

Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*

Carlos Barata^{a,*}, Arun Solayan^b, Cinta Porte^b

^a Laboratory of Environmental Toxicology, INTEXTER-UPC, CN 150, Km 14.5, 08220 Terrassa, Barcelona 08220, Spain

^b Department of Environmental Chemistry, IIQAB-CSIC, Jordi Girona 18, Barcelona 08034, Spain

Received 14 April 2003; received in revised form 6 July 2003; accepted 11 July 2003

Abstract

In this study, the cladoceran *Daphnia magna* was exposed to two model organophosphorous and one carbamate pesticides including malathion, chlorpyrifos and carbofuran to assess acetylcholinesterase (AChE) and carboxylesterase (CbE) inhibition and recovery patterns and relate those responses with individual level effects. Our results revealed differences in enzyme inhibition and recovery patterns among the studied esterase enzymes and pesticides. CbE was more sensitive to organophosphorous than AChE, whereas both CbE and AChE showed equivalent sensitivities to the carbamate carbofuran. Recovery patterns of AChE and CbE activities following exposure to the studied pesticides were similar with 80–100% recoveries taking place 12 and 96 h after exposure to organophosphorous and carbamates pesticides, respectively. The physiological role of AChE and CbE inhibition patterns in *Daphnia* was examined by using organophosphorous and carbamate compounds alone and with specific inhibitors of CbE. Under exposure to organophosphorous pesticides, survival of *Daphnia* juveniles was impaired at AChE inhibition levels higher than 50% whereas under exposure to the carbamate carbofuran low levels of AChE inhibition affected mortality. Inhibition of CbE by 80–90% increased toxicity to organophosphorous and carbamate pesticides by up to two- and four-fold, respectively. Our results suggest that both AChE and CbE enzymes are involved in determining toxicity of *Daphnia* to the studied chemicals and that AChE inhibition levels higher than 50% can be considered of environmental concern to *Daphnia* species.

© 2003 Elsevier B.V. All rights reserved.

Keywords: *Daphnia*; Chlorpyrifos; Malathion; Carbofuran; Acetylcholinesterase; Carboxylesterase

1. Introduction

Organophosphorous and carbamates pesticides are among the most commonly used pesticides in the western countries. Their success rely on their

high toxicity and rapid environmental degradation (Eto, 1974). Unfortunately, organophosphorous and carbamates pesticides lack of target specificity and can cause severe, long lasting population effects on aquatic non-target species, particularly invertebrates (Schulz and Liess, 1999; Fulton and Key, 2001). Thus appropriate monitoring strategies are required to protect sensitive non-target invertebrate species against the adverse effects of organophosphorous pesticides

* Corresponding author. Tel.: +34-93-7398396; fax: +34-93-7398392.

E-mail address: barata@intexter.upc.es (C. Barata).

and carbamates. Evaluating environmental risks associated to organophosphorous and carbamates pesticides is not an easy task since their low octanol/water partition coefficients and high biotransformation rates prevent these chemicals to accumulate through the food chain and hence to be detected by conventional analytical techniques (Chambers et al., 2002). In relation to this the biomarker approach is particularly suitable for evaluating the exposure to or effects of this class of compounds (Chambers et al., 2002).

Organophosphorous and carbamates pesticides are known to inhibit type “B” esterases including cholinesterases and carboxylesterases, by binding to the active site and phosphorylating the enzyme. Among cholinesterases, acetylcholinesterases (AChE; EC 3.1.1.7) are considered to be the target site of organophosphorous and carbamates pesticides. AChE preferentially hydrolyze acetylcholine and their persistent inhibition causes neurotoxic effects (O'Brien, 1960). In mammals, death is caused by asphyxiation (Murphy, 1966), but the action in aquatic organisms including fish and invertebrates is less clear since as long as water crosses respiratory organs (i.e. gills), transfer of oxygen occurs (Day and Scott, 1990; Boone and Chambers, 1996). CbE are known to hydrolyze a wide range of exogenous and endogenous esters, and they are assumed to provide protection against organophosphorous and carbamate pesticides poisoning through two main mechanisms: (1) direct hydrolysis of ester bonds in organophosphorous and carbamates pesticides and (2) stoichiometric phosphorylation with resultant destruction of the oxon, carbamate, which reduces the amount of organophosphorous and carbamate pesticide available for AChE inhibition (Jokanovic et al., 1996).

Despite of B-esterases being used to evaluate exposure organophosphorous and carbamates pesticides in aquatic invertebrates, their patterns of inhibition, recovery and relationship with individual level effects are unknown for most species (Day and Scott, 1990; Fulton and Key, 2001). Patterns of AChE inhibition and its correspondence with the likelihood of survival have only been reported in a few species (Day and Scott, 1990; Kuhn and Streit, 1994; Sturm and Hansen, 1999; Barata et al., 2001; Fulton and Key, 2001); and less information is available on the role of CbE on aquatic invertebrates (Escatin and Porte, 1997; Galloway et al., 2002).

The cladoceran species *Daphnia magna* offers an excellent model system to relate B-esterase inhibition patterns with individual level effects. *D. magna* is very sensitive to organophosphorous pesticides (Guilhermino et al., 1996; Barata et al., 2001), is widely used in aquatic risk assessment and often inhabits small water bodies in and around agricultural fields receiving organophosphorous and carbamates pesticide treatments (Barata et al., 2000a). Thus, the studied relationships between B-esterase inhibition and toxicity responses may help to understand the physiological role of AChE and CbE in related species. Although AChE inhibition responses in *D. magna* have been previously used as a tool to diagnose organophosphorous pesticide poisoning (Guilhermino et al., 1996; Barata et al., 2001), the kinetics of inhibition and recovery have never been conducted. Furthermore, information regarding the role of other esterases that may also be involved in determining toxicity differences to organophosphorous and carbamate pesticides such as CbE is lacking.

The specific objectives of this research are: (1) to assess the patterns of inhibition and recovery of AChE and CbE in *D. magna* exposed to model organophosphorous and carbamate pesticides and (2) to assess the physiological role of B-esterases in determining toxicity to these pesticides. The study will be conducted with two different anticholinergic acting chemical families: the organophosphorous pesticides chlorpyrifos and malathion and the carbamate pesticide carbofuran. The selected organophosphorous pesticides need to be activated into their oxon metabolites by cytochrome P450 enzymes to become toxic and inhibit AChE and CbE (Chambers et al., 1990; Thompson, 1992). Recovery of oxon-inhibited AChE is very slow, thus reestablishment of enzyme activity will depend mainly on de novo synthesis (Yuan and Chambers, 1996). Conversely, the carbamate pesticide carbofuran does not need to be activated to exert its toxicity and recovery of carbamate-inhibited AChE is relatively fast (Zinkl et al., 1991). Thus, by using organophosphorous and carbamate pesticides it would be possible to compare enzymatic and toxicity patterns in two different acting chemicals. Toxicity bioassays with and without pesticide synergists coupled with enzyme assays will be used to achieve our goals (Yuan and Chambers, 1996).

2. Material and methods

2.1. Chemicals

Chemicals used for toxicity bioassays were chlorpyrifos (99% purity), malathion (95% purity), triphenyl phosphate (TPP; 99% purity) from Aldrich Chemicals (Gillingham, UK); carbofuran (95% purity) from Polyscience Corp. (Niles, IL, USA); 2-(*O*-cresyl)-4H-1,3,2-benzodioxaphosphorin-2-oxide (CBDP; 97% purity) kindly provided by Oksana Lockridge (Nebraska Medical Center, Omaha, NE). B-esterase and protein assays were performed with α -naphthyl acetate, acetylthiocholine iodine, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and serum albumin from Sigma (St. Louis, MO, USA). Extraction and analysis of organophosphorous and carbamate pesticides were conducted with pesticide grade acetone, methanol and ethyl acetate purchased from Merck (Darmstadt, Germany).

