Altered Cholinesterase and Monooxygenase Levels in
*Daphnia magna* and *Chironomus riparius* Exposed to
Environmental Pollutants

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Biochemical indices were investigated for their potential use as variables of sublethal toxicity in *Daphnia* (cholinesterase) and *Chironomus* (cholinesterase and biotransformation enzymes). Parathion, dichlorvos, and aldicarb caused dose-related inhibition of cholinesterase (ChE) in 24-h bioassays with both species. Ratios of *Daphnia* and *Chironomus* ChE IC₅₀ values to corresponding immotility EC₅₀ values derived from the same experiment covered the range 0.26 to 1.2. Estimates of the ChE inhibition caused by the immotility EC₅₀ were in the range 53–99% below control activity. ChE IC₅₀ values of dichlorvos, parathion, and aldicarb were 0.17, 0.61, and 95 µg/liter in *Daphnia* and 6.2, 2.9, and 27 µg/liter in *Chironomus*, respectively. Cytochrome P₄₅₀-dependent monooxygenase activities (ethoxyresorufin-O-deethylase, methoxyresorufin-O-deethylase, and ethoxycoumarin-O-deethylase) were detectable in *Chironomus* but not in *Daphnia*. *Chironomus* monooxygenase activities were significantly inhibited to about 30% of control values after 4 days of exposure to 50 µg/liter 3,4-dichloroaniline but remained unchanged by 0.5 µg/liter parathion. An approximately 1.3-fold induction of monooxygenase activities was caused by the model inducer naphthalene (0.1 mg/liter). These results suggest that cytochrome P₄₅₀-dependent monooxygenase activities may be useful variables in toxicity tests with aquatic insects.

Key Words: aquatic invertebrate; toxicity; pesticide; organophosphate; cholinesterase; cytochrome P₄₅₀; biomarker.

INTRODUCTION

The protection of aquatic ecosystems against adverse effects of chemicals released by humans is of great concern as these ecosystems are highly valuable in terms of economical, genetic, and recreational resources. Several European Community council directives lay down mandatory toxicology testing with aquatic organisms, e.g., 88/379/EEC (Classification, Packaging, and Labeling of Dangerous Substances) and 91/414/EEC (Use, Marketing, and Registration of Pesticides). Under the current protocols, however, the application of these tests is limited as they are restricted to lethal responses and/or do not take into account different routes of exposure. Therefore, there is a clear need within the European Community for applied methodologies in ecotoxicology that consider both sublethal responses and realistic scenarios of exposure. The research project METIER (Modular Ecotoxicity Tests Incorporating Ecological Relevance) addresses the development of a series of ecotoxicity tests that assess the effects of chemical contaminants on aquatic organisms, striving for environmental realism in both above-mentioned aspects (Ribero et al., 1995).

Biochemical variables have been suggested as indicators of chemical exposure and sublethal effects for different reasons. Assuming that toxicity is ultimately mediated by biochemical processes, a response at the biochemical level should theoretically precede effects on higher levels of organization such as organisms (Adams et al., 1989). Furthermore, the response of specific biochemical variables may allow conclusions about mechanisms of toxicity (Di Giulio et al., 1995). In aquatic toxicology, most research on potential biochemical indicators and biochemical mechanisms underlying toxic action was conducted on fish or marine invertebrates (Di Giulio et al., 1995). Given the ecological importance of limnic invertebrates, their high susceptibility to many pollutants, and their common use as test species, the present knowledge of biochemical effects of toxicants in these groups is disproportionately small. There is obviously an urgent need for a better understanding of the biochemical aspects of toxicology in limnic invertebrates.

Cholinesterases (ChEs) represent the site of action for organophosphorus and organocarboximate insecticides, the anticholinesterase compounds (anti-ChEs) (Fukuto, 1987).
The use of anti-ChEs markedly increased after the ban of organochlorine insecticides in most industrialized countries. In fish (Weiss, 1959) and aquatic invertebrates (Bocquené et al., 1991; Day and Scott, 1990) decrease of ChE was related to exposure to anti-ChEs and suggested as a bioindicator of water contamination. It is well established that exposure of fish to low, sublethal levels of anti-ChEs can result in significant decrease of ChE (Weiss, 1959). After exposure of aquatic invertebrates to anti-ChEs, however, some studies reported no ChE inhibition below levels causing acute toxicity (Day and Scott, 1990), whereas others found significant inhibition at clearly sublethal concentrations (Abdullah et al., 1994; Kuhn and Streit, 1994). This may be due to differences in the general susceptibility of different taxa to anti-ChEs and interspecific differences in the toxicology of a given compound (Day and Scott, 1990). Further research is needed to determine to what extent toxicity and inhibition of ChE are linked in invertebrates.

