as nominal water concentrations. The values in parentheses are toxic units (TUs), which are defined as $100/EC_{50}$ (the concentration at which 50% of the population exhibit an effect).

[b]The mean mortality in two replicates of five neonate *C. dubia*. The standard deviations for all samples were zero. Esterase (−) samples contain no added enzyme. Esterase (+) samples contain commercial porcine carboxylesterase spiked at 2.5×10^{-3} U/ml (purchased from Sigma Chemical). Data are from Wheelock et al. (2004).

[c]Controls were performed with and without the addition of the esterase to account for potential esterase-associated toxicity.

[d]Data are from Weston and Amweg (2007).

[e]Toxicant concentrations are not given as studies were performed with a range of values to determine LC_{50}s in the presence and absence of esterase. The LC_{50}s were measured for cadmium (control: 71.9 mg/kg, esterase 267 mg/kg), chlorpyrifos [control: 2.96 µg/g organic carbon (oc); esterase 4.20 µg/g oc], and DDT (control: 147 µg/g oc, esterase 174 µg/g oc).

because toxicity has been observed at increased concentrations of the enzyme (most likely from the presence of ammonia). The carboxylesterase is added based upon units of enzyme activity, which are defined by the supplier. In this case, one unit (U) is defined as the amount of enzyme required to hydrolyze 1.0 µmole of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25°C. We have developed a dilution nomenclature based upon enzyme activity, wherein "X" units of enzyme activity equals 0.0025 U/mL sample; therefore, at 500 X, 1.25 U are added per milliliter (mL) sample. Typically, *H. azteca* can tolerate an enzyme strength of 500 X without showing toxicity. Enzyme activity is unique for each lot purchased and should be defined on a case-by-case basis (Wheelock et al. 2006). The enzyme should be added to the water overlying the sediment on the day of test initiation at least 6 hr before the organism addition. This interval provides the enzyme with sufficient time to hydrolyze pyrethroids present in the sample. Specific activity assays performed with the porcine esterase showed that hydrolysis was pyrethroid structure dependent, with Type II pyrethroids hydrolyzed more slowly than those of Type I. However, given the rate of hydrolysis (160 nmol/min/mg protein for

permethrin vs. 23 nmol/min/mg protein for cyfluthrin), it is likely that the majority of pyrethroid present in the sample will be hydrolyzed within 6 hr. If the samples are renewed, for example, during a 96-hr testing cycle, then the esterase is also added with the new water sample.

One concern in the use of the esterase in toxicity assays is the specificity of the observed reduction in toxicity. As already discussed, pyrethroids are extremely hydrophobic and may adsorb to the hydrophobic enzyme surface without undergoing hydrolysis and subsequent detoxification. In addition, it is possible that other hydrophobic contaminants in the system could also undergo nonspecific absorption interactions with the enzyme, thereby eliminating the selectivity of the method. It is therefore suggested that a separate test be performed to control for nonspecific reductions in observed toxicity. To accomplish this, BSA can be added to a separate set of sample replicates. BSA is a noncatalytically active protein that is approximately the same size as carboxylesterase (~67 kDa for BSA vs. ~65 kDa for carboxylesterase). The BSA stock solution is prepared using the same mass of protein and volume of Nanopure water as used with the enzyme, and the same volume of stock solution is added to the test solution. Accordingly, if a reduction in toxicity is observed in the BSA samples, it can be attributed to nonspecific adsorption effects instead of specific catalytic reductions in pyrethroid levels. However, it should be cautioned that carboxylesterase activity has occasionally been found in commercial BSA preparations. It is recommended that the enzyme be added daily to the water column exposures and to the water overlying the sediment to ensure complete hydrolysis of pyrethroids. This procedure may also be used in a combined treatment with the addition of PBO to determine if both OPs and pyrethroids are contributing to the observed toxicity; however, results can be difficult to interpret because of the counterindicative effects.

5 Applications of Carboxylesterase Activity in TIEs

The following examples of the use of carboxylesterase additions in freshwater and marine ambient waters, sediment, and interstitial waters are summarized in Table 7.

