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USE OF ACETYLCHOLINESTERASE ACTIVITY TO DETECT SUBLETHAL TOXICITY IN STREAM INVERTEBRATES EXPOSED TO LOW CONCENTRATIONS OF ORGANOPHOSPHATE INSECTICIDES

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ABSTRACT

Acetylcholinesterase (AChE) activity was measured in selected species of aquatic invertebrates using two methodologies based on the Ellman technique - a modified kit procedure using the spectrophotometer (BMC procedure) and a microplate assay. Levels of AChE ranged from 12.7 mU/mg protein for Daphnia magna to 96.9 mU/mg protein for Hydropsyche spp. The coefficients of variation (CVs) for the microplate technique were much lower than the CVs for the BMC procedure (e.g., 6.9 to 25.5% vs. 34.6 to 55.4%) and allowed measurements within single whole organisms or individual head capsules.

Exposure of invertebrates to low concentrations of the organophosphate insecticides, azinphosmethyl and fenitrothion, did not result in a significant depression in AChE levels with one exception i.e., levels of AChE in Hyalella azteca declined to 55.2% of control levels following 24 h exposure to 2.0 µg azinophosmethyl/l. Exposure for longer periods (48 to 96 h) did not result in reduced levels. Exposure of the stonefly, Claassenia sp., to chlorpyrifos significantly lowered AChE levels by 30.7-45.1% at concentrations approaching lethality (≥ 40 µg/L). The results indicate that measurement of the activity of AChE in aquatic invertebrates exposed to field concentrations of organophosphate insecticides may be a useful biochemical technique but only for detecting acute toxicity following exposure in the field. In addition, choice of species may be important in the detection of sublethal effects.

RÉSUMÉ POUR LA DIRECTION

On a étudié une méthode biochimique permettant de déceler les effets de concentrations ambiantes d'insecticides organophosphatés sur les invertébrés aquatiques. Il est bien connu que les organophosphates se fixent sur le site actif de l'enzyme acétylcholinestérase (AChE), qui est à l'origine de la décomposition d'un neuro-transmetteur, l'acétylcholine (ACh). Une baisse des concentrations de l'AChe a été observée chez des oiseaux et des poissons exposés à des organophosphates en laboratoire et sur le terrain, mais on possède très peu de renseignements sur les invertébrés aquatiques.

L'activité de l'AChE a été mesurée chez des invertébrés aquatiques exposés à de faibles concentrations de trois insecticides organophosphatés, l'azinphosméthyl, le chlorpyrifos et le fénitrothion, grâce à deux méthodes s'inspirant de la technique Ellman - procédé normalisé (méthode BMC), avec essai sur microplaque. Les concentrations d'AChE variaient de 12,7 mU/mg de protéine pour Daphnia Magnia à 96,9 mU/mg de protéine pour Hydropsyche spp. Les coefficients de variation (CV) pour la technique de la microplaque étaient beaucoup plus faibles que les CV correspondant à la méthode BMC (par ex. 6,9 à 25,5 % contre 34,6 à 55,4 %), ce qui a permis d'effectuer des mesures sur des organismes individuels entiers ou sur des capsules céphaliques individuelles. Les résultats montrent que la baisse de l'activité de l'AChE chez les invertébrés aquatiques exposés à des concentrations d'insecticides organophosphatés peut représenter une méthode biochimique très utile pour déceler un niveau de toxicité aiguë après une exposition; mais, elle ne permet pas de déceler les effets sublétaux chez tous les invertébrés.

EXECUTIVE SUMMARY

A biochemical method to detect the effects of ambient concentrations of organophosphate insecticides on aquatic invertebrates was examined. Organophosphate are known to bind to the active site of the enzyme, acetylcholinesterase (AChE), which is responsible for the breakdown of the neurotransmitter, acetylcholine (ACh). Decreases in levels of AChE have been observed in avian and fish species exposed in the laboratory and field to organophosphate insecticides but little information on aquatic invertebrates is available.

AChe activity was measured in aquatic invertebrates exposed to low concentrations of three organophosphate insecticides, azinphosmethyl, chlorpyrifos and fenitrothion using two methodologies based on the Ellman technique - a modified kit procedure (BMC procedure) and a microplate assay. Levels of AChE ranged from 12.7 mU/mg protein for Daphnia magna to 96.9 mU/mg protein for Hydropsyche spp. The coefficients of variation for the microplate technique were much lower than the CVs for the BMC procedure e.g., 6.9 to 25.5% vs. 34.6 to 55.4% and allowed measurements within single whole organisms or individual head capsules. Significant decreases in brain AChE were observed when levels approaching acute lethality were reached but few changes occurred at sublethal concentrations. The results indicate that depression of AChE activity in aquatic invertebrates exposed to organophosphate insecticides may be a useful biochemical technique for detecting acute toxicity following exposure but not for the

detection of sublethal effects and not in all species of invertebrates.

RÉSUMÉ

L'activité de l'acétylcholinestérase (AChE) a été mesurée chez des espèces choisies d'invertébrés aquatiques grâce à deux méthodes s'inspirant de la technique d'Ellman - procédé normalisé, utilisant un spectrophotomètre (méthode BMC) et un essai sur microplaque. Les concentrations d'AChE variaient de 12,7 mU/mg de protéine pour Daphnia Magna à 96,9 mU/mg de protéine pour Hydropsyche spp. Les coefficients de variation (CV) pour la technique de la microplaque étaient beaucoup plus faibles que les CV correspondant à la méthode BMC (par ex. 6,9 à 25,5 % contre 34,6 à 55,4 %), et ils permettaient d'effectuer des mesures sur des organismes individuels entiers ou sur des capsules céphaliques individuelles.

