BIOMARKERS, 2002, VOL. 7, NO. 2, 138–150

Effects of dimethoate on snail B-esterase and growth as a function of dose, time and exposure route in a laboratory bioassay

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Received 18 June 2001, revised 17 September 2001

The aim was to study the effects of dimethoate on enzymatic targets and on the growth of *Helix aspersa* for different times and modes of exposure under laboratory conditions. Young snails were exposed to increasing dimethoate concentrations in the food (D.exp) or in an artificial substrate (S.exp) for 1, 2, 7 and 14 days. Both acetylcholinesterase (AChE) and carboxylesterase (CaE) activities were measured in the foot of the snails for each concentration and exposure time tested. Growth was evaluated after 7 days of exposure. AChE inhibition, dose-dependent for all lengths of exposure, was stronger in S.exp. AChE was more sensitive than CaE for both modes of exposure. IC_{50}-7 days was 38.3 $\mu g\,g^{-1}$ in D.exp and 11.7 $\mu g\,g^{-1}$ in S.exp for AChE and was higher than 150 $\mu g\,g^{-1}$ in two exposure modes for CaE. AChE activity decreased from the first day to reach maximum inhibition after 7 days of exposure. As noted for B-esterase activities, growth inhibition was stronger in S.exp and was only significant for AChE inhibition of >90%. The present results show that AChE activity could be used to give early warning of toxic effects of dimethoate in terrestrial gastropods.

Keywords: acetylcholinesterase, carboxylesterase, snail, dimethoate, exposure route

Introduction

The generalized use of chemical pesticides may present risks to terrestrial and aquatic ecosystems. To evaluate and predict ecosystem degradation, ecotoxicologists increasingly use animals as indicators of exposure and/or to reveal the deleterious biological effects of organic chemicals. Among the endpoints generally investigated, biochemical responses have been reported to be early and sensitive markers that can predict the expression of toxic effects in the higher levels of biological organization (Lagadic *et al*. 1994, Peakall 1994, Lagadic 1999).

Organophosphorus insecticides (OPs) are commonly used in numerous countries because they are less persistent than organochlorines in the various environmental compartments. OPs typically inhibit B-esterase activities by binding to the active site and phosphorylating the enzyme, disrupting the nervous system

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Biomarkers ISSN 1354–750X print/ISSN 1366–5804 online © 2002 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500110099672

function not only of insects, but also of non-target organisms. Such a mode of action has suggested the use of B-esterase inhibition as a biochemical marker of OP exposure in man and wild fauna (Mineau 1991).

B-esterase, including cholinesterases (ChE) and carboxylesterases (CaE), are widely distributed in the animal kingdom. ChE are an important group of enzymes typically subdivided into two major classes, acetylcholinesterases (AChE, EC 3.1.1.7) and butyrylcholinesterases (EC 3.1.1.8), in vertebrate species, but this nomenclature is not so clear in invertebrates (Bocquéné *et al.* 1997). The specific role of AChE is to hydrolyse acetylcholine during neurotransmission. A significant reduction in AChE activity in soil invertebrates may indicate exposure to OP residues and, therefore, may provide an early warning for potential adverse effects that may occur later at population and community level (Ribeiro *et al.* 1999).

The physiological role of CaE (EC 3.1.1.1) is not clear, but they are known to hydrolyse a wide range of endogenous and exogenous esters (Walker and Thompson 1991). They are assumed to provide protection against OP poisoning through two main mechanisms: (1) hydrolysis of ester bonds in OPs, and (2) stoichiometric phosphorylation with resulting destruction of the oxon of OPs, which reduces the amount of active OPs form available for AChE inhibition (Jokanovic *et al.* 1996). In addition, the CaE of insects (Poirié 1991) and bivalve molluscs (Escartin and Porte 1997, Basack *et al.* 1998) have shown higher sensitivity to OPs than AChE and can be considered good biomarkers candidates.