2.2. Experimental animals and toxicity tests

To minimise variability in response to the studied chemicals a single laboratory clone of *D. magna* Straus cultured in the laboratory since 1995 was selected for this study (Baird and Barata, 1998). Bulk cultures of 15 animals each were maintained in ASTM hard synthetic water (APHA et al., 1995) as described by Barata et al. (2000b). Animals were fed daily with *Chorella vulgaris* Beijerinck (10^6 cells/nl, corresponding to 3.6 μ g C/ml; Barata et al., 2000b). The culture medium was changed every other day, and neonates were removed within 24 h. From 200 to 250 neonates were then transferred to 41 tanks and reared under the same conditions as their mothers until they reached their fourth instar (4–5 days at 20 °C). At this stage groups of juveniles were used for enzymatic and toxicity studies.

2.3. Toxicity bioassays

Exposure to the studied pesticides were conducted by using two types of bioassays:

1. Acute toxicity bioassays, with and without the presence of synergists, to determine toxicity and B-esterase sensitivities to the studied organophosphorous and carbamate pesticides. Lethal and en-

zymatic responses were obtained after 24 h exposures of 20 juveniles in 200 ml ASTM hard water to 4–5 concentrations of the studied pesticides in the absence of food. The degree of synergism of two known CbE inhibitors (TPP, CBDP; Maxwell, 1992; Shao-nan and De-Fang, 1997) was investigated by exposing *D. magna* to the studied pesticides with the highest dose of synergist that had no measurable effect on AChE activity in control treatments (7 and 30 nM for CBDP and TPP, respectively). Thus, allowing to assess the role that CbE play in determining toxicity to organophosphorous and carbamate pesticides. Acetone (HPLC grade < 1 ml/l) was used as a carrier for all treatments including controls. Preliminary experiments showed that acetone at this concentration did not affect survival neither B-esterase activity. Experiments were performed in triplicate following established OECD protocols (Barata et al., 2001). At the end of experiments, animals were counted and collected to determine enzymatic activities and lethal concentration effects.

2. Time course experiments were conducted to determine the kinetics of inhibition and recovery of B-esterases, and to relate those responses with toxicity responses. Inhibition of AChE and CbE activities, and mortality responses were determined during exposure to two concentration levels of the studied pesticides selected to cause severe (24 h-LC₅₀) and moderate (50% of the acute dose 24 h-LC₅₀) effects on survival. Groups of 200–250 juveniles were exposed to the selected pesticide concentrations in 41 ASTM hard water without food. Experimental concentrations were renewed every 24 h to minimise pesticide losses during exposure (Barata et al., 2001) and experiments lasted 48 h. Survival and enzymatic responses were assessed at 1, 3, 6, 12, 24 and 48 h time intervals. Recovery patterns were determined by transferring live juveniles previously exposed to severe organophosphorous and carbamate pesticides concentrations to clean ASTM hard water. Following transfer to clean water, enzymatic activities and acute responses were assessed at 1, 12, 24, 48, 72 and 96 h time intervals. Thus, it was possible to relate enzymatic recovery levels with toxic responses to successive pulse acute exposures to organophosphorous and carbamate pesticides, a common

situation in the field (Naddy et al., 2000). Control treatments were always included and sampled at the same time as exposure and recovery treatments.

2.4. Enzyme preparation

In each experiment groups of 20 juveniles surviving pesticide exposure were pooled in an eppendorf and immediately frozen at -80°C until further enzyme analysis. Since the rate of deterioration of B-esterases following death is not known, only live daphnids were used, even though it is likely that the measured percentage inhibition would be biased to a lesser value. Juveniles were homogenized in 500 μl ice-cold 100 mM phosphate buffer, pH 7.4 containing 100 mM KCl and 1 mM EDTA. Homogenates were centrifuged at $10,000 \times g$ for 10 min and the supernatants were immediately used as enzyme sources for AChE and CbE assays.

2.5. Enzyme assays

Biochemical measurements were carried out on Uvikon 941 Plus dual-beam and Spectra-max Plus microplate reader spectrophotometers. AChE was determined by a modification of the Ellman method (Ellman et al., 1961) adapted to microplate (Barata et al., 2001). AChE activity was measured in the presence of 1 mM acetylthiocholine and 0.1 mM 5,5'-dithiobis-2-dinitrobenzoic acid (DTNB), and the increase of absorbance was measured at 405 nm. A previous study showed that the contribution of other esterases on the hydrolysis of acetylthiocholine was negligible, thus all enzymatic activity will be referred to AChE (Barata et al., 2001). Carboxylesterase activity was measured by the UV method of Mastropaolo and Yourno (1981) in the presence of 0.25 mM α -naphthyl acetate, and the formation of naphthol monitored by the increase in absorbance at 235 nm. Homogenate proteins were measured by the method of Lowry et al. (1951).

2.6. Chemical analyses

Water samples of freshly prepared test solutions of the three studied pesticides were collected from experimental vessels at the beginning of the experiment to ensure that initial measured concentrations were close

to nominal levels. Although not measured, previous studies conducted with closely related compounds indicated that pesticide losses during 24 h were expected to be low in our system (Barata et al., 2001). Organophosphorous and carbamate analyses were restricted to the test concentrations used in the time course inhibition experiments: 0.7, 1.4 nM for chlorpyrifos, 6, 12 nM for malathion, 339, 678 nM for carbofuran. Pesticides were extracted from water samples and analysed by solid phase extraction followed by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) following Lacorte and Barceló (1996) procedures. Briefly, chlorpyrifos, malathion and carbofuran water samples of 500, 100 and 50 ml, respectively, were extracted and pre-concentrated in methanol (HPLC grade) using solid-phase OSP-2 C-18 extraction columns (Merck, Whitehouse Station, NJ, USA). Pesticide residues were eluted from solid-phase cartridges with acetyl acetate followed by methanol. The obtained extract was then evaporated under a stream of N_2 and re-suspended in methanol. Actual concentrations of the three chemicals were determined by liquid chromatography (LC) coupled with an APCI interface mass spectrometry. LC conditions were: a symmetry column (250 mm \times 4.6 mm) from Supelco (Sigma-Aldrich Quimica, Madrid, Spain) packed with 4 μm of C8; an elution gradient set from 40% methanol and 60% water to 90% methanol and 10% water in 20 min, followed by 10 min at 90% methanol and 10% water; a flow rate of 1 ml/min. The amount of sample injected was 20 μl . Mass spectrometric characteristics were: a VG platform from Micromass (Manchester, UK) equipped with APCI interface; cone and corona voltages were set at values of 20–40 V and 3–4 kV, respectively; the HV lens voltage was set at 0.32 kV and the focus voltage varied between 27 and 47 V; ion surface and probe temperatures were set to 200 and 400 $^{\circ}\text{C}$, respectively. Chromatograms were recorded under time-schedule SIM conditions using retention times of 11.3, 17.2 and 23.8 min for carbofuran, malathion and chlorpyrifos, respectively. Calibration graphs of eight standard concentrations were used for each chemical examined. Spiked water samples of known standards were pre-concentrated, eluted and analysed at the same time as test samples to determine recoveries. Average \pm S.D. recoveries for the studied pesticides from water samples were

Table 1

Nominal and measured concentrations (mean \pm S.E.M.) of chlorpyrifos, malathion and carbofuran in duplicate water samples taken at the beginning of experiments

| Nominal (nM) | Measured (nM) |
|--------------|-------------------|
| Chlorpyrifos | |
| 0.7 | 0.74 \pm 0.11 |
| 1.4 | 1.57 \pm 0.09 |
| Malathion | |
| 6 | 5.69 \pm 0.16 |
| 12 | 11.50 \pm 0.21 |
| Carbofuran | |
| 339 | 354.13 \pm 51.2 |
| 678 | 708.27 \pm 85.7 |

100 \pm 16% ($n = 12$).