L'exposition d'invertébrés à de faibles concentrations d'insecticides organophosphatés, l'azinphosméthyl et le fénitrothion, n'a pas entraîné une baisse significative des concentrations d'AChE, à une seule exception près, les concentrations d'AChE chez Hyalella azteca, qui ont diminué de 55,2 % par rapport à des témoins, après une exposition à 2,0 ?g d'azinphosméthyl/L pendant 24 h. Des expositions pendant des périodes prolongées (48 à 96 h) n'ont pas résulté en concentrations réduites. L'exposition de la perle, Claassenia sp., au chlorpyrifos, a réduit de façon significative, soit de 30,7-45,1 %, les concentrations, qui se rapprochaient ainsi de la teneur létale (? 40 ?g/L). Les résultats montrent que la mesure de l'activité de l'AChE chez les invertébrés aquatiques exposés à des concentrations d'insecticides organophosphatés (comparables à celles du terrain) peut représenter une méthode biochimique très utile, mais seulement aux fins de détection d'une toxicité aiguë résultant d'une exposition sur le terrain. De plus, le choix des espèces peut se révéler très important pour la détection des effets sublétaux.

INTRODUCTION

Organophosphate insecticides can contaminate surface waters through intentional application during control of biting insects, or unintentionally through drift of aerial spray, watershed drainage and/or accidental spillage. Aquatic invertebrates living in surface waters can thus be exposed to insecticide levels which range from acutely lethal to sublethal. Traditionally, insecticide residues of organophosphates occurring in water near areas of application have been difficult to correlate with sublethal effects on invertebrates. Large-scale studies have examined the effects of these insecticides on the population dynamics of both aquatic plants and animals and have correlated the direct and indirect effects of these pesticides with plant-herbivore or predator-prey interactions (Hurlbert, 1975). Approaches to the problem from a physiological and/or biochemical perspective are still rare (Edwards and Fisher, in press).

Organophosphate insecticides act as nerve poisons by blocking synaptic transmission in the cholinergic portions of the nervous system (O'Brien, 1976). The disruption of nerve impulses is caused by excessive accumulation of the neurotransmitter, acetylcholine (ACh) which is normally broken down by the enzyme acetylcholinesterase (AChE). Organophosphates bind to the active site of AChE by phosphorylation and prevent the breakdown of ACh. This results in repeated stimulation of the nerve fibres by ACh and the eventual failure of the nervous system.

The inhibition of AChE activity has been used successfully in

combination with other measurements of chemical residues, behavior and toxic response, as a tool in diagnosing organophosphate poisoning in birds and fish (Macek et al., 1972; Hill and Fleming, 1982; Jarvinen et al., 1983; Lockhart et al., 1985; Zinkl et al., 1987; Busby et al., 1987). However, few studies (Karnak and Collins, 1974; Flannagan et al., 1978 and Srinivasulu Reddy and Ramana, 1988) have examined the effects of these insecticides on AChE levels in invertebrates as a means of detecting sublethal toxicity due to the contamination of aquatic ecosystems with organophosphate insecticides.

The objectives of this research are 1) to measure levels of AChE in several species of aquatic invertebrates 2) observe any significant changes in such measurements following exposure to sublethal levels of organophosphate insecticides and 3) determine if such measurements can be a useful diagnostic tool in detecting sublethal toxicity to low concentrations of organophosphate insecticides.

MATERIALS AND METHODS

Levels of acetylcholinesterase were measured in several species of aquatic invertebrates (nymphs or juvenile instars) obtained either from laboratory cultures maintained at Canada Centre for Inland Waters, Burlington, Ontario or local rivers and streams. Animals ranged in size from the cladoceran, Daphnia magna (≤2.8 mm; laboratory culture), to the stonefly, Claassenia sp.(≥3.0 cm), and included the amphipod, Hyalella azteca (laboratory

culture), the mayfly, Ephemerella sp., and the caddisfly, Hydropsyche slossonae/betteni. Animals were collected in the field by dislodging bottom substrate and capturing drifting insects in a D-net held immediately downstream (kick sampling) in riffle areas of Bronte Creek and the Upper Forks of the Credit River in southern Ontario. Animals were returned to the laboratory, placed in 10-L recirculating stainless steel laboratory bioassay units (modified from Rodrigues and Kaushik, 1984) containing a pebble substrate and acclimated in Lake Ontario water at 10 °C for 2-3 days prior to use in exposure studies. Two strains of house flies (Musca domestica; both resistant and susceptible to organophosphate insecticides) were also obtained from the University of Guelph, Guelph, Ontario and analysed for levels of AChE.

Groups of aquatic invertebrates were exposed in separate expseriments to three organophosphate insecticides (azinphosmethyl, O,O-dimethyl S-((4-oxo-1,2,3-benzotriazin-3(4H)-yl)methyl) phosphorodithioate; chlorpyrifos, O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate; and fenitrothion, O,O-dimethyl O-(4-nitro-m-tolyl) phosphorothioate) at several lethal and sublethal concentrations in duplicate recirculating tanks. All pesticides were technical grade (≥ 95% purity) and concentrations in each tank were obtained by addition of a stock solution in acetone. Addition of acetone to the tanks never exceeded 0.05%. At various time intervals (24-96 h), single organisms or groups of animals (5-15) were removed in triplicate from the tanks. Whenever possible, analysis of AChE was immediate or within 1-2 weeks using frozen

(-20°C) organisms. Karnak and Collins (1974) and Zinkl et al., (1987) reported no significant loss of AChE activity in the heads of larval midges and fish brain tissue frozen at -20°C for extended periods of time.