Most of the research concerning the use of B-esterases as biomarkers has focused on vertebrates: fish (Zinkl *et al.* 1991, Fulton and Key 2001), birds and mammals (Grue *et al.* 1991), and aquatic invertebrates (Edwards and Fisher 1991, Fulton and Key 2001). In recent years, there has been increasing interest in the use of esterase markers in terrestrial invertebrates such as earthworms (Edwards and Fisher 1991, O'Halloran *et al.* 1999) and arthropods (Edwards and Fisher 1991, Ribeiro *et al.* 1999) for the assessment of the potential adverse effects of OPs on soil biocenoses. Indeed, OPs have some effects on target organisms (insects) and also on non-target species (e.g. other arthropods, earthworms and gastropods) that play a major role in the functioning of the soil ecosystem (Hopkin 1989). Snails are well known as suitable indicators of metal contamination (Berger and Dallinger 1993) and they can be used as bioindicators of the effects (Gomot-de Vaufleury and Bispo 2000, NF X 31-255-1 and 2) and of bioaccumulation (Cœurdassier *et al.* 2000, Gomot-de Vaufleury and Pihan 2000). However, the effects of OPs on land snails are less documented. Schuytema *et al.* (1994) focused on the lethal, behavioural and morphological effects of OPs in the garden snail *Helix aspersa*. A preliminary study by Rorke *et al.* (1974) underlined that the AChE activity of *H. aspersa* can be affected by exposure to OPs. Recently, a specific study about the effects of dimethoate (reference pesticide in the European Community Research and Development project, 'Sublethal Effects of Chemicals on Fauna in the Soil Ecosystem') in the snail has shown that growth and *in vivo* AChE activity in *H. aspersa* are inhibited after 4 weeks of exposure to dimethoate (diM) by digestive route (Cœurdassier *et al.* 2001). AChE activity was four times higher in the foot compared with viscera of the control snails and was 80% inhibited in both tissues for $250\,\mu g\,g^{-1}$, the lowest concentration tested. AChE was more sensitive than growth ($EC_{50} = 665\,\mu g\,g^{-1}$) to diM (Cœurdassier *et al.* 2001). The results show the need to develop experiments about the effects of OPs on snail B-esterase activities

during shorter exposures and for lower dimethoate concentrations to validate the use of this enzyme inhibition as a potential biomarker of OP exposure in the field.

The aim of the study was to evaluate the effects of diM on B-esterase (AChE and CaE) in the foot of *H. aspersa* as a function of the pesticide concentration and the duration of exposure in laboratory bioassays. Moreover, two modes of exposure, similar to the natural modes of contamination, were used: by food or in the artificial substrate (ISO/DIS 11268-1, 1994). At the same time, the effects of diM on snail growth were measured to determine possible correlations between biochemical and physiological perturbations.

Materials and methods

Chemicals

DiM technical (*O,O*-dimethyl *S*-(*N*-methylcarbamoyl-methyl) phosphorodithioate, CAS 60-51-5, 96–98% pure) was graciously provided by Cheminova Agro (Lemvig, Denmark). The following chemicals were purchased from Sigma-Aldrich Chimie S.A.R.L. (Saint Quentin-Fallavier, France): acetylthiocholine iodide (AThCh), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and α-naphthyl acetate. Other chemicals of analysis grade were obtained from Prolabo (Gradignan, France).

Exposure conditions

The snails *H. aspersa* descended from a strain collected in the region of Cavaillon (France). The individuals used in the tests, aged 1 month and weighing 1 ± 0.3 g, were reared in the laboratory as described previously (Gomot-de Vaufleury 2000).

Two modes of exposure, in accordance with those proposed in standards NF X 31-255-1 and 2, were used. (1) In the first, the snails were exposed to diM in a vegetable snail diet Helixal® (Philicot S.A., Clairvaux-les-Lacs, France) for 7 days at 0, 3.91, 15.6, 31.2, 62.5, 125 and 250 $\mu g\,g^{-1}$ (dietary exposure, D.exp). Seven days was the shortest length of exposure that allowed evaluation of the effects of diM on growth. For three concentrations of diM (0, 15.6, 250 $\mu g\,g^{-1}$), snails were also exposed for 1, 2 and 14 days to study (a) the onset and the persistence of the B-esterase inhibitions, and (b) any inhibition increase after 7 days of exposure. Contamination of the Helixal food was performed by spraying ultrapure water solutions contaminated at different diM concentrations permitting the food contamination at the diM concentrations cited above. For both control and contaminated food, the volume of solution sprayed on was equivalent to 20% of its dry weight. The food was prepared at the beginning of the experiments and stored at −20°C in Petri dishes. For each concentration, one group of five young snails was placed in transparent polystyrene containers (ref. E1DBBAC001, IFFA-CREDO, 69 L'Arbresle, France) of 1600 cm^3 (24 cm length × 10.5 cm width × 8 cm height). The bottom of the containers was filled with 140 g dry ISO substrate humidified with ultrapure water to 50% of the water-holding capacity. The containers were cleaned with ultrapure water and the food, thawed just before being given to the snails, was renewed three times a week. The ISO substrate was renewed after 1 week. Snail faeces were also removed before mixing the substrate. The humidity of ISO substrate was maintained at its initial level by spraying with ultrapure water every 2 days. The snails were reared under 18 h light for 6 h dark, at a temperature of 20 ± 2°C and a relative humidity of 80–95%.