2.7. Statistical procedures

All measurements were performed by triplicate and the results reported as means \pm S.E.M. Significant differences between exposure and control treatments were assessed by the Student's *t*-test corrected with sequential Benferroni procedure for simultaneous tests (Rice, 1989). Lethal concentration values were determined from probit analysis (Finney, 1971). Concentration enzymatic and time dependent response values (i.e. I_{50} , LC_{50}) were determined from non linear regression analysis to account for exponential and threshold biological responses (Barata et al., 2000b). Prior to analysis exposure concentrations X were $\ln(X + 1)$ to include control treatments and data were tested for ANOVA assumptions of normality and variance homocedasticity (Zar, 1996).

3. Results

3.1. Test concentrations

Measured concentrations of the studied pesticides at the beginning of experiments were within 10% of nominal levels, thus indicating that initial exposure levels were similar to nominal concentrations (Table 1). For clarity, hereafter all concentrations will be referred as nominal.

Table 2

Toxicities of three pesticides and the degree of synergism by two CbE inhibitors (CBDP, TPP)

| Treatment | LC_{50} (nM) | Degree of synergism |
|---------------------|------------------------|---------------------|
| Chlorpyrifos | 1.28 (1.06–1.48) | |
| Chlorpyrifos + CBDP | 0.64 (0.47–0.87) | 2 |
| Chlorpyrifos + TPP | 0.722 (0.60–0.85) | 1.8 |
| Malathion | 12.38 (7.34–15.74) | |
| Malathion + CBDP | 6.76 (4.8–9.56) | 1.8 |
| Malathion + TPP | 8.93 (6.16–12.7) | 1.4 |
| Carbofuran | 762.93 (632.76–903.95) | |
| Carbofuran + CBDP | 156.12 (93.95–250.7) | 4.9 |
| Carbofuran + TPP | 111.45 (92.34–134.68) | 6.8 |

Results are reported in 24 h- LC_{50} determined by probit analysis. 95% CI are depicted between brackets. Degree of synergism was determined by LC_{50} of the insecticide/ LC_{50} of the mixture combination of the insecticide and the CbE inhibitor.

3.2. Toxicity responses

D. magna juveniles showed significant differences in sensitivities (non-overlapping 95% CI of LC_{50}) to the studied pesticides (Table 2). Chlorpyrifos was one and almost three-orders of magnitude more toxic to *Daphnia* than malathion and carbofuran, respectively. The LC_{50} of the three pesticides all decreased appreciably when combined with TPP and CBDP exposure levels that inhibited CbE 80–90% (Fig. 1D and E). The synergism was greater for carbofuran, which had low toxicities alone, and was less for chlorpyrifos and malathion.

3.3. Enzyme sensitivities

Acetylcholinesterase (AChE) and carboxylesterase (CbE) activities measured in whole *Daphnia* tissues and their sensitivity to the studied pesticides and synergists are depicted in Fig. 1 and Table 3. Unexposed *Daphnia* showed over 50-fold more CbE activity (200 nmol/(min mg protein)) than AChE (3.5 nmol/(min mg protein)). AChE and CbE activities were inhibited by organophosphorous and carbamate pesticides in a concentration dependent manner following the allosteric decay model which describes a period of no response at low concentrations followed by an accelerating negative response as concentration increased (Fig. 1A–C). Absolute and relative sensitivity of AChE and CbE varied among the

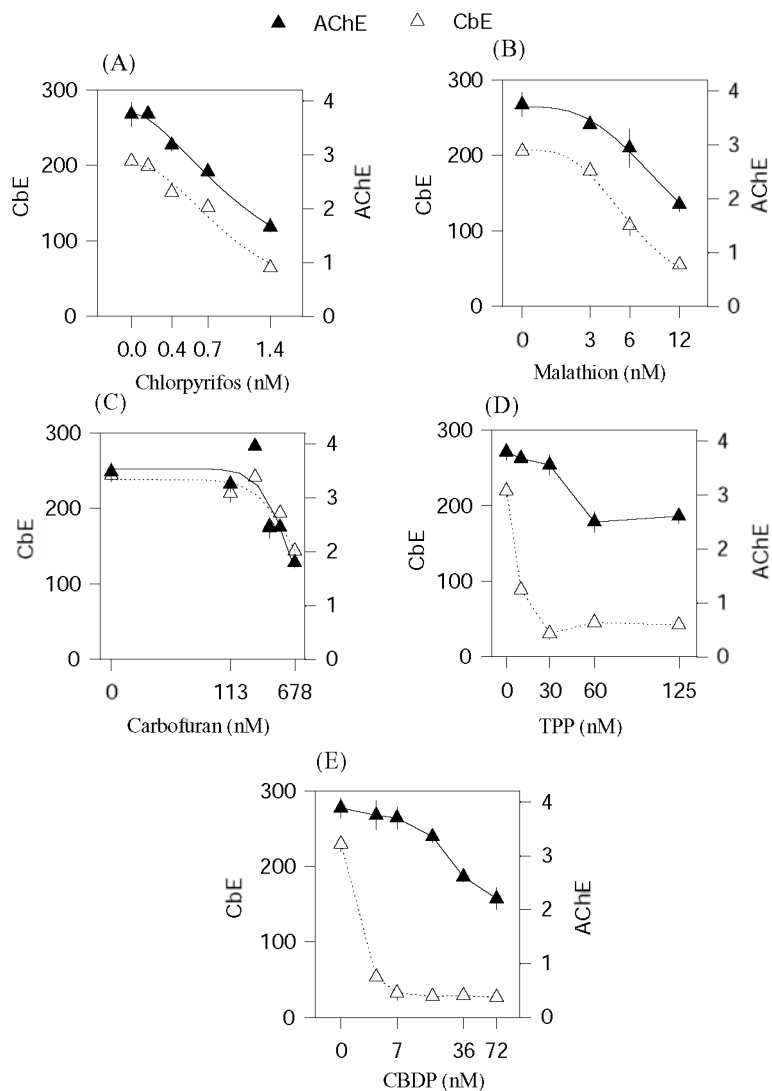


Fig. 1. AChE and CbE activities (nmol/(min mg protein)) in whole *D. magna* tissues as a function of chlorpyrifos (A), malathion (B), carbofuran (C), TPP (D) and CBDP (E) exposure levels. For clarity exposure levels are depicted in log scale. Responses to organophosphorous and carbamate pesticides were fitted to an allosteric decay model. Error bars indicate S.E.M. In some treatments error bars were smaller than symbol size.

studied pesticides (I_{50} , Table 3). AChE and CbE were equally inhibited by chlorpyrifos at concentrations that were over 6- and 500-fold greater than malathion and carbofuran, respectively. Under malathion exposure CbE were about two fold more sensitive than AChE, whereas both AChE and CbE were inhibited at

equivalent concentrations of carbofuran (overlapping 95 CI of I_{50}). TPP and CBDP alone had little or no anti-AChE effect at concentrations as high as 30 and 7 nM, respectively (Fig. 1D and E). Conversely CbE inhibition was near maximum (80–90%) at the previous mentioned exposure levels of TPP and CBDP.