The tanks were monitored during each experiment for mortality of the invertebrates, water temperature and dissolved oxygen. Samples of water (1.5 l) from each exposure and control tanks were analysed for pesticide content at the end of each of experiment. Water samples were extracted in dichloromethane and analysed using a Varian 3400 gas chromatograph (GC) according to Batchelor et al. (1990).

Sample preparation

Animal tissues were homogenized in either 1.0 ml of 0.25 M sucrose or 52.0 mM phosphate buffer (pH 7.0 - 7.4) using an automated Fisher Scientific homogenizer (Model 7265) at high speed for 30 s. Whole bodies of Daphnia and Hyalella were homogenized; larger species of invertebrates (e.g., mayflies, stoneflies, caddisflies, etc.) were decapitated and only head capsules were analysed for AChE. The homogenized sample was transferred quantitatively to a centrifuge tube and centrifuged for 10 min at 8500 rpm. The samples were kept on ice throughout the homogenization process and the centrifuge temperature was 2-4°C.

Analysis of Acetylcholinesterase

The AChE assay was investigated using a technique which measures a colorimetric reaction between the hydrolyzed acetylcholine (ACh) analogue, thiocholine, and the reagent dithiobisnitrobenzoate (DNTB)

(Ellman et al., 1961). In early experiments, the reaction was measured on a spectrophotometer (Philips Pye Unicam UV/VMS) at 405 nm in microcuvettes using 100 µl of acetylthiocholine iodide (156 mM), 3.0 ml of DNTB/phosphate buffer (0.26 mM/52.0 mM) (Boehringer Mannheim Corporation, Montreal) and 20 µl sample (the modified BMC technique). The sample was added last and 30 s was allowed before the first absorbance reading was taken; absorbances were then recorded at 30 s intervals for 90 s. Quality control was maintained using Precitrol N, a lyophilized control serum. A blank (buffer + DTNB + substrate) was subtracted from the absorbance increase per minute.

It was determined early in the research that the above procedure, although accurate for AChE activity in the control serum and groups of pooled (5-15) animals, was not sensitive enough to measure the enzyme levels in small invertebrates such as Hyalella or Daphnia or in single head capsules of the larger species. Several methodologies based on adaptations of the Ellman procedure have utilized a microassay technique and a microplate reader to analyse for AChE; these methods have been shown to increase the sensitivity of the assay (Brogdon and Dickinson, 1983; Moores et al., 1988). Therefore, in later experiments, a microplate reader (Molecular Devices Kinetics) was utilized to analyse the enzyme levels. 50 µl samples were transferred to each microplate well (total volume 300 µl) and diluted with 50 µl of 52.0 mM phosphate buffer. After an incubation period of 10 min., 100 µl of the substrate (2.6 mM ATCh) was added to each well. 100 µl of DTNB

(0.326 mM) was then placed in each well following a second incubation period of 10 min. The optical density was read at 405 nm on the microplate reader for 10 min. and the instrument blank was 100 µl buffer + 100 µl of ATCh and 100 µl of DTNB.

Analysis of protein

Initially, protein was assayed using the bicinchoninic acid (BCA) microtechnique (Smith et al., 1985) based on the colorimetric reaction between protein and Cu^{++}. Protein standards were prepared with Bovine Serum Albumin (BSA; Pierce). Absorbance was measured at 562 nm on a Philips Pye Unicam UV/VMS spectrophotometer.

Measurement of protein was later adapted to the microplate technique following the procedure of Bradford (1976) which measures the absorbance of a dye, Coomassie Brilliant Blue G-250) which binds to protein present in the sample. This reaction is measured at 595 nm. Protein standards ranging from 5 to 200 µg/ml in distilled water were made from BSA.

AChE levels were standardized to protein concentrations for each sample and activity is expressed as mU/mg protein; a mU is 1 µmole of substrate hydrolysed/l/min.

The results were analysed using analysis of variance (ANOVA; Steel and Torrie, 1980) to compare within each group of species the activities for the different treatments. The groups that were significantly different ($P \leq 0.05$) were analysed with Duncan's New Multiple Range Test using Parastat, a program for the personal computer developed and assembled by T. James, Department of

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RESULTS

The activities of AChE in several species of invertebrates were generally higher for the same species using the modified BMC technique compared to the microplate technique (Table I). The coefficients of variation (CVs) were also much higher for this method e.g., values range from 34.6-55.4% compared to 6.9-25.5% for the microplate technique. In addition, the microplate technique detected measurable levels of AChE in single head capsules or single organisms for any given species.

Both resistant and susceptible strains of houseflies (Musca domestica) had the highest activities of AChE for all invertebrates tested using the microplate technique. Within the aquatic invertebrates, Hyalella azteca had the highest levels of AChE activity (74.8 ± 5.8) and Daphnia magna the lowest (12.7 ± 2.2).

Acute lethality tests were not conducted with the invertebrates used in this study due to the large numbers of organisms required from the field to carry out such bioassays. Instead, the concentrations for the lethal and sublethal exposure of organisms to the three organophosphate insecticides were based on a range of values obtained from the literature for aquatic invertebrates, particularly stream organisms (Table II).

The activity of AChE in caddisflies (Hydropsyche spp.) and mayflies (Ephemerella sp.) exposed to nominal concentrations of 0.5 and 5.0 µg/l of azinphosmethyl (measured concentrations 6.99 and 0.20

µg/l) were not found to be significantly different from those in the control organisms at both concentrations and periods of exposure (Fig. 1); however, activities in all organisms were found to decrease over the experiment in both control and treated animals. No moribund or dead animals were observed during the experiment.