(2) In the second mode of exposure, the snails were exposed to diM in an ISO substrate for 7 days at 0, 0.98, 3.91, 15.6, 62.5 and 250 $\mu g\,g^{-1}$ (substrate exposure, S.exp). Snails were also exposed to 0, 15.6 and 250 $\mu g\,g^{-1}$ for 1, 2 and 14 days. The artificial substrate was contaminated by spraying diM in ultrapure water solutions to reach 50% of the water-holding capacity. Contaminated substrates were prepared at the beginning of the experiment and stored at −20°C. The substrate was changed after 1 week. Uncontaminated Helixal diet was humidified (20%) and stored at −20°C in Petri dishes as described previously. During all the tests, the groups of five snails were kept in the same conditions and followed as described in the D.exp.

The stability of diM in ISO substrate was determined for 62.5 $\mu g\,g^{-1}$ at 0 and 7 days.

Sample preparation and analysis

Snails were killed by deep freezing at −80°C. All procedures were carried out at 4°C.

Preparation of subcellular fractions. After thawing, the foot and the viscera of each animal were separated. The enzyme activities were measured in the snail feet after homogenization for 1 min in Tris buffer (100 mM, pH 7.5) at a 1:4 w/v ratio using an Ultra-Turrax T25 homogenizer at 12 000 rpm. The homogenate was centrifuged at 9000 g for 30 min to yield the post-mitochondrial (supernatant: S9) fraction.

Measurement of protein. Total protein contents were determined according to Lowry *et al.* (1951).

Measurement of acetylcholinesterase activity. AChE activity was determined in the S9 fraction according to Saint-Denis *et al.* (1999). Acetylthiocholine (AthCh) was hydrolysed by AChE, yielding thiocholine and acetic acid. The thiocholine released reacts with 5,5′-dithio-bis-2-nitrobenzoate (DTNB) to produce 5-thio-2-nitro-benzoate (TNB), a yellow compound, that absorbs light at 412 nm (absorbance 13.6 mM^{-1} cm^{-1}). The reaction conditions used were 95 mM Tris, pH 7.4, 0.4 mM DTNB, 2.25 mM AthCh and 200-µg sample proteins per ml incubate. The reaction was maintained at 25°C and initiated by addition of AThCh.

Measurement of carboxylesterase activity. CaE activity was also measured in the feet of the snails. The method was adapted to the use UV technique of Mastropaolo and Yourno (1981) in the presence of 0.25 mM α-naphthyl acetate and the formation of naphthol monitored by the increase of absorbance at 235 nm (absorbance 23.4 mM Tris, pH 7.4, 25°C, 0.25 mM α-naphthyl acetate and 40-µg sample proteins per ml incubate.

Growth assessment

Snail growth was measured after 1 week of exposure by using the largest diameter of the shell as previously proposed in the case of snail intoxication with diM (Cœurdassier *et al.* 2001). The growth coefficient of the snails at each concentration was calculated using:

$$Y_t = [(w_t - w_0)/w_0] \times 100,$$

where Y_t is the growth coefficient of snails exposed to $C\,\mu g\,g^{-1}$, w_t is the mean shell diameter of snails exposed to $C\,\mu g\,g^{-1}$ and w_0 is the mean shell diameter of snails exposed to $C\,\mu g\,g^{-1}$ when $t = 0$.

Statistical analysis

The results were compared with the non-parametric Mann–Whitney or Kruskal–Wallis tests (Sokal and Rohlf 1997). The relationships between AChE and CaE activities or growth inhibition and the concentration of diM in the food or the substrate were fitted by using a logistic dose-response model and the 95% confidence intervals of IC_{10} and IC_{50} (concentrations of diM that inhibit respectively 10 and 50% of enzymatic activities versus control) for both B-esterase activities and of EC_{10} and EC_{50} (respectively 10 and 50% effect concentrations) for growth were determined (Haanstra *et al.* 1985). NOEC (no observed effect concentration), LOEC (lowest effect concentration), IC_{10} and IC_{50}, EC_{10} and EC_{50} were exclusively evaluated at 7 days. Regression analyses were used to determine the relationships between concentrations of diM in the diet or ISO substrate, AChE and CaE activities and growth coefficient after 7 days of exposure.

Results

Concentrations of diM were reported to be stable in Helixal food for 1 month (Cœurdassier *et al.* 2001). However, the concentration decreased by 10% in the substrate after 1 week under the present experimental conditions (table 1).

Inhibition of AChE activity

AChE activities of control snails were higher after 2 days of experiment than after longer times of exposure, 7 and 14 days (table 2, $p > 0.05$).

With both modes of exposure, the inhibition of AChE activity increased with the diM concentrations (figure 1) whatever the length of exposure (table 2).