Table 3

Concentration—CbE and AChE inhibition levels (I_{50}) for *D. magna* juveniles to in vivo exposures to chlorpyrifos, malathion, and carbofuran

| Chemical | CbE (I_{50}) | AChE (I_{50}) | | | N | r^2 |
|--------------|----------------------|-------------------|-------|---------------------|-----|-------|
| Chlorpyrifos | 1.0 (0.9–1.1) | 15 | 0.96* | 1.2 (1.0–1.5) | 15 | 0.94* |
| Malathion | 6.5 (5.7–7.5) | 15 | 0.97* | 12.6 (8.5–18.2) | 15 | 0.82* |
| Carbofuran | 896.3 (613.8–1309.3) | 18 | 0.75* | 516.6 (504.8–841.6) | 18 | 0.72* |

I_{50} values are depicted in nM, 95% CI are showed between brackets, r^2 : coefficient of determination, N : sample size. I_{50} values were determined assuming an allosteric decay non-linear model.

* $P < 0.05$.

3.4. Kinetics of inhibition and toxicity

Inhibition dynamic patterns of B-esterases and the relationship between anti-AChE activity and survival

responses was assessed considering severe (LC_{50}) and moderate (50% LC_{50}) levels of exposure to the three studied pesticides (Figs. 2 and 3, Table 4). Time course B-esterase inhibition responses varied among

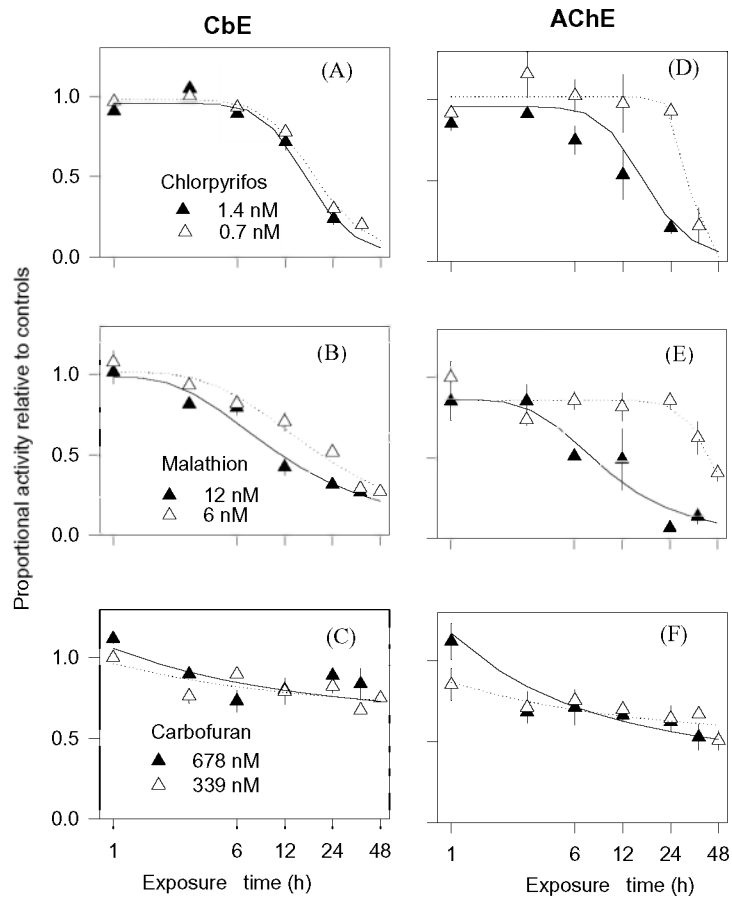


Fig. 2. Time course inhibition of AChE (A–C) and CbE (D–F) responses in *D. magna* following exposures to severe and moderate levels of chlorpyrifos, malathion and carbofuran. Exposure time is depicted in log scale. Error bars indicate S.E.M. In same treatments error bars were smaller than symbol size. Responses to organophosphorous and carbamate pesticides and C were fitted to allosteric decay and exponential models, respectively.

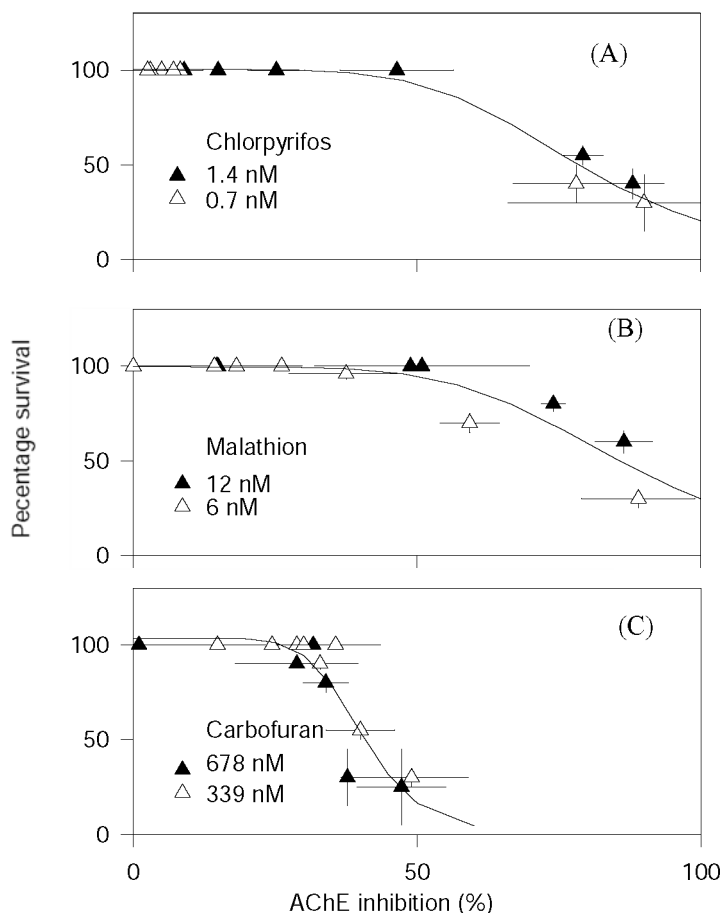


Fig. 3. Time course survival responses of *D. magna* juveniles following exposures to severe and moderate levels of chlorpyrifos (A), malathion (B) and carbofuran (C). Survival is plotted against AChE inhibition responses. Error bars indicate S.E.M. In same treatments error bars were smaller than symbol size. Responses to organophosphorous and carbamate pesticides were fitted to an allosteric decay model.

enzymes, pesticides and exposure levels. AChE and CbE responses to single dose exposures to organophosphorous pesticides followed the allosteric decay model with a period of no response, which was shorter at severe exposure levels, followed by an accelerating negative response as exposure time increased (Fig. 2A, B, D and E). At moderate exposure levels, 10–50% inhibition of CbE activities occurred between 4.8 and 19.7 h, whereas AChE achieved equivalent inhibition levels at greater exposure periods >24 h (Table 4). At severe exposure levels, 10–50% inhibition of AChE and CbE activities occurred at equivalent periods (between 3 and 16.6 h, Table 4). Under exposure to the carbamate carbofuran, CbE and AChE activities were

inhibited immediately after exposure (<2 h) irrespectively of exposure level (Table 4, Fig. 2C and F). The relatively low anti AChE–CbE activity achieved by carbofuran prevented good estimates of the exposure time needed to inhibit those enzymes 50%.