The exposure of Hyalella azteca to nominal concentrations of 0, 0.05, 0.5 and 2.0 µg azinphosmethyl/l resulted in a signficant increase (130%) at the medium concentration of 0.5 µg/l and a significant decrease (55.2%) at the highest concentration of 2.0 µg/l after 24 h exposure (Fig. 2). Further exposure had no significant effects on levels of AChE compared to those of the control animals.

In contrast, stoneflies (Claassenia sp.) exposed to several concentrations of chlorpyrifos showed significant reductions in AChE activity at concentrations as low as 20 µg/l (Fig. 3). For example, concentrations of AChE were reduced to 30.7 to 45.1% of the controls at levels of chlorpyrifos ≥ 20 µg/l. However, over continuous exposure for 72 h, only concentrations ≥ 60 µg/l consistently caused reductions in AChE (4.3 to 35.2% of controls). Concentrations ≥ 40 µg/L resulted in a significant percentage of moribund or dead organisms (Table III).

Exposure of the same species (Claassenia sp.) to concentrations of fenitrothion ranging from 0 - 10 µg/L (measured concentration 0.87-10.53 µg/L) resulted in increases in AChE in treated animals following exposure for 24 h (Fig. 4). Further exposure resulted in a decrease in AChE but results were not significantly different from controls.

DISCUSSION

The AChE activities reported in the present study for several species of aquatic invertebrates are in the range of values reported for other invertebrates. For example, Flannagan et al. (1978) analysed the head capsules of the stonefly, Acroneuria spp., and found values to range from approximately 12.2 to 90.0 mU/mg protein for control animals and those exposed to fenitrothion. Pree et al. (1987) found that the rate of hydrolysis of acetycholine bromide for the nematode, Aphelenchus avenae and the housefly, Musca domestic, was 40.9 ± 17.1 and 226 ± 46.1 mU/mg protein, values which compare favourably with those in this study. Past observations of terrestrial insects have revealed an apparent correlation between physical activity and levels of AChE (Metcalf et al., 1955); for example, active insects such as houseflies have higher AChE activity than less active insects such as lepidopterous larvae. Aquatic invertebrates such as Hyalella azteca may be more active that sedentary organisms such as the net-spinning, filter-feeding caddisfly and this may explain differences in their activities as measured by the microplate technique.

As early as 1959, Weiss and Weiss and Gakstatter (1964) suggested that very low concentrations of organophosphate insecticides in natural waters could be detected by measuring the degree of inhibition of AChE activity in the brains of fish and invertebrates. In laboratory studies with sheepshead minnows, Coppage (1972) reported that a reduction in brain cholinesterase activity to about 18% of normal could be used to predict impending

death due to poisoning by parathion, azinphosmethyl and phorate. Similar results have been reported with invertebrates; for example, Flannagan et al. (1978) exposed nymphs of the stoneflies, Acroneuria lycorias and A. abnormis to 1, 2, and 40 µg/l fenitrothion and reported significant reductions in AChE activity with time at all three concentrations. Srinivasulu Reddy and Ramana (1988) found that the AChE activity of nervous tissue of the penaeid prawn, Metapenaeus monoceros, was significantly inhibited in animals exposed to both lethal and sublethal concentrations of phosphamidon and methyl parathion for up to 48 h.

It has been generally accepted that a 20% or greater depression in AChE activity in either birds, fish or invertebrates indicates exposure to organophosphate insecticides; a 50% or greater depression is indicative of a life-threatening situation although some animals have been shown to survive higher levels of inhibition and others succomb at levels below 50% (Ludke et al., 1975; Zinkl et al., 1987; Busby et al., 1989).

In the present study, only chlorpyrifos at concentrations ≥ 40 µg/l and azinphosmethyl at a concentration of 2.0 µg/l resulted in greater than 50% reductions in AChE activities. These concentrations are well within the range of acute lethalities for bioassays conducted with benthic invertebrates, particularly stoneflies (Table II), which exhibited signs of morbidity and mortality during the experiment.

Exposure of Claassenia sp. to fenitrothion and other species of invertebrates to azinphosmethyl did not result in significant

depressions of AChE activity even though the concentrations tested were within the lethal and sublethal ranges from the literature for invertebrates. In fact, levels of AChE increased in stoneflies exposed to low concentrations of fenitrothion at 24 and 48 h with a gradual non-significant decrease after 72 h exposure. Similar results were observed for Hyalella azteca exposed to sublethal concentrations of azinphosmethyl. Exposure of the red American crayfish, Procambarus clarkii, to low concentrations of the organophosphate triclorfon resulted in an increase in AChE followed by an abrupt reduction in the enzyme after four days (Repetto et al., 1988).

The lack of a significant depression in AChE levels in some invertebrates exposed to organophospate insecticides at supposedly lethal and sublethal concentrations and the occasional increase in levels may be explained by the following. First of all, aquatic invertebrates exhibit a wide range of sensitivities to organophosphate insecticides. For example, 24 h LC_{50}'s for fenitrothion can be as low as 0.4 µg/L for Culex tarsalis and 2.0 µg/L for Acroneuria sp. but other invertebrates such as the caddisfly, Brachycentrus numerosus, have been shown to be sensitive only at concentrations ≥ 20000 µg/L (Flannagan, 1973; Symons and Metcalfe, 1978). The results from the present study indicate that choice of species of invertebrate and a knowledge of their sensitivities to organophosphate insecticides would be important in any hazard assessment program which uses changes in AChE activities as an indication of sublethal toxicity.