In D.exp-7 days, $IC_{50} = 38.3\,\mu g\,g^{-1}$ (table 3), whereas NOEC and LOEC were respectively 3.91 and $15.6\,\mu g\,g^{-1}$ (figure 1). The AChE activity was inhibited from day 1 and decreased until the day 14. At $250\,\mu g\,g^{-1}$, inhibition was three times higher than at $15.6\,\mu g\,g^{-1}$ from the second day and was almost total after 1 week of exposure (table 2).

In S.exp-7 days, the inhibition of the AChE activity was greater compared with D.exp: $IC_{50} = 11.7\,\mu g\,g^{-1}$ (table 3) and NOEC and LOEC, 3.91 and $15.6\,\mu g\,g^{-1}$ respectively (figure 1). For the first day, inhibition was twice as high for $15.6\,\mu g\,g^{-1}$

Table 1. Nominal and measured concentrations of dimethoate in the substrate ($\mu g\,g^{-1}$) after 0 and 7 days.

Nominal	62.5
Measured	
0 day	75
7 days	67

Table 2. Mean AChE activity (nmoles min^{-1} mg $protein^{-1}$) (±SD) and corresponding percentage of inhibition in the foot of *H. aspersa* exposed to increasing concentrations of dimethoate in the food (D.exp) or substrate (S.exp) for 1, 2, 7 and 14 days.

[diM] $\mu g\,g^{-1}$	0	15.6		250	
time (days)	AChE activity	AChE activity	% Inhibition	AChE activity	% Inhibition
Contaminated food (D.exp)					
1	51.5 ± 12.3	40.5 ± 5.9	21.3	33.9 ± 6.7*	34.2
2	55.2 ± 3.3	41.1 ± 4.9*	25.6	12.3 ± 4.1**	77.7
7	41.7 ± 3.6	28.7 ± 4.4**	31.0	2.6 ± 1.4**	93.8
14	41.2 ± 2.6	26.3 ± 4.2**	36.2	0.4 ± 0.3**	99.0
Contaminated substrate (S.exp)					
1	51.5 ± 12.3	31.4 ± 4.9*	39.1	0.2 ± 0.0**	99.6
2	55.2 ± 3.3	34.9 ± 3.9**	36.7	n.d.	n.d.
7	41.7 ± 3.6	15.6 ± 2.9**	62.7	1.2 ± 0.7**	97.0
14	41.2 ± 2.6	17.6 ± 2.3**	57.4	n.d.	n.d.

n.d., Not determined. *, ** Significantly different from the control (Mann–Whitney test; *$p < 0.05$; **$p < 0.01$).

than in D.exp and the activity was totally inhibited at 250 $\mu g\,g^{-1}$ (table 2). The inhibition increased after 2 days in the S.exp at 15.6 $\mu g\,g^{-1}$ and remained constant during the last week of exposure (table 2).

Inhibition of CaE activity

The CaE of snails exposed to diM for 7 days were less sensitive than AChE in D.exp and S.exp (figure 1 and table 4). The corresponding inhibitions of both activities were not significantly correlated.

In D.exp-7 days, the CaE activity was significantly higher in snails exposed to 3.91 $\mu g\,g^{-1}$ than in controls (Mann–Whitney, $p < 0.05$) (figure 1). CaE inhibition appeared from 62.5 $\mu g\,g^{-1}$ (Mann–Whitney, $p < 0.05$) and increased for the following diM concentrations 125 and 250 $\mu g\,g^{-1}$ with a mean inhibition of 35.2 ± 2.8% (Mann–Whitney, $p < 0.01$). The $IC_{50} > 250\,\mu g\,g^{-1}$ (table 3) and NOEC and LOEC were respectively 31.2 and 62.5 $\mu g\,g^{-1}$ (figure 1). For different durations of D.exp, CaE activities decreased significantly at 250 $\mu g\,g^{-1}$ for 2 and 7 days and recovered after 14 days.

As noted for AChE activity, CaE was more inhibited in S.exp, particularly for 62.5 and 250 $\mu g\,g^{-1}$ (figure 1). IC_{50} was evaluated at 150.6 $\mu g\,g^{-1}$ (table 3). NOEC and LOEC were 15.6 and 62.5 $\mu g\,g^{-1}$ respectively (figure 1). However, the CaE inhibition noted at 250 $\mu g\,g^{-1}$ in S.exp-7 days did not increase significantly