In vivo inhibition of AChE plotted against survival of *D. magna* juveniles denoted differences in the relationship between toxicity and antitarget enzyme potency (Fig. 3, Table 4). Survival of *D. magna* juveniles exposed to organophosphorous pesticides was impaired at inhibition levels of AChE higher than 50%. More specifically, 56 and 80% AChE inhibition was needed to impair survival 10 and 50%, respectively. Conversely, low AChE inhibition levels (<40%) were

Table 4

Time course survival, AChE and CbE inhibition responses of *D. magna* juveniles exposed to chlorpyrifos, malathion and carbofuran

| B-esterases | t_{10} (h) | t_{50} (h) | N | r^2 |
|-------------------------|--------------------------------------|--------------------------------------|-----|-------|
| Chlorpyrifos 0.7 nM | | | | |
| AChE a | 24.4 (17.5–33.9) | 30.9 (26.0–36.6) | 18 | 0.72* |
| CarbE a | 9.0 (7.8–10.4) | 18.7 (17.2–20.1) | 18 | 0.98* |
| Chlorpyrifos 1.4 nM | | | | |
| AChE a | 6.0 (3.2–11.0) | 14.0 (10.0–19.5) | 15 | 0.81* |
| CarbE a | 8.7 (6.8–11.2) | 16.6 (14.4–19.0) | 15 | 0.94* |
| Malathion 6 nM | | | | |
| AChE a | 28.5 (11.6–42.5) | 46.3 (39.1–54.6) | 21 | 0.65* |
| CarbE a | 4.8 (3.2–7.2) | 19.7 (15.3–25.5) | 21 | 0.93* |
| Malathion 12 nM | | | | |
| AChE a | 3.5 (1.6–7.6) | 9.3 (5.3–16.4) | 18 | 0.75* |
| CarbE a | 3.1 (2.1–4.6) | 11.6 (8.4–16.0) | 18 | 0.91* |
| Carbofuran 339 nM | | | | |
| AChE e | 0.7 (0.4–1.1) | – | 21 | 0.52* |
| CarbE e | 1.1 (0.5–1.8) | – | 21 | 0.51* |
| Carbofuran 678 nM | | | | |
| AChE e | 1.2 (0.8–1.8) | – | 18 | 0.70* |
| CarbE e | 1.6 (0.8–3.1) | – | 18 | 0.52* |
| Survival a | LI ₁₀ (% AChE inhibition) | LI ₅₀ (% AChE inhibition) | | |
| Chlorpyrifos 0.7–1.4 nM | 56.1 (41.4–69.1) | 78.1 (74.0–82.2) | 33 | 0.97* |
| Malathion 6–12 nM | 57.2 (42.5–73.2) | 86.2 (78.0–93.1) | 39 | 0.86* |
| Carbofuran 339–678 nM | 25.3 (20.1–32.0) | 38.1 (34.1–42.1) | 39 | 0.80* |

t_{10-50} : exposure time (h) needed to impair B-esterase responses 10–50%, LI₁₀₋₅₀: AChE inhibition level (%) needed to impair survival 10–50%; 95% CI are depicted between brackets; r^2 : coefficient of determination, N : sample size; –: undetermined, * $P < 0.05$. t_{10-50} and LI₁₀₋₅₀ values were determined assuming an (a) allosteric decay non-linear and (e) exponential regression model.

needed to impair *D. magna* survival under carbamate exposure.

3.5. Kinetics of recovery and toxicity

Recovery rates of B-esterases following 24 exposures to severe levels of the studied pesticides varied between organophosphorous and carbamate pesticides, but not between enzymes. B-esterase activities inhibited by organophosphorous pesticides took longer to recover after transfer to clean medium than those inhibited by carbofuran (Fig. 4A–C). More specifically, CbE and AChE activities inhibited by carbofuran recovered to control levels shortly after transfer (<12 h), whereas those inhibited by organophosphorous pesticides took about 24 and 96 h to achieve 50% and near complete recovery levels, respectively.

Toxic responses of *D. magna* juveniles to pulse exposures during recovery allowed determining the re-

lationship between enzymatic recovery and the health of pre-exposed individuals. Mortality of *D. magna* juveniles was negligible (<5%) during the 4 days following termination of severe exposures to organophosphorous pesticides (data not shown). Subsequent pulse acute exposure of *D. magna* juveniles during recovery denoted only significant detrimental effects (lower LC₅₀ than controls) for organophosphorous pesticides within the first 24 h (Fig. 4D and E).

4. Discussion

Sensitivity of *Daphnia* AChE to *in vivo* exposures to the studied pesticides were similar to reported toxicity differences with chlorpyrifos being about 10 and 500 times more toxic than malathion and carbofuran, respectively. Observed differences in sensitivity to organophosphorous and carbamate pesticides were in

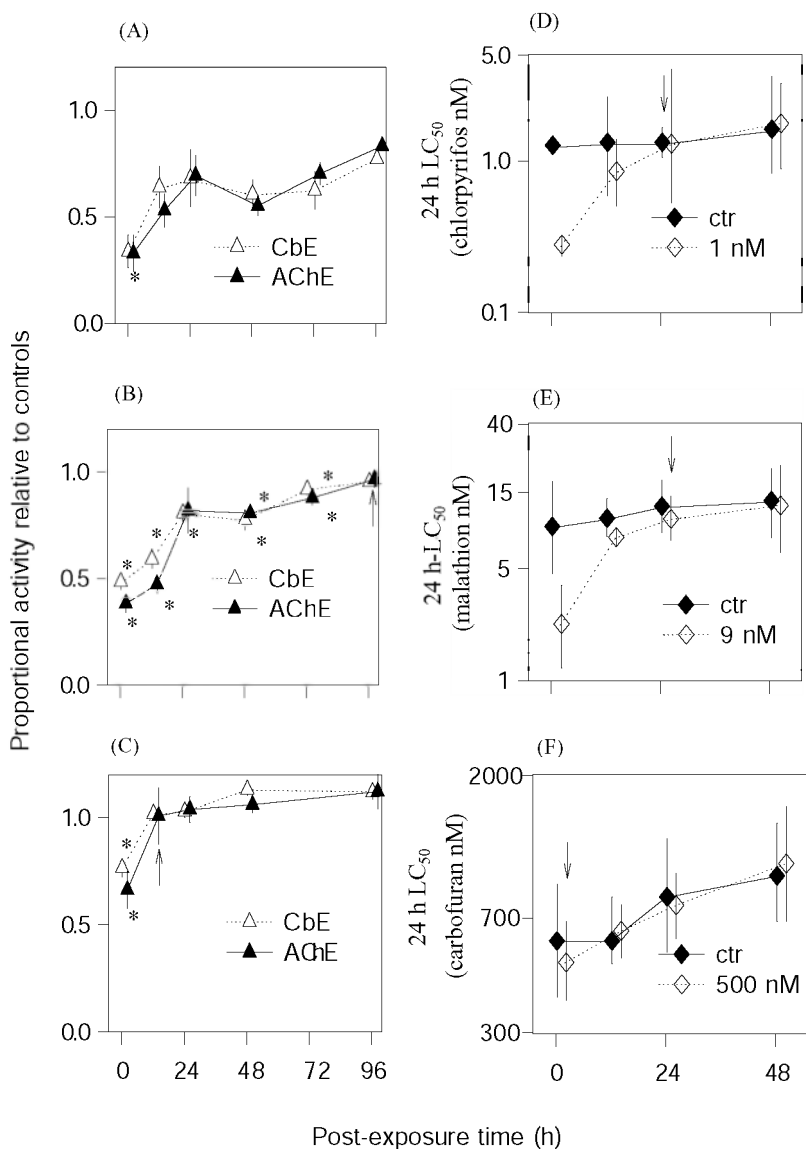


Fig. 4. Time course recovery of AChE and CbE and acute responses of *D. magna* (24 h LC₅₀) following exposures to 1, 9 and 500 nM of chlorpyrifos, malathion and carbofuran, respectively. Error bars in graphs A–C indicate S.E.M. whereas those in graphs D–F denote 95% CI. For clarity, LC₅₀ from graphs D–F are depicted in log scale and those from pre-exposed *Daphnia* are switched slightly to the right. Arrows indicate post exposure periods above which there were non significant ($P < 0.05$, Student *t*-tests) differences between treated and control (ctr) animals.