Studies on bird and fish populations exposed to low concentrations of organophosphates have shown great variability in levels of AChE even amongst individuals of the same species. For example, Busby et al. (1987) noted control birds which exhibited brain cholinesterase activity indicative of exposure to a cholinesterase-inhibiting agent. Ernst and Julien (1984) observed a 19-26% depression in brain cholinesterase of caged brooktrout compared with control animals in streams aerially oversprayed with fenitrothion at field application rates of 210 g a.i./ha; however, due to the high variability of individual measurements, the treatment samples were not significantly different from the controls. Brain AChE has been shown to vary in relation to such factors as age, sex, physiological condition and route of exposure of pesticides to the animal (Fairbrother et al., 1989). These results make it difficult to achieve statistically significant differences between organisms exhibiting a response and those considered to be controls. It therefore becomes very important to establish the "normal" range of values for AChE levels within a species before attempting to document exposure. It also is necessary to select an analytical method for the biochemical determination of AChE levels which is as precise as possible i.e., the microplate technique utilized in the later portion of this study.

A final factor to consider in the depression of AChE levels as a biochemical indicator of toxicity to organophosphate insecticides is the importance of cholinesterase inhibition as the sole lesion in invertebrate poisoning. Although the primary mode of action of

organophosphates is thought to be AChE inhibition, other effects such as the stimulation of oxygen consumption, the liberation of toxins other than AChE and the inhibition of other enzymes e.g., aliphatic esterase (Ali-E), have all been reported during exposure of insects to organophosphates (Winteringham and Lewis, 1959). Thus, sites of action other than AChE inhibition may be involved in toxicity and measurements of other biochemical and/or physiological parameters may be more important indicators of toxicity.

The main purpose of acetylcholinesterase monitoring is to reliably detect sublethal responses to pesticide applications at the biochemical level before more detrimental effects in terms of reductions in growth, reproduction and survival manifest themselves. In the present study, low concentrations of two organophosphate insecticides i.e., azinophosmethyl and fenitrothion, did not result in significant reductions in AChE in several species of aquatic invertebrates at sublethal concentrations likely to occur in natural bodies of water during operational spraying (Fairchild et al., 1989). Only exposure to chlorpyrifos significantly depressed AChE levels and this occurred mainly at concentrations approaching lethality.

These results suggest that only certain species of aquatic invertebrates may be good candidates as indicator organisms in hazard assessment using AChE inhibition as a parameter of toxicity. In addition, the use of AChE levels in aquatic invertebrates exposed to low concentrations of organophosphate insecticides may not be sensitive enough for detecting sublethal toxicity but could be useful in cases where acute lethality is likely to occur.

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TABLE I. ACTIVITY OF ACETYLCHOLINESTERASE IN SELECTED SPECIES OF INVERTEBRATES (mU/mg protein).

Species	AChE Activity (mU . mg protein)	
	Modified BMC[a]	Microplate[b]
Hydropsyche spp. (caddisfly)	92.6 ± 38.0 (40.9%)[c]	17.4 ± 2.6 (14.9%)
	96.9 ± 31.3 (34.6%)	-
Claassenia sp. (stonefly)	78.5 ± 29.5 (37.6%)	26.9 ± 2.5 (9.3%)
	41.5 ± 23.0 (55.4)	41.2 ± 10.5 (25.5%)
Ephemerella sp. (mayfly)	25.9 ± 9.2 (35.5%)	42.2 ± 6.7 (15.9%)
Musca domestic		
(R)	-	74.9 ± 8.9 (11.1%)
	-	69.0 ± 10.6 (15.0%)
(S)	-	105.5 ± 22.4 (21.2%)
	-	92.4 ± 6.4 (6.9%)
Daphnia magna	-	12.7 ± 2.2 (17.3%)
Hyalella azteca	-	74.8 ± 5.8 (7.8%)

[a] replicate (n = 3-9) groups of (5-15) animals
[b] replicate (n = 4-15) single head capsules or whole organisms
[c] coefficient of variation

(R) = resistant strain (S) = susceptible strain

TABLE II. ACUTE TOXICITIES OF ORGANOPHOSPHATE INSECTICIDES TO INVERTEBRATES.

Compound	LC_{50} µg/l	Exposure Time (h)	Species	Reference
Azinophosmethyl	8.0	48	Pteronarcys sp. (stonefly)	Sanders & Cope, 1968
	3.2	48	Daphnia magna (cladoceran)	"
Chlorpyrifos	50.0	48	Pteronarcys sp. (stonefly)	Marshall & Roberts, (1978)
	8.2	24	Claassenia sp. (stonefly)	"
	50.0	22	caddisfly	"
	10-30	-	dragonfly	"
	25.0	24	Daphnia magna	
Fenitrothion	2.0	-	Acroneuria sp. (stonefly)	Flannagan (1973)
	28.0	48	Pteronarcys sp.	Sanders & Cope, 1968
	12.0	48	Gammarus lacustris	"

TABLE III. MORBIDITY AND MORTALITY OF STONEFLIES (Claassenia sp.) EXPOSED TO CHLORPYRIFOS.