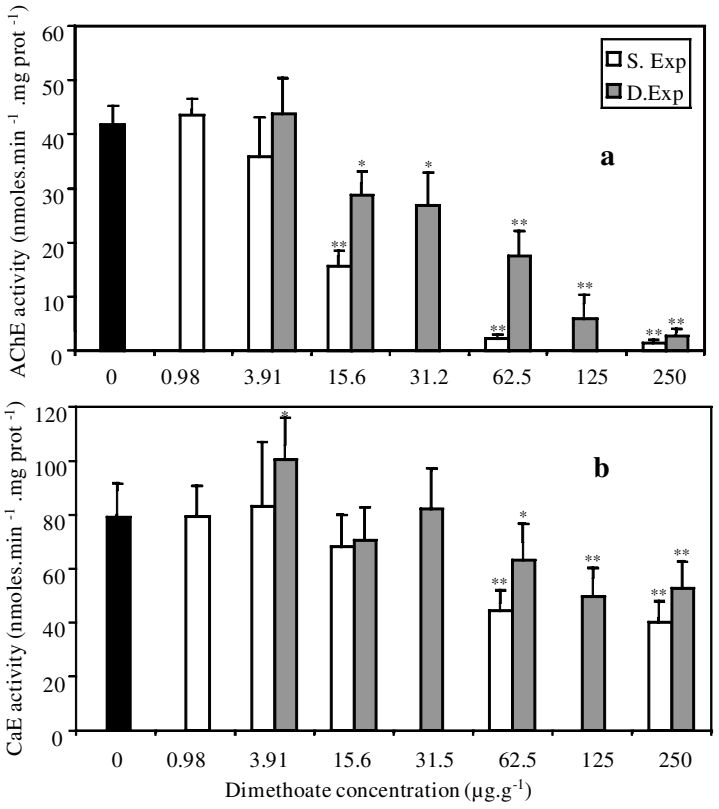

Figure 1. Effect of increasing dimethoate concentrations on the AChE and CaE activities (±standard deviation) in the foot of *H. aspersa* after (a) dietary (D.Exp) or (b) substrate (S.Exp) exposure for seven days. (* and ** indicate that the value is significantly different from the control value; Mann-Whitney test, $*p < 0.05$, $**p < 0.01$).

compared with those observed at 62.5 µg g^{-1} (figure 1). CaE inhibition was significant from the first day at 250 µg g^{-1} (table 4).

Growth inhibition and relation between growth and B-esterase activities

In S.exp-7 days, growth inhibition was greater in the snails exposed to diM in the substrate than in those exposed via the food (figure 2). Indeed, $EC_{50} > 250$µg g^{-1} in D.exp and was calculated as 171.7 µg g^{-1} in S.exp (figure 3). Moreover, in D.exp, the NOEC for growth inhibition was 125 µg g^{-1} and LOEC was the highest concentration 250 µg g^{-1} (figure 2). NOEC and LOEC were lower in S.exp at respectively 15.6 and 62.5 µg g^{-1} (figure 2).

In snails, the inhibition of growth and of AChE activity was strongly related. This relation is well described by a logarithmic relationship (figure 4). From measured data, it was observed that a significant effect on growth occurred ($p < 0.05$) when the AChE activity was inhibited by >90%. On the other hand, there was no significant relationship between growth inhibition and the decrease of CaE activity. For 125 and 250 µg g^{-1} of D.exp-7 days, similar levels of CaE inhibition (figure 1) were noted for different responses in growth effect (figure 2).

Table 3. Effects of progressive concentrations of dimethoate on AChE and CaE activities in the foot of *H. aspersa* exposed to the pesticide in food (D.exp) or substrate (S.exp) for 7 days.

	Mode of exposure	
Enzymatic activity	D.exp	S.exp
AChE	IC50 = 38.3 (29.3–48.2) IC10 = 7.0 (3.4–11.8) NOEC = 3.91; LOEC = 15.6	IC50 = 11.7 (10.2–13.7) IC10 = 3.1 (2.1–4.3) NOEC = 3.91; LOEC = 15.6
CaE	IC50 = n.d. IC10 = 16.0 (1.2–68.3) NOEC = 31.2; LOEC = 62.5	IC50 = 150.6 (74.9– 393.9) IC10 = 5.0 (0.4–37.5) NOEC = 15.6; LOEC = 62.5

n.d. Not determinable; IC_x ($\mu g\, g^{-1}$ diM) and 95% confidence interval in parentheses.

Table 4. Mean CaE activity (nmoles. min^{-1}. mg prot.$^{-1}$) (± standard deviation) and corresponding percentage of inhibition in the foot of *H. aspersa* exposed to increasing concentrations of dimethoate in the food (D.exp) or in the substrate (S.exp) for one, two, seven and fourteen days.