concern with previous studies conducted in *Daphnia* and other aquatic invertebrates. Kikuchi et al. (2000) screening *Daphnia* toxicity to 18 organophosphate and carbamate pesticides reported that chlorpyrifos was about one- and two-orders of magnitude

toxic than malathion and the carbamate fenobucard, respectively. Moore et al. (1998) also found that chlorpyrifos was about three-orders of magnitude more toxic to *Daphnia* and *Hyalella azteca* than the carbamate aldicarb. Similarly, studies conducted in

Daphnia exposed to the organophosphorous pesticide ethyl parathion also found that AChE and survival responses were impaired at equivalent exposure levels (Sturm and Hansen, 1999; Barata et al., 2001).

Interestingly, CbE showed over 50-fold more activity than AChE and showed equivalent or higher levels of sensitivity to the studied pesticides, thus indicating a great potential for sequestering available organophosphorous and carbamate pesticides and hence protecting AChE of being inhibited (Boone and Chambers, 1996). Certainly, an inhibition of CbE increased toxicity of organophosphorous and carbamate pesticides by two and over five folds, respectively. CbE are assumed to provide protection against organophosphorous and carbamate pesticides through two main mechanisms: (1) direct hydrolysis of carboxyl esters and (2) a binding protein for oxygen analogs of organophosphorous compounds (Maxwell, 1992; Jokanovic, 2001). Both roles will protect the intrinsic target, AChE, for intoxication. Chambers et al. (1990) and Yuan and Chambers (1996) provided convincing arguments for this latter mechanism of detoxication in vitro and in vivo experiments on the ability of rat liver and boll weevil CbE to non catalytically detoxify the oxones of organophosphorous pesticides. In the present study, the parallel nature of dose-dependent increases of CbE and AChE inhibition, and mortality responses suggested that inhibition of CbE by TPP and CDPB may have increased organophosphorous and carbamate pesticide availability for inhibiting AChE, thus resulting in an enhance sensitivity.

Time course inhibition experiments denoted differing esterase responses across organophosphorous and carbamate toxicants and exposure levels. B-esterases were inhibited by malathion and chlorpyrifos following a typical threshold model response (Barata et al., 2000b) which considers a period of no respond followed by an inhibition response when bioactivated organophosphorous pesticide reached its target enzyme. Observed enzymatic responses were in concern with the fact that the studied organophosphorous pesticides required oxidation to their oxons to be potent inhibitors of B-esterases (Jokanovic, 2001; Barata et al., 2001). This reaction is catalyzed by the cytochrome P450 enzyme system. At high exposure levels, inhibition of CbE and AChE began shortly after exposure (<3–9 h) reaching 50% inhibition levels at 9–17 h. The observed rapid inhibition of CbE

and AChE enzymes are in agreement with previous studies and reflected the rapid oxidation of malathion and chlorpyrifos to the oxon form. Boone and Chambers (1996) reported that high levels of brain AChE and liver CbE inhibition of mosquito fish took place 12 h after exposure to chlorpyrifos. For aquatic crustacea species including *Gammarus* and *Procambarus*, high levels of AChE inhibition were also reached soon (<24 h) after exposure to high doses of fenitroton and parathion-methyl (Kuhn and Streit, 1994; Escatin and Porte, 1996a).

At moderate levels of exposure (50% of the acute dose), CbE were inhibited by organophosphorous pesticides much earlier than AChE, thus suggesting that CbE may have a higher affinity for bioactivated malathion and chlorpyrifos (malaaxon and chlorpyrifos-oxon, respectively) and hence, could afford substantial protection from the oxons as they are generated within the organism. Higher affinity of CbE for oxon metabolites relative to AChE have also been reported for rats, fish, and mussels (Chambers et al., 1990; Boone and Chambers, 1996; Escatin and Porte, 1997). Alternatively, differences in tissue locations of the studied enzymes in *Daphnia* could also have contributed to their susceptibility to inhibition. Chambers and Chambers (1990) and Boone and Chambers (1996) studying B-esterase inhibition patterns in rats and mosquitofish exposed to organophosphorous pesticides, found that liver CbE was less sensitive than plasma CbE to paraoxon and that muscle AChE was more sensitive than brain AChE. Unfortunately, the small size of *Daphnia* individuals prevented to discriminate enzymatic activities across *Daphnia* tissues.

Inhibition responses of B-esterases to carbofuran followed a different pattern. AChE and CbE were inhibited immediately after exposure, irrespectively of the concentration used with AChE reaching higher levels of inhibition than CbE. In eels Fernández-Vega et al. (2002) also found high levels (30–50%) of brain and muscle AChE inhibition 2 h after exposure to the carbamate thiobencarb. Studies in mussels reported that the relative sensitivities of B-esterases varied across carbamates with CbE being equally and less sensitive than AChE to carbofuran and eserine, respectively (Escatin and Porte, 1997; Galloway et al., 2002). Thus, our results agree with previous studies and support the view that carbamates are expected to induce toxicity faster than organophosphorous

pesticides such as malathion and chlorpyrifis since do not need to be bioactivated to be potent inhibitors of AChE and that CbE (Smith, 1987).

AChE of *Daphnia* juveniles inhibited by organophosphorous pesticides took 24 and 96 h to recover 50% and almost 100%, respectively, whereas complete recovery of AChE to carbofuran was achieved within the first 12 h. The fact that recovery enzymatic rates were slower for organophosphorous pesticides than for carbamates is not surprising since organophosphorous pesticides are considered irreversible inhibitors of AChE when compared with carbamate pesticides, thus reactivation of inhibited enzyme takes place very slowly in comparison with AChE inhibited by carbamates (Smith, 1987). Indeed in most cases AChE inhibited by organophosphorous pesticides can only be recovered by de novo synthesis of the enzyme (Boone and Chambers, 1996). In relation with this, observed recovery rates of AChE inhibited by organophosphorous pesticides in *Daphnia* were high compared with other aquatic invertebrate and vertebrate species. Reported studies in aquatic crustacea and fish species indicate that AChE inhibited by organophosphorous pesticides took from 2 to >4 weeks to recover to control levels (Kuhn and Streit, 1994; Mc Henerey et al., 1996; Escatin and Porte, 1996b; Boone and Chambers, 1996). The fast recovery period noted in this study for AChE inhibited by carbofuran agree with reported values on midges but not on the snail *Pomacea patula*. In *Chironomus riparius* AChE inhibited by the carbamate carbaryl recovered to control levels 6 h after animals were placed in clean medium (Kallander et al., 1997). Alternatively, Ramirez et al. (2000) observed that although *P. patula* snails took 7 h to eliminate carbaryl, inhibited AChE did not recovered after 72 h.