5.1 Water Column

Water samples collected from the New River (CA, USA), where it crosses the border with Mexico, contain complex mixtures of contaminants from agriculture, and municipal, and industrial effluents. A series of TIEs implicated several classes of contaminants as the cause of observed toxicity (Phillips et al. 2007), but the most recent TIEs using carboxylesterase, along with SPE and subsequent elution of the SPE column, identified cypermethrin as the actual cause of toxicity. At the time of analysis, the laboratory detection limit for cypermethrin was well above the toxicity threshold of the test organism, but after the enzyme characterized the cause of

Table 7 Summary of reported water and sediment (solid phase and interstitial water) TIEs that have utilized carboxylesterase addition

Location and reference	Carboxylesterase success	Additional TIE evidence	Chemical analysis
Water			
New River, CA, USA (Phillips et al. 2007)	Reduced toxicity	Return of toxicity with Phase II elution of SPE column	Detection of toxic concentration of cypermethrin in SPE eluate
Central Valley, CA, USA (AQUA-Science, Inc.)	Reduced toxicity	Increased toxicity with PBO addition	None
Sediment			
Solid phase:			
Orcutt Creek, CA, USA (Phillips et al. 2006)	Reduced toxicity	Increased toxicity with PBO addition	Concentrations of pyrethroids in sediment were below LC_{50} values
Westley Wasteway, CA, USA (Anderson et al., in press)	Reduced toxicity	Some evidence in concurrent interstitial water TIE	Detection of toxic concentration of lambda-cyhalothrin in sediment and resin eluate
Agricultural Tailwater Pond SV-03, Salinas, CA, USA (Anderson et al., in press)	Reduced toxicity	Some evidence in concurrent interstitial water TIE	Detection of toxic concentrations of cypermethrin and lambda-cyhalothrin in sediment
Grayson Drain, CA, USA (MPSL 2006b)	Reduced toxicity, but also had reduction with BSA addition	None	No pyrethroids detected in sediment
Del Puerto Creek, CA, USA (MPSL 2006a)	Reduced toxicity	Some evidence in concurrent interstitial water TIE	Detection of toxic concentration of bifenthrin in sediment and resin eluate
Alisal Creek, CA, USA (Anderson et al. 2007b)	Reduced toxicity	Some evidence in concurrent interstitial water TIE	Detection of toxic concentration of pyrethroids in sediment and resin eluate
Indiana Harbor, IN, USA (Anderson et al. 2007b)	Reduced toxicity, but also had reduction with BSA addition	None	No pyrethroids detected in sediment
San Diego Creek, CA, USA (Anderson et al. 2007b)	Reduced toxicity	Increased toxicity with PBO addition	Detection of toxic concentrations of bifenthrin and permethrin in resin eluates
Upper Newport Harbor, CA, USA (Anderson et al. 2007b)	None	None	Detection of toxic concentration of bifenthrin in sediment

Interstitial water:

Santa Maria River, CA, USA (Anderson et al. 2006b)	Reduced toxicity	Increased toxicity with lower temperature and PBO addition	Detection of toxic concentration of lambda-cyhalothrin in sediment
Westley Wasteway, CA, USA	Reduced toxicity	Increased toxicity with PBO addition	Detection of toxic concentration of lambda-cyhalothrin in sediment
Agricultural Tailwater Pond SV-03, Salinas, CA, USA (Anderson et al., in press)	Reduced toxicity	Increased toxicity with PBO addition	Detection of toxic concentrations of cypermethrin and lambda-cyhalothrin in sediment
Del Puerto Creek, CA, USA (MPSL 2006a)	Reduced toxicity	Increased toxicity with PBO addition	Detection of toxic concentration of bifenthrin in sediment
Alisal Creek, CA, USA (Anderson et al. 2007b)	Reduced toxicity	Increased toxicity with PBO addition	Detection of toxic concentration of pyrethroids in interstitial water
Indiana Harbor, IN, USA (Anderson et al. 2007b)	Reduced toxicity, but also had reduction with BSA addition	None	No pyrethroids detected in interstitial water
Upper Newport Harbor, CA, USA (Anderson et al. 2007b)	Reduced toxicity	Increased toxicity with PBO addition	Detection of toxic concentration of bifenthrin in sediment

PBO, piperonyl butoxide; BSA, bovine serum albumin.

toxicity as a pyrethroid, the solvent elution of the SPE was concentrated and analyzed directly. The results of this direct analysis demonstrated the presence of cypermethrin at a toxic concentration.

In another ambient water sample collected from the central valley of California, addition of PBO synergized toxicity as much as 30 fold (AQUA-Science, Inc., personal communication). The addition of carboxylesterase completely removed toxicity. The enzyme was also used in a stacked treatment with PBO to determine if OPs contributed to observed toxicity. PBO was added several hours after the enzyme, but no mortality was observed, suggesting pyrethroids were the sole cause of toxicity. However, the sample was not chemically analyzed for pyrethroids, so the authors were not able to positively identify the cause of toxicity.