[diM] $\mu g\, g^{-1}$	0	15.6		250	
time (days)	CaE activity	CaE activity	% inhibition	CaE activity	% inhibition
Contaminated food (D.Exp)					
1	67.4 ± 8.3	81.5 ± 9.9	0	69.4 ± 15.3	0
2	73.7 ± 15.3	66.8 ± 6.7	9.3	49.8 ± 8.5*	32.4
7	79.0 ± 12.7	70.5 ± 12.2	10.8	52.8 ± 9.8*	33.3
14	55.5 ± 3.4	59.3 ± 4.8	0	51.8 ± 4.5	6.8
Contaminated substrate (S.exp)					
1	67.4 ± 8.3	67.5 ± 16.1	0	51.3 ± 9.2*	23.9
2	73.7 ± 15.3	68.3 ± 7.2	7.4	n.d.	n.d.
7	79.0 ± 12.7	68.1 ± 12.0	13.8	39.8 ± 8.1*	49.6
14	55.6 ± 3.4	49.4 ± 10.0	11	n.d.	n.d.

n.d., Not determined. * Significantly different from the control (Mann–Whitney test; *$p < 0.05$).

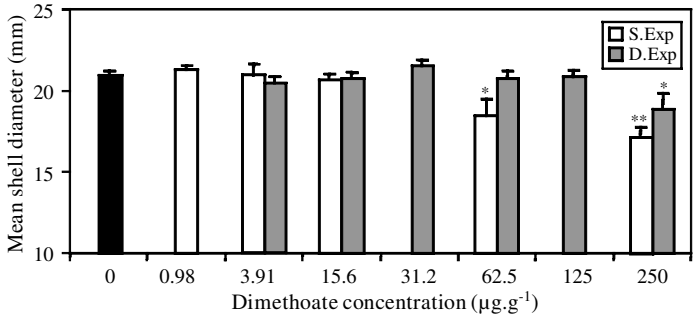

Figure 2. Effect of increasing dimethoate concentrations on the growth coefficient (shell diameter ± standard deviation) of the snail *H.aspersa* after dietary or substrate exposure for seven days. (* and ** indicate that the value is significantly different from the control value; test of Kruskal-Wallis, *$p < 0.05$, **$p < 0.01$).

Discussion

The present work provides original data on AChE and CaE activities in snails exposed to diM in laboratory bioassays. This represents a useful step in the development of sensitive biomarkers that can be used in the field as early warnings of toxic stress in *H. aspersa*. Estimation of *B-esterase* inhibition adds to the set of molecular targets of toxicants already studied in terrestrial gastropods in experimental conditions and *in situ* (Kammenga *et al.* 2000). Moreover, the standardized designs used in this work reveal correlations occurring between biochemical endpoints and effects on a biological parameter such as growth of juvenile snails.

The present study shows that AChE activity was inhibited in the foot of *H. aspersa* exposed to diM via the food or the substrate. This inhibition was dose and time dependent. After 1 and 2 weeks of exposure to 250 $\mu g\, g^{-1}$ in D.exp, inhibition of AChE activity (94 and 99% respectively) was already as important as noted in the foot of *H. aspersa* (87.4%) when exposed to the same diM concentration for 4 weeks (Cœurdassier *et al.* 2001). Presently, AChE activity decreases from the first day of exposure. Such an early anticholinesterasic effect was also reported in the earthworm *Apporectodea caliginosa* exposed to OP pesticides for 1 day in a soil with inhibitions of 75% for 60 $\mu g\, g^{-1}$ diazinon and 86% for 28 $\mu g\, g^{-1}$ chlorpyrifos (O'Halloran *et al.* 1999).

In snail foot, CaE activity was inhibited *in vivo* with diM as noted *in vitro* in *Eisenia fetida* exposed to 0.01 M diM (Kammenga *et al.* 2000). CaE of *H. aspersa* appears to be less sensitive than AChE to diM as proven by NOEC, LOEC and IC50. The opposite was observed *in vivo* in the bivalve molluscs *Corbicula fluminea* (Basack *et al.* 1998) and *in vitro* in the gills and the digestive gland of *Mytilus galloprovincialis* (Escartin and Porte 1997). Our results suggest that CaE does not provide efficient protection of AChE in the foot of the snails but the sensitivity of CaE to diM should be explored in the viscera, which are the main tissue of accumulation of diM in *H. aspersa* (Cœurdassier *et al.* 2001). Indeed, Triebskorn (1991) reported inhibition of non-specific esterases (including CaE) in the cells of the alimentary tract (oesophagus, crop stomach, gut) of the slug *Deroceras reticulatum* after application of carbamate molluscicides.