Concentration µg/l	Time (h) 24	48	72
0	0	0	0
20	0	0	0
40	0	80* (20**)	100**
60	80*	60* (40**)	100**
80	60* (40**)	60* (40**)	100**

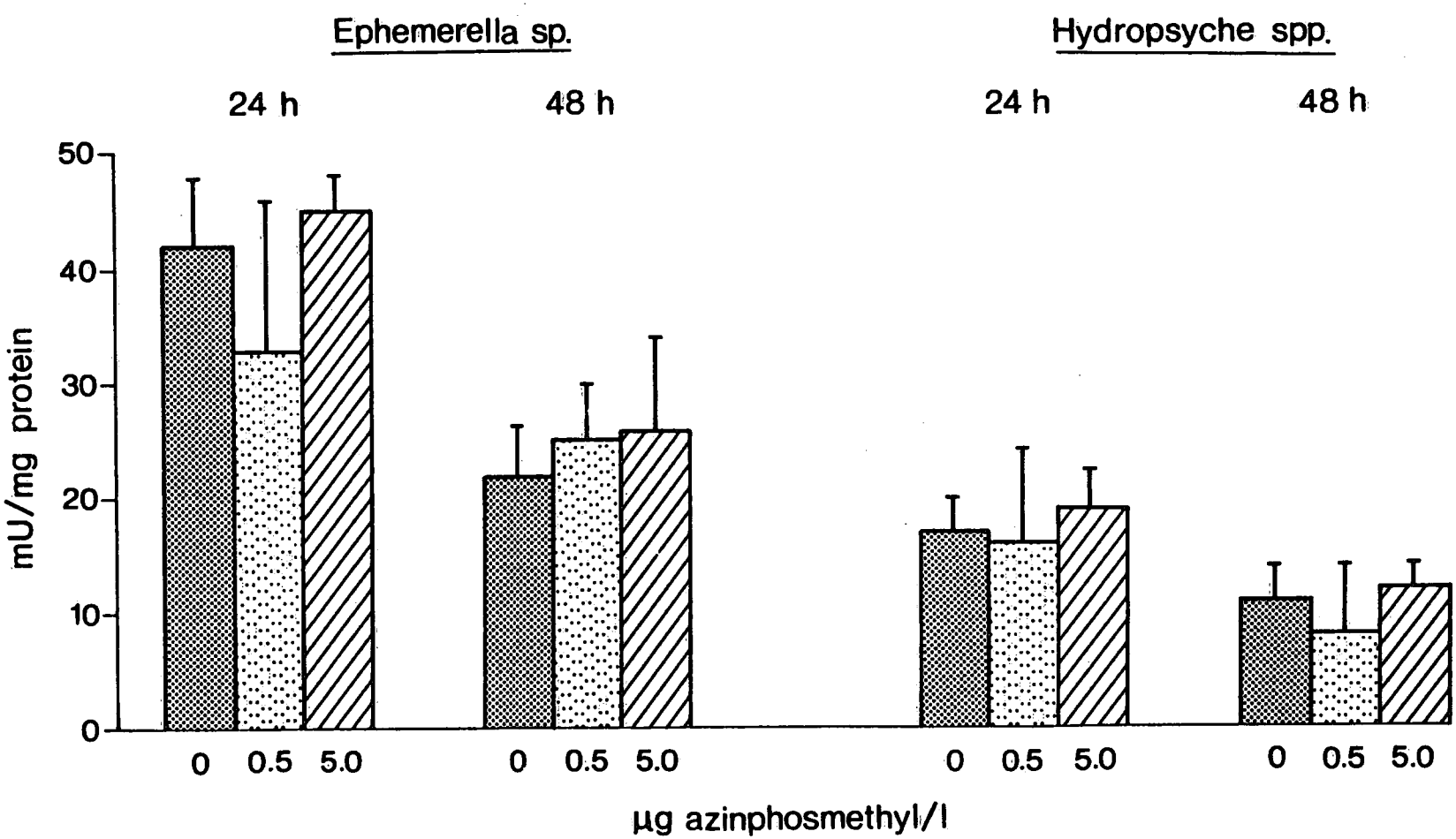
* percentage moribund
** percentage dead

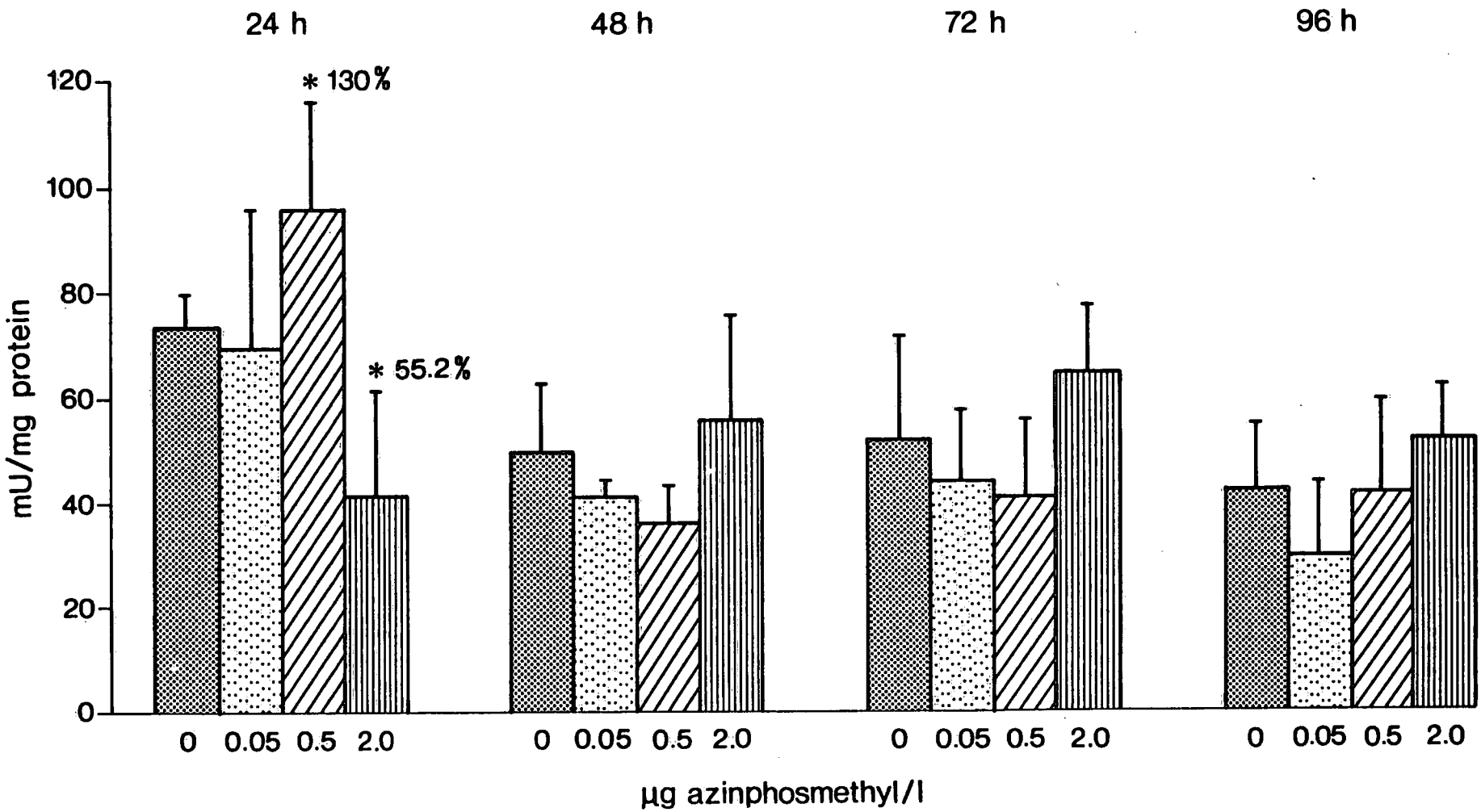
FIGURE 1. AChE activity in mayflies (Ephemerella sp.) and caddisflies (Hydropysche spp.) exposed to sublethal concentrations of azinophosmethyl.