5.2 Sediment

Because pyrethroids are hydrophobic, they deposit in sediments, and there is growing evidence of their presence in sediments associated with agricultural and urban areas (Weston et al. 2004, 2005; Gan et al. 2005; Amweg et al. 2006). Although pyrethroids have been attributed to observed toxicity in several studies based on concentration alone (Weston et al. 2005), few comprehensive sediment TIE studies have been conducted. Through the use of carboxylesterase, several of these studies have demonstrated that pyrethroids contributed to toxicity.

Results from Anderson et al. (2006b) suggested that pyrethroids were contributing to interstitial water toxicity in a sample from the lower Santa Maria River (CA, USA), a watershed heavily impacted by agriculture. The results of the carboxylesterase treatment in this TIE were inconclusive because of an elevated concentration of chlorpyrifos. It was assumed that the addition of enzyme reduced pyrethroid-associated toxicity, but no reduction of toxicity was observed because of the presence of a toxic concentration of chlorpyrifos. However, addition of PBO did not reduce chlorpyrifos toxicity because of the presence of a pyrethroid and resulting synergism. Although interstitial water chemistry was not conducted, the concentration of lambda-cyhalothrin in the sediment exceeded the solid-phase LC_{50} for the test organism. In a companion paper, the TIE results of Phillips et al. (2006) suggested that pyrethroids contributed to toxicity in sediment from Orcutt Creek, a tributary of the Santa Maria River. This study used carboxylesterase in the overlying water of a standard solid-phase toxicity test to determine if pyrethroids were contributing to the toxicity of *H. azteca*. Carboxylesterase addition increased organism survival from 15% to 60%.

Anderson et al. (in press) provide an up-to-date review of current sediment TIE methods for pyrethroids. They also present two sediment toxicity case studies that illustrate the successful use of carboxylesterase. Both case study sediments are from agricultural drainages in California and include the application of carboxylesterase in both solid-phase and interstitial water exposures. In the first case study, toxicity in the solid-phase exposures from station WWNCR (Westley Wasteway) was not

reduced with the addition of an organic-binding resin, but the addition of the enzyme increased survival from 0% to 48%. Addition of carboxylesterase to interstitial water decreased toxicity from 5.6 toxic units (TUs; a TU is defined as $100/EC_{50}$, where EC_{50} is the concentration at which 50% of the population exhibits an effect) in the untreated baseline sample to 1.7 TUs. Addition of BSA to water overlying sediment and interstitial water did not significantly affect toxicity relative to the baseline sample. Chemical analysis of the sediment detected 14 µg/g organic carbon of lambda-cyhalothrin [approximately 30 TUs based on the LC_{50} of Amweg et al. (2005)]. Addition of the carbonaceous resin Ambersorb did not reduce toxicity, but when the resin was recovered from the sediment and eluted with acetone, the eluate was toxic. Analysis of the eluate detected 1279 ng/L lambda-cyhalothrin. When combined with evidence of reduced toxicity from esterase addition, these results provide compelling evidence that this pyrethroid was the primary cause of toxicity.

In the second case study presented by Anderson et al. (in press), solid-phase and interstitial water TIEs were conducted on sediment collected from an agricultural tailwater pond. Addition of carboxylesterase increased survival of *H. azteca* in both the water overlying the sediment and the interstitial water. The addition of BSA did not reduce toxicity, supporting pyrethroids as the cause of toxicity. Analysis of the sediment identified toxic concentrations of cypermethrin and lambda-cyhalothrin (18 TUs, and 2 TUs, respectively).

Carboxylesterase addition was also used successfully in two studies conducted by the University of California, Davis, Marine Pollution Studies Laboratory (MPSL 2006a,b). Sediment from two agricultural drainages, Del Puerto Creek and Grayson Drain, caused high mortality in initial toxicity tests. Addition of the enzyme to overlying water in solid-phase exposures with Grayson Drain sediment reduced toxicity, but additions of BSA also completely removed toxicity. Analysis of the sediment did not detect any pyrethroids, but concentrations of DDT metabolites in the resin eluate exceeded published LC_{50} values for *H. azteca*. The toxicity of Del Puerto Creek sediment was also reduced by the addition of carboxylesterase to the water overlying the sediment, and BSA addition did not reduce toxicity. A TIE with interstitial water was also conducted with similar results: addition of the enzyme reduced toxicity and BSA addition did not. Analysis of Del Puerto Creek sediment and the resin eluate detected toxic concentrations of bifenthrin.