Recovery patterns of CbE were similar to those observed for AChE, thus suggesting that CbE could be inhibited irreversible and reversible by the studied organophosphorous and carbamate pesticides, respectively. Little is known about the underlying mechanisms of inhibition and recovery of CbE in mammals and even less information exist in invertebrate species. In a study of mechanisms of interaction of CbE with some organophosphorous pesticides in vitro, it was found that this reaction was reversible due to a rapid spontaneous reactivation of inhibited enzyme (Jokanovic et al., 1996). Nevertheless, it would be wrong to con-

clude that spontaneous reactivation is a common feature of CbE since there are examples, where this reaction is clearly irreversible (Boone and Chambers, 1996).

There is some controversy in the literature relative to the extent of AChE depression required to cause death in aquatic animals. In studies with aquatic invertebrates including *Daphnia*, AChE inhibition after exposure to lethal concentrations of anti-AChE was usually in the range of 70–100% (Bocquené et al., 1991; Detra and Collins, 1991; Barata et al., 2001). In some cases, however, lethal effects of anti-AChE occurred at a AChE inhibition of 40% or below (Bocquené et al., 1991; Escatin and Porte, 1996a). In the present study, we found that lethal effects of organophosphorous pesticides and carbamate in *Daphnia* juveniles occurred at high (50–80%) and low (20–40%) AChE inhibition levels, respectively. It should be noted, however, that *Daphnia* juveniles surviving the treatments were transferred to clean water for 5 min to allow removal of pesticide residues and then used in the enzymatic assays. Thus, anti-AChE activity causing lethality responses are likely to be underestimated and partial reactivation of CbE and AChE inhibited by carbofuran could have occurred.

Common field crop insect management regimens include successive and periodic applications of large amount of pesticides (Glogoza et al., 2002), of which several hundred tons eventually end up in adjacent waterways and further diluted and washed out downstream (Richards and Baker, 1993). Thus, aquatic organisms living nearby agricultural fields treated with pesticides are potentially exposed to elevated concentrations for short periods of time. In relation to this animals exposed to multiple pulses, a common situation in the field, may be more likely to survive acute doses, compared with continuous exposures, because organisms can potentially detoxify or depurate any accumulated contaminants during the exposure intervals, that is the recovery phase (Naddy et al., 2000). For organophosphorous and carbamate pesticides, the extent to which AChE had recovered from previous exposure is considered to be a determining factor in determining the susceptibility to subsequent pulse exposures (Kallander et al., 1997). In our study, there was a close correspondence between AChE recovery rates and *Daphnia* sensitivity to successive acute exposures.

Following exposure to organophosphorous and carbamate pesticides, toxicity responses to successive acute exposures elapsed 12 and 24 h, respectively, did not differ significantly from those of unexposed controls. Thus, indicating that 12 and 24 h time intervals were needed for *Daphnia* juveniles to recover, in toxicity terms, following acute exposures. Observed recovery periods were in concern with those reported for midges exposed to carbamates (6 h) and organophosphorous pesticides (>24) but were shorter than those depicted for *D. magna* exposed to chlorpyrifos (72 h) (Kallander et al., 1997; Naddy and Klaine, 2001). *Daphnia* AChE inhibited by organophosphorous pesticides took 24 h to recover 50% of its activity reaching low inhibition levels relative to unexposed controls (30%), whereas complete recovery of AChE to carbofuran was achieved within the first 12 h. Therefore, our results indicated that the extent to which AChE had recovered from previous exposures determined toxicity responses to successive acute pulses. This means that due to their fast recovery rates, *Daphnia* species living nearby agricultural fields treated with organophosphorous and carbamate compounds are likely to withstand acutely lethal pesticide pulse exposures, provided that there is an adequately time for recovery between exposures.

In conclusion our results indicate that CbE and AChE followed similar patterns of inhibition and recovery responses to organophosphorous and carbamate pesticides, with CbE being more or equally sensitive, thus indicating a great potential of CbE for sequestering available organophosphorous and carbamate pesticides and hence protecting AChE of being inhibited. Certainly, an inhibition of CbE increased toxicity of organophosphorous and carbamate pesticides by two and over five fold, respectively, thus indicating that CbE can afford protection in *Daphnia* against AChE inhibition and toxicity to organophosphorous and carbamate pesticides. The close correspondence between inhibition of AChE and lethal responses across the studied toxicants denotes that AChE inhibition levels higher than 50% should be considered of environmental concern to *Daphnia* related species. Furthermore, the observed relatively rapid enzymatic AChE and CbE recovery patterns following pulse exposures to organophosphorous and carbamate pesticides may allow *Daphnia* species to

withstand acutely lethal pesticide pulse exposures, provided that there is an adequately time for recovery between exposures.

Acknowledgements

This study was funded by the MCyT Spanish project REN 2002-01709. Carlos Barata was supported by a I3P-PC2001-1 contract from the Consejo Superior de Investigaciones Científicas (Spain) and Arun Solayan by a postdoctoral MCyT grant while carrying out this research. The authors thanks Dr. Oksana Lockridge for kindly providing CBDP and Dr. Silvia Lacorte and Alain Hildebrandt for conducting the chemical analysis.

References

- APHA-AWWA-WEF, 1995. Standard Methods for the Examination of Water and Wastewater, 19th ed. American Public Association/America Water Works Association/Water Environment Federation, Washington, DC.
- Baird, D.J., Barata, C., 1998. Genetic variability in the response of *Daphnia* to toxic substances: implications for risk assessment. In: Forbes, V.E. (Ed.), Genetics in Ecotoxicology. Taylor and Francis, New York, pp. 207–221.
- Barata, C., Baird, D.J., Amat, F., Soares, A.M.V.M., 2000a. Population responses to contaminants, between laboratory and field: an approach using *Daphnia magna* ephippial egg banks. *Funct. Ecol.* 14, 513–523.
- Barata, C., Baird, D.J., Miñarro, A., Soares, A.M.V.M., 2000b. Do genotype responses always converge from lethal to non-lethal toxicant exposure levels? A hypothesis tested using laboratory *Daphnia magna* Straus clones. *Environ. Toxicol. Chem.* 19, 2314–2322.
- Barata, C., Baird, D.J., Soares, A.M.V.M., Guilhermino, L., 2001. Biochemical factors contributing to response variation among resistant and sensitive clones of *Daphnia magna* straus exposed to ethyl parathion. *Ecotox. Environ. Safe.* 49, 155–163.
- Bocquené, G., Galgani, F., Truquet, P., 1991. Acetylcholinesterase activity in common prawn (*Palaemon serratus*) contaminated by carbaryl and phosalone: choice of a method for detection of effects. *Ecotox. Environ. Safe.* 22, 248–254.
- Boone, J.S., Chambers, J.E., 1996. Time course of inhibition of cholinesterase and aliesterase activities, an nonprotein sulphydryl levels following exposure to organophosphorus insecticides in Mosquitofish (*Gambusia affinis*). *Fund. Appl. Toxicol.* 29, 202–207.
- Chambers, J.E., Chambers, H.W., 1990. Time course of inhibition of acetylcholinesterase and aliesterases following parathion and paraoxon exposure in rats. *Toxicol. Appl. Pharmacol.* 103, 420–429.