DiM was generally reported to be a moderately persistent pesticide with a DT_{50} of 21 days in the soil (van Straalen and van Rijn 1998), 83 days in humidified ISO substrate (Martikainen 1996) and stable for 1 month in the Helixal food (Cœurdassier *et al.* 2001). So, it can be assumed that the native molecule penetrates into the organism via the digestive and epithelial route and is bioactivated in its toxic oxon omethoate and certain of its oxidative analogues which were at least 1000 times more potent inhibitors of cholinesterase (Lucier and Menzer 1970). The biotransformation of diM may take place in the digestive gland of snails as previously noted for OPs in aquatic gastropods (Mahendru and Agarwal 1983, Takimoto *et al.* 1987). A rapid and efficient biotransformation in *H. aspersa* is supported by the low bioaccumulation of diM (Cœurdassier *et al.* 2001) and should be investigated in complementary studies about the toxicokinetics of diM and omethoate in different tissues of *H. aspersa*.

As noted about the sensitivity of AChE and CaE, the different modes of exposure used in this study show the snail growth is more affected in S.exp. These results are in agreement with Vink *et al.* (1995) who observed that carbofuran, benomyl or diazinon mixed into soil were more toxic than when mixed with food in isopods. The generally higher toxicity of diM via the substrate in snails may be

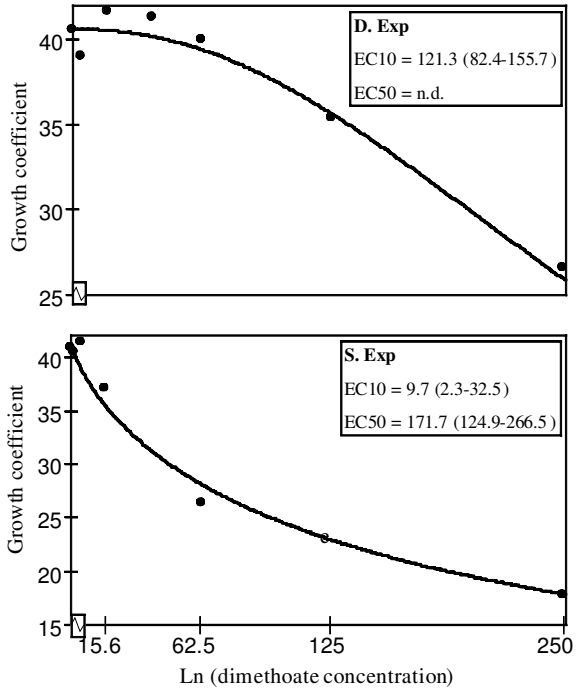

Figure 3. Evaluation of dose-dependent growth effects (EC50 and EC10, 95% confidence interval in parentheses) in *H.aspersa* exposed to dimethoate in the food or the substrate for seven days by using a logistic model.

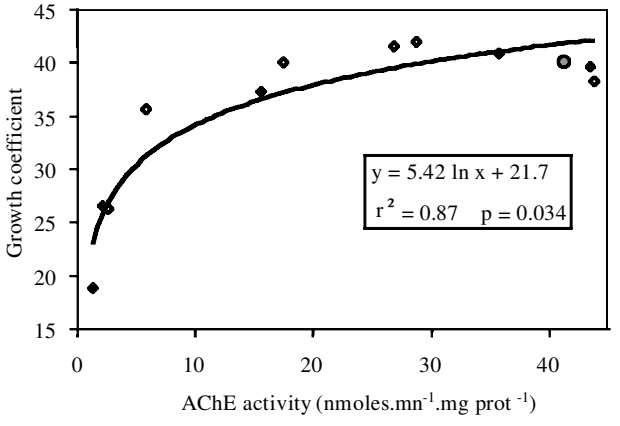

Figure 4. Relationship between growth coefficient and AChE activity in the foot of *H. aspersa* exposed to dimethoate in the food (◇) or in the substrate (◆) for seven days (●: control snails).

explained by efficient uptake via the epithelium. Two main mechanisms may be involved in: (1) endocytosis by the foot epithelium as described for large molecules in the slug *A. reticulatus* (Ryder and Bowen 1977), and (2) water absorption routes because diM is a hydrophilic compound (log $K_{ow} = 0.69$). Moreover, pedal mucus produced by snails may increase the exposure (dose and/or time) because it has a high affinity for pesticides, capable of sorbing OPs to a far greater degree than soil

(Brereton *et al.* 1999). Considering ICx in S.exp-7 days, it appears that the diM concentration which inhibited 10% of the AChE activity in snails after 1 week of exposure is close to the recommended dose in soil ($1.1\,\mu g\,g^{-1}$; Van Straalen and Van Rijn 1998). This suggest that *H. aspersa* is a good candidate for integration into a set of tests involving representative soil invertebrates, e.g. earthworms, collembola and isopods, as indicators of early perturbations due to OPs in terrestrial ecosystems.