As part of a larger study to evaluate current and emerging sediment TIE methods, carboxylesterase was used as a standard treatment in six ambient sample TIEs utilizing solid-phase and interstitial water matrices (Anderson et al. 2007b). Carboxylesterase was evaluated using three freshwater samples and one marine sample. The toxicity of sediment and interstitial water from Alisal Creek (CA, USA) was reduced by the addition of the enzyme. A toxic concentration of lambda-cyhalothrin was detected in the sediment, and additional pyrethroids were identified in the interstitial water and in the solvent eluate of the solid-phase resin. Two additional stations from southern California included enzyme additions in TIEs: San Diego Creek and Upper Newport Harbor. Both stations are heavily

influenced by urban inputs and a small percentage of agriculture, and San Diego Creek is a tributary to Newport Bay. Addition of carboxylesterase reduced the toxicity of San Diego Creek sediment, but the enzyme treatment was apparently saturated by the toxicity of Upper Newport sediment. Addition of the enzyme to Upper Newport interstitial water reduced toxicity. Bifenthrin and permethrin were detected in resin eluates from the solid-phase treatments from both samples, and in the sediment from Upper Newport, indicating that pyrethroids contributed to the toxicity of these samples.

Weston and Ameg (2007) formally evaluated the solid-phase TIE treatment by adding carboxylesterase to the overlying water of spiked and ambient sediments in tests with *H. azteca*. Addition of enzyme to the water overlying the sediment successfully reduced the toxicity of bifenthrin-spiked sediment. The enzyme also reduced the toxicity of cadmium-spiked sediment but did not reduce the toxicity of sediments spiked with DDT or chlorpyrifos. The authors also evaluated the enzyme by adding it to the overlying water of 12 ambient samples containing known toxic concentrations of various pyrethroids. The addition of carboxylesterase significantly increased survival in all the samples, but in 3 of these samples the increase was not significantly different from that observed following the addition of BSA.

5.3 Method Limitations

As discussed, carboxylesterase addition can help identify pyrethroid-associated toxicity in water and sediment samples. However, there are limitations with the current method that should be addressed to improve this tool. One of the main limitations is that the carboxylesterase preparation, either liquid or lyophilized powder, is heterogeneous. The commercial product is prepared from pig or rabbit livers and undergoes a simple acetone precipitation before sale. Subsequently, a large amount of undefined noncatalytically active protein is added to the test system, greatly increasing the likelihood of nonspecific interactions with hydrophobic contaminants. To overcome this problem, it would be ideal to use a recombinant protein selected for a high specific activity for pyrethroid hydrolysis in these assays, along the lines of that identified by Stok et al. (2004a). If available, such a preparation would provide high enzymatic activity while minimizing the concentration of nonspecific proteins in the assay. However, making this preparation would require protein expression and purification on a commercial scale to obtain sufficient quantities of purified protein.

Another problem with the commercial preparation is the reproducibility of the preparation. Experiments in which small amounts of the liquid esterase preparation were aliquotted into 20-mL borosilicate glass containers exhibited high variability (21% relative standard deviation; RSD) in contrast to tests with larger amounts of esterase (10% RSD) (Wheelock et al. 2006). In addition, vial-to-vial variability was ~11% among five different bottles examined. Another issue is variability among

lots. Each vial of carboxylesterase has a certain level of activity, which is defined by the supplier/manufacturer. However, this activity can vary greatly among lots. This variability requires adjusting the total amount of protein or the total amount of enzyme activity added with each new lot purchased. Although none of these points prevents the use of the enzyme in TIEs, identifying an alternative enzyme source could greatly increase the utility of this method. In particular, different aquatic testing laboratories could more easily compare results if they used an identical enzyme preparation.

A number of other concerns regarding the esterase preparation were identified by Weston and Amweg (2007), who performed an extensive evaluation of the use of carboxylesterase activity in whole-sediment testing. One major concern is the physical amount of material added to the test system. The mass of carboxylesterase used in this method has been demonstrated to be sufficient to decrease the oxygen levels in testing systems, with dissolved oxygen dropping by more than 50% within 24 hr of test initiation. Studies performed with mercuric chloride addition eliminated the oxygen depletion, strongly suggesting that the observed reductions were the result of increased microbial activity. These observations were further supported by control studies with BSA, which also caused a drop in oxygen levels (albeit slower than that following esterase addition). However, similar concerns have not been noted by other researchers.