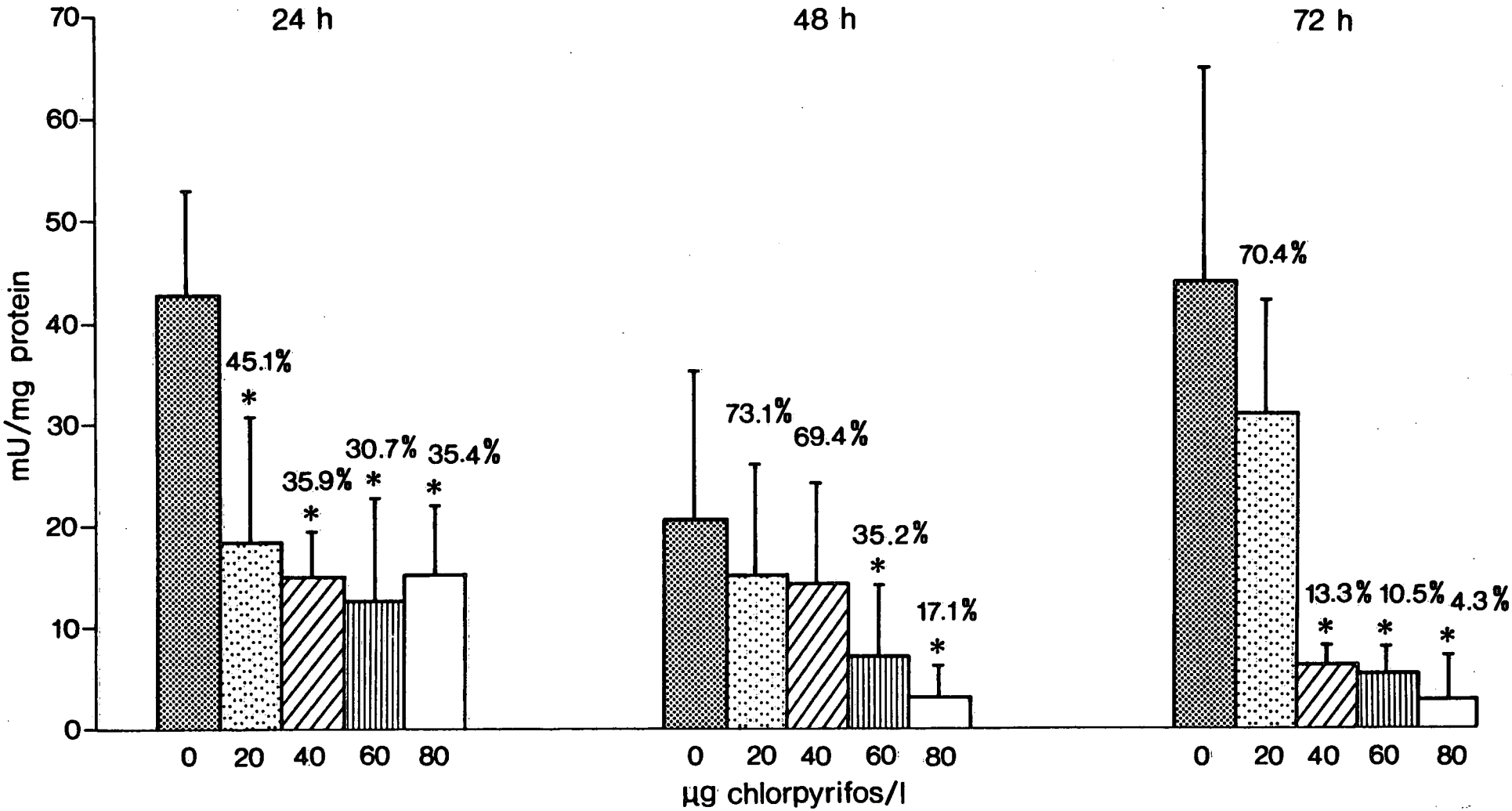
FIGURE 2. AChE activity in the amphipod, Hyalella azteca, exposed to sublethal concentrations of azinphosmethyl.