In agreement with previous observations in *H. aspersa* exposed to diM in the food for four weeks (Cœurdassier *et al.* 2001), the present study points out that growth is less sensitive to diM than the AChE activity of snails. A lack of correlation between ChE inhibition and different types of biological effects has been generally reported for different invertebrate phyla (Edwards and Fisher 1991, Fulton and Key 2001). For gastropods, snails did not show any physiological disturbances when exposed to fenitrothion despite a drop in ChE ranging from 13 to 77% (Edwards and Fisher 1991). However, a logarithmic relationship between the inhibition of AChE activity in the foot and the toxic effect of diM on snail growth after 1 week of exposure is noted in the present work. This constitutes a promising result to find a relation between biochemical and adverse physiological effects. However, according to Edwards and Fisher (1991), more investigations about the sensitivity of different isoforms of ChE, as studied by Stenersen *et al.* (1992) in earthworms, in some localized tissues would be helpful to clarify the involvement of ChE inhibition in the expression of toxic effects of OPs and other anticholinesterasic pesticides in *H. aspersa*.

Cholinergic poisons have biological target sites other than B-esterase that contribute to the overall toxicity of the molecule. Several species of molluscs have been reported to lose protein from the mantle, gonad and foot in response to OP treatment (Mane *et al.* 1986). Presently, a significant decrease in the level of foot proteins is observed in S.exp snails exposed to the highest diM concentration for 7 days and in D.exp snails for $250\,\mu g\,g^{-1}$ for 2 and 14 days (Mann–Whitney, $p < 0.01$). Another effect of OP contamination in snails is the decrease of oxygen uptake which did not result from ChE depression in the respiratory centres (Bharathi and Prasada Rao 1989). Brimijon and Koenigsberger (1999) suggested that OPs adversely affect the process of neural development in vertebrates and insects (*Drosophila*), leading to permanent deficits in the architecture of the nervous systems, and consequently, leading to deferred biological effects.

Establishing a relationship between cholinesterase inhibition and toxic effects other than growth at the individual level during several weeks or months will be an interesting possibility. Moreover, such long investigations have to take into account the kinetics of diM metabolism in the soil. This brings some complementary information about the level and the duration of OP contamination which allowed the physiological homeostatic recovery of snails in spite of esterasic or other biochemical perturbations (Depledge 1994).

Conclusion

Terrestrial snails are commonly described as suitable bioindicators of environmental pollution because some physiological responses are induced by metallic or organic pollutants in laboratory bioassays (effects on growth or reproduction) and *in situ* (bioaccumulation of metals). Such endpoints are generally assessed after 4

weeks of exposure in subchronic tests. The present results constitute a promising perspective for developing more rapid tests using early biochemical responses in *H. aspersa* exposed to OP chemicals. The B-esterases of the garden snail *H. aspersa* are rapidly inhibited when it is exposed to diM in laboratory bioassays. Moreover, AChE appears to be more sensitive than CaE in the snail foot. When intoxication occurs via the substrate, a realistic and immediate route of exposure in the case of diM spraying in the field, AChE activity is inhibited at a low and ecologically representative concentration of diM. These results show that the decrease of snail AChE may be a suitable indicator of OP contamination in terrestrial ecosystems. The development of a battery of complementary tests with different endpoints (biochemical, physiological, ecological) using endogenous (earthworms), litter (collembola and isopods) and above-ground (snails) invertebrates would allow a better assessment of soil disturbances by pollution, as suggested by Hopkin (1993) for metal pollution. Other studies, in the laboratory and *in situ* (e.g. effects of environmental parameters on B-esterase activities as in earthworms; Booth *et al.* 2000), would help to develop the use of snail B-esterase as a forward marker of OP contamination in the natural environment.

Under controlled conditions, toxic effects on the growth of *H. aspersa* only appear at a strong inhibition of AChE and CaE for a short period of exposure, i.e. 1 week (present study) and 1 month (Cœurdassier *et al.* 2001). However, numerous environmental stress factors exist in the field and lead to slower growth for juvenile snails (18–24 months to reach adult size instead of 2 months in the present experimental conditions). The decrease of AChE activity in wild snails exposed to OP compounds can present more detrimental effects on individual (i.e. moving and feeding behaviour) and, so, have deleterious consequences on whole population.

Acknowledgements

The authors express their gratitude to Pr. L. Gomot and R. Scheifler for helpful scientific discussions. They also thank M. Daubèze and C. Lovy for technical assistance. Furthermore, they are indebted to two anonymous referees for useful suggestions that improved the paper. This project was supported by the French Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (Biotechnologies, Convention 98C 0266), the ADEME (Agence De l'Environnement et de la Maîtrise de l'Energie) (Contract No. 9893021) and the Conseil Régional de Franche-Comté.

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