One potential way to increase the efficacy of the assay is to increase the incubation time of the esterase with the sample to be tested before organism addition. Current methods use an 1- to 6-hr incubation; however, it may be better to incubate for 12–24 hr to ensure significant hydrolysis of pyrethroids before organism addition. Assays could be formatted such that carboxylesterase was introduced to the sample the evening before test initiation/organism addition. The increased incubation time may increase the efficiency of pyrethroid removal/hydrolysis from the testing solution.

6 Summary

This review has examined a number of issues surrounding the use of carboxylesterase activity in environmental monitoring. It is clear that carboxylesterases are important enzymes that deserve increased study. This class of enzymes appears to have promise for employment in environmental monitoring with a number of organisms and testing scenarios, and it is appropriate for inclusion in standard monitoring assays. Given the ease of most activity assays, it is logical to report carboxylesterase activity levels as well as other esterases (e.g., acetylcholinesterase). Although it is still unclear as to whether acetylcholinesterase or carboxylesterase is the most “appropriate” biomarker, there are sufficient data to suggest that at the very least further studies should be performed with carboxylesterases. Most likely, data will show that it is optimal to measure activity for both enzymes whenever possible. Acetylcholinesterase has the distinct advantage of a clear biological

function, whereas the endogenous role of carboxylesterases is still unclear. However, a combination of activity measurements for the two enzyme systems will provide a much more detailed picture of organism health and insecticide exposure. The main outstanding issues are the choice of substrate for activity assays and which tissues/organisms are most appropriate for monitoring studies. Substrate choice is very important, because carboxylesterase activity consists of multiple isozymes that most likely fluctuate on an organism- and tissue-specific basis. It is therefore difficult to compare work in one organism with a specific substrate with work performed in a different organism with a different substrate. An attempt should therefore be made to standardize the method. The most logical choice is PNPA (*p*-nitrophenyl acetate), as this substrate is commercially available, requires inexpensive optics for assay measurements, and has been used extensively in the literature. However, none of these beneficial properties indicates that the substrate is an appropriate surrogate for a specific compound, e.g., pyrethroid-hydrolyzing activity. It will most likely be necessary to have more specific surrogate substrates for use in assays that require information on the ability to detoxify/hydrolyze specific environmental contaminants.

The use of carboxylesterase activity in TIE protocols appears to have excellent promise, but there are further technical issues that should be addressed to increase the utility of the method. The main concerns include the large amount of nonspecific protein added to the testing system, which can lead to undesirable side effects including nonspecific reductions in observed toxicity, decrease in dissolved oxygen content, and organism growth. It is probable that these issues can be resolved with further assay development. The ideal solution would be to have a commercial recombinant carboxylesterase that possessed elevated pyrethroid-hydrolysis activity and which was readily available, homogeneous, and inexpensive. The availability of such an enzyme would address nearly all the current method shortcomings. Such a preparation would be extremely useful for the aquatic toxicology community. Further work should focus on screening available esterases for stability, cost, and activity on pyrethroids, with specific focus on esterases capable of distinguishing type I from type II pyrethroids. It would also be beneficial to identify esterases that are not sensitive to OP insecticides. Many esterases and lipases are available as sets to test chemical reactions for green chemistry, enabling large-scale screening. Other potential approaches to increase the utility of the enzyme include derivatization with polyethylene glycol (PEG) or cyanuric acid chloride to increase stability and reduce microbial degradation. It is also possible that the enzyme could be formulated in a sol gel preparation to increase stability. It is likely that the use of carboxylesterase addition will increase for applications in sediment TIEs.

Carboxylesterases are an interesting and useful enzyme family that deserves further study for applications in environmental monitoring as well as to increase our understanding of the fundamental biological role(s) of these enzymes. There are, of course, other enzymes that show high esterase activity on pyrethroids but are not technically carboxylesterases in the α/β-hydrolase fold protein family. These enzymes should also be examined for use in TIE protocols and “esterase” arrays as well as for general applications in environmental monitoring. One can envision

the creation of a standardized screen of enzymes with esterase activity to (1) identify environmental contaminants, (2) estimate the potential toxic effects of new compounds on a range of organisms, and (3) monitor organism exposure to agrochemicals (and potentially other contaminants). This approach would provide a multibiomarker integrative assessment of esterase-inhibiting potential of a compound or mixture. In conclusion, much is still unknown about this enzyme family, indicating that this area is still wide open to researchers interested in the applications of carboxylesterase activity as well as basic biological questions into the nature of enzyme activity and the endogenous role of the enzyme.

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