- Chambers, H.W., Brown, B., Chambers, J.E., 1990. Noncatalytic detoxication of six organophosphorus compounds by rat liver homogenates. *Pest. Biochem. Physiol.* 36, 162–169.
- Chambers, J.E., Boone, J.S., Carr, R.L., Chambers, H.W., Straus, D.L., 2002. Biomarkers as predictors in health and ecological risk assessment. *Hum. Ecol. Risk Assess.* 8, 165–176.
- Day, K.E., Scott, I.M., 1990. Use of acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to low concentrations of organophosphate insecticides. *Aquat. Toxicol.* 18, 101–114.
- Detra, R.L., Collins, W.J., 1991. The relationship of parathion concentration environ, exposure time, cholinesterase inhibition and symptoms of toxicity in midge larvae (Chironomidae: Diptera). *Toxicol. Chem.* 10, 1089–1095.
- Ellman, G.L., Courtney, K.D., Andres, V.J., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95.
- Escatin, E., Porte, C., 1996a. Acetylcholinesterase inhibition in the crayfish *Procambarus clarkii* exposed to fenitrothion. *Ecotox. Environ. Safe.* 34, 160–164.
- Escatin, E., Porte, C., 1996b. Bioaccumulation, metabolism, and biochemical effects of the organophosphorus pesticide fenitrothion in *Procambarus clarkii*. *Environ. Toxicol. Chem.* 15, 915–920.
- Escatin, E., Porte, C., 1997. The use of cholinesterase and carboxylesterase activities from *Mytilus galloprovincialis* in pollution monitoring. *Environ. Toxicol. Chem.* 16, 2090–2095.
- Eto, M., 1974. Organophosphorus pesticides: organic and biological chemistry. CRC Press, Ohio.
- Fernández-Vega, C., Sancho, E., Ferrando, M.D., Andreu, E., 2002. Thiocarb-induced changes in acetylcholinesterase activity of the fish *Anguilla anguilla*. *Pestic. Biochem. Phys.* 72, 55–63.
- Finney, D.J., 1971. Probit Analysis, third ed. Cambridge University Press, Cambridge.
- Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environ. Toxicol. Chem.* 20, 37–45.
- Galloway, T.M., Millward, N., Browne, M.A., Depledge, M.H., 2002. Rapid assessment of organophosphorus/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. *Aquat. Toxicol.* 61, 169–180.
- Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. *Chemosphere* 32, 727–738.
- Glogoza, P., Knodel, J., Boetel, M., Olson, D., Brewer, G., 2002. Field Crop Insect Management Recommendations. North Dakota Insect Management Publications, North Dakota State University, Bismarck, ND.
- Jokanovic, M., 2001. Biotransformation of organophosphorus compounds. *Toxicology* 166, 139–160.
- Jokanovic, M., Kosanovi, M., Maksimovic, M., 1996. Interaction of organophosphorus compounds with carboxylesterases in rat. *Arch. Toxicol.* 70, 444–450.
- Kallander, D.B., Fisher, S.W., Lydy, M.J., 1997. Recovery following pulse exposures to organophosphorus and carbamate insecticides in the midge, *Chironomus riparius*. *Arch. Environ. Con. Tox.* 33, 29–33.
- Kikuchi, M., Sasaki, Y., Wakabayashi, M., 2000. Screening of organophosphate insecticide pollution in water by using *Daphnia magna*. *Ecotox. Environ. Safe.* 47, 239–245.
- Kuhn, K., Streit, B., 1994. Detecting sublethal effects of organophosphates by measuring acetylcholinesterase activity in *Gammarus*. *Bull. Environ. Con. Toxicol.* 53, 398–404.
- Lacorte, S., Barceló, D., 1996. Determination of parts per trillion levels of organophosphorus pesticides in groundwater by automated on line liquid solid extraction followed by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry using positive and negative ion modes of operation. *Anal. Chem.* 68, 2464–2470.
- Lowry, O., Rosebrough, N.J., Farr, N.J., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 93, 265–275.
- Mastropaolo, W., Yourno, J., 1981. An ultraviolet spectrophotometric assay for alpha-naphthyl butyrate esterases. *Anal. Biochem.* 115, 188–193.
- Maxwell, D.M., 1992. The specificity of carboxylesterase protection against the toxicity of organophosphorus compounds. *Toxicol. Appl. Pharm.* 114, 306–312.
- Mc Henerey, J.G., Francis, C., Davies, I.M., 1996. Threshold toxicity and repeated studies of dichlorvos to the larvae of the common lobster (*Homarus gammarus* L.). *Aquat. Toxicol.* 34, 237–251.
- Moore, M.T., Huggett, D.B., Gillespie, W.B., Rodgers, J.H., Cooper, C.M., 1998. Comparative toxicity of chlordane, chlorpyrifos, and aldicarb to four aquatic testing organisms. *Arch. Environ. Con. Toxicol.* 34, 152–157.
- Murphy, S.D., 1966. Liver metabolism and toxicity of thiophosphate insecticides in mammalian, avian, and piscine species. *Proc. Soc. Exp. Biol. Med.* 123, 392–398.
- Naddy, R.B., Klaine, S., 2001. Effect of pulse frequency and interval on the toxicity of chlorpyrifos to *Daphnia magna*. *Chemosphere* 45, 497–506.
- Naddy, R.B., Johnson, K.A., Klaine, S., 2000. Response of *Daphnia magna* to pulse exposures of chlorpyrifos. *Environ. Toxicol. Chem.* 19, 423–431.
- O'Brien, R.D., 1960. Toxic Phosphorus Esters. Chemistry, Metabolism, and Biological Effects. Academic Press, New York.
- Ramirez, B., Martinez, L., Sanchez, E., Castañeda, G., Gutierrez, M.C., Flores, F., 2000. Relationship between toxicokinetics of carbaryl and effect on acetylcholinesterase activity in *Pomacea paula* snail. *Ecotox. Environ. Safe.* 46, 234–239.
- Rice, W.R., 1989. Analysing tables of statistical tests. *Evolution* 43, 223–225.
- Richards, R.P., Baker, D.B., 1993. Pesticide concentration patterns in agricultural drainage networks in the lake Erie basin. *Environ. Toxicol. Chem.* 12, 13–26.
- Schulz, R., Liess, M., 1999. A field study of the effects of agriculturally derived input on stream macroinvertebrate dynamics. *Aquat. Toxicol.* 46, 155–176.
- Shao-nan, L., De-Fang, F., 1997. Activity of esterases from different tissues of freshwater fish and responses of their

- isoenzymes to inhibitors. *J. Toxicol. Env. Health* 51, 149–157.
- Smith, G.J., 1987. Pesticide Use and Toxicity in Relation to Wildlife: Organophosphorous and Carbamate Compounds. United States Department of the Interior, Fish and Wildlife Service, Resource Publication 170, Washington, DC.
- Sturm, A., Hansen, P.D., 1999. Altered cholinesterase and monoxygenase levels in *Daphnia magna* and *Chironomus riparius* exposed to environmental pollutants. *Ecotox. Environ. Safe.* 42, 9–15.
- Thompson, C.M., 1992. Preparations, analysis, and toxicity of phosphorothionates. In: Chambers, J.E., Levi, P.E. (Eds.), *Organophosphates: Chemistry, Fate, and Effects*. Academic, San Diego, pp. 19–46.
- Yuan, J., Chambers, H.W., 1996. Toxicology and biochemistry of two aliesterase inhibitors as synergists of four organophosphorus insecticides in Boll Weevils (Coleoptera: Curculionidae). *Pestic. Biochem. Phys.* 54, 210–219.
- Zar, J.H., 1996. *Biostatistical Analysis*. Prentice-Hall, New Jersey.
- Zinkl, J.G., Lockhart, W.L., Kenny, S.A., Ward, F.J., 1991. The effects of cholinesterase inhibiting insecticides on fish. In: Mineau, P. (Ed.), *Cholinesterase-Inhibiting Insecticides*. Elsevier, New York, pp. 234–254.