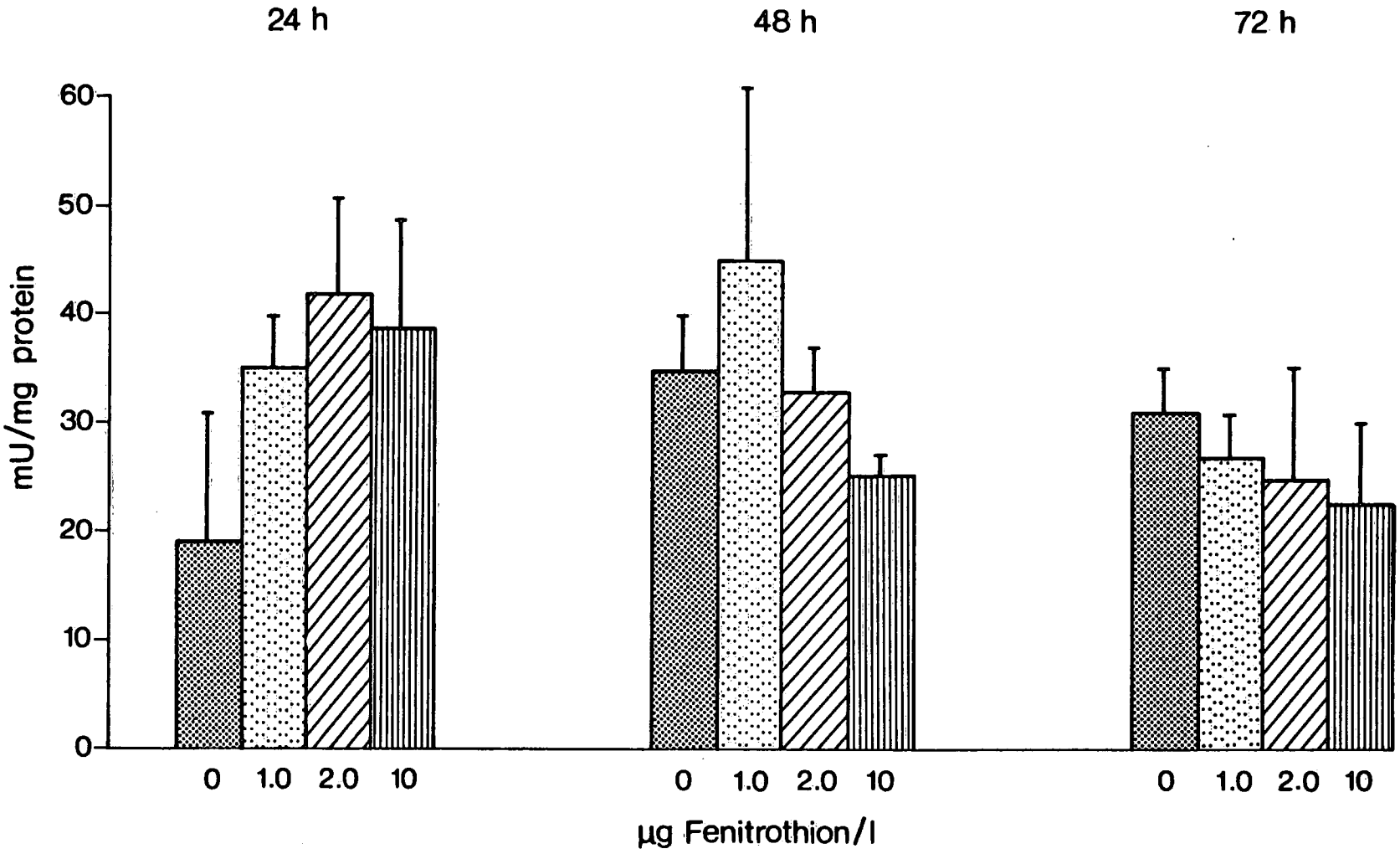
FIGURE 3. AChE activity in the stonefly, Claassenia sp., exposed to lethal and sublethal concentrations of chlorpyrifos.

FIGURE 4. AChE activity in the stonefly, Claassenia sp., exposed to sublethal concentrations of fenitrothion.


Ephemerella sp.
Hydropsyche spp.
24 h
48 h
24 h
48 h
50
40
30
20
10
0
mU/mg protein
0
0.5
5.0
0
0.5
5.0
0
0.5
5.0
0
0.5
5.0
µg azinphosmethyl/l


24 h
48 h
72 h
96 h
120
100
80
60
40
20
0
mU/mg protein
* 130%
* 55.2%
0 0.05 0.5 2.0
0 0.05 0.5 2.0
0 0.05 0.5 2.0
0 0.05 0.5 2.0
µg azinphosmethyl/l


24 h
48 h
72 h
mU/mg protein
70
60
50
40
30
20
10
0
45.1%
35.9%
30.7%
35.4%
73.1%
69.4%
35.2%
17.1%
70.4%
13.3%
10.5%
4.3%
*
0 20 40 60 80
µg chlorpyrifos/l


24 h
48 h
72 h
60
50
40
30
20
10
0
mU/mg protein
0
1.0
2.0
10
µg Fenitrothion/l