Cytochrome P450 plays a central role in the metabolism of structurally diverse organic xenobiotics, mostly, but not necessarily, initiating detoxification of toxic compounds (Di Giulio et al., 1995). The inducibility of the fish isoenzyme cytochrome P450IA by such environmental pollutants as PAHs, PCBs, and PCDDs is the basis for its usefulness in field monitoring (Di Giulio et al., 1995). Insect cytochrome P450 differs from its vertebrate counterpart in the spectrum of catalyzed reactions and in its response to model inducers (Ronis and Hodgson, 1989). Inducers of vertebrate cytochrome P450I are not effective in insects, but several inducers of the mammalian P450 gene families II and IV induce insect cytochrome P450 (Ronis and Hodgson, 1989).

The objective of this study was to investigate ChE and selected enzymes of biotransformation metabolism, including cytochrome P450-dependent enzymes, for their potential use as indicators of sublethal toxicity in Chironomus riparius larvae and neonate Daphnia magna. Cytochrome P450-dependent enzymes could not be detected in Daphnia. Therefore, experiments on biotransformation metabolism were limited to Chironomus. In experiments with anti-ChEs, a response of ChE was expected and the aim of the study was to investigate the relationship between acute toxic response and ChE inhibition. The organophosphorus compounds parathion and dichlorvos and the carbamate compound aldicarb were selected as anti-ChEs covering different modes of action. By contrast with dichlorvos, parathion requires metabolic activation for toxicity. Experiments with 3,4 dichloroaniline (3,4-DCA), a degradation product of several herbicides with no reported anti-ChE activity, served to control the specificity of the response. The question behind the experiments on Chironomus biotransformation metabolism was whether sublethal levels of environmental chemicals will affect enzyme activities. Three O-dealkylation reactions—ethoxyresorufin-O-deethylase (EROD), methoxyresorufin-O-deethylase (MROD), and ethoxyxoumarin-O-deethylase (ECOD)—were selected as indices of cytochrome P450-dependent monoxygenase (MO) metabolism. Glutathione-S-transferase (GST) was selected as a major phase II pathway in invertebrates. Parathion and 3,4-DCA were selected as model environmental contaminants. Naphthalene was included in experiments as a model inducer of insect cytochrome P450 (Agosin, 1976).

**MATERIALS AND METHODS**

**Animal Culture and Exposure Experiments**

*Daphnia magna* Straus was cultured in ADaM medium (Klüttgen et al., 1994) and was fed *Scenedesmus vacuolatus* from synchronized culture. *Chironomus riparius* larvae were cultured in plastic compartments containing quartz sand as a substrate and M4 medium (Elendt and Bias, 1990) and were fed ground fish food. Culture and toxicity testing took place at 20°C. *Daphnia* toxicity tests were carried out with ADaM medium as dilution water. Aerated artificial freshwater (294 mg/liter CaCl_2, 2H_2O, 123 mg/liter MgSO_4, 7H_2O, 5.8 mg/liter KCl, 64.8 mg/liter NaHCO_3) was used as dilution water in *Chironomus* toxicity tests. Aldicarb (2-methyl-2[methylthio]propioaldehyde O-[methyl carbamoyl]-oxime), parathion-ethyl (parathion; O,O-diethyl O,P-nitrophenyl phosphorothioate), dichlorvos (2,2-dichlorovinyl dimethyl phosphate), and 3,4-DCA were certified pure compounds from Dr. Ehrensdorfer, Germany. *Daphnia* tests, in which both immotility and cholinesterase inhibition were recorded, were performed in crystallization dishes containing 40 ml of testing solution. Fifty neonate *Daphnia* were used per replicate. At least five concentrations were tested per compound. After 24 h of exposure, daphnids were classified as motile or immotile. Animals were rinsed twice with fresh medium prior to homogenization for later biochemical analyses. Tests with *Chironomus* larvae, in which both immotility and cholinesterase inhibition were recorded, were carried out using 25 fourth-instar larvae per 100 ml crystallization dish containing 40 g quartz sand and 60 ml of testing solution or artificial freshwater (controls). Containers were gently aerated. After introduction of animals and prior to the addition of toxicants, animals were fed 20 mg of fish food per container and allowed to acclimate for 24 h. After 24 h of exposure to toxicants, animals were removed from test containers and classified as motile or immotile. Animals were then rinsed, randomly distributed to pools of five, and stored at -20°C until enzyme analysis.

At least five concentrations were tested per chemical. Methanol was used as a carrier for toxicants at a final concentration of 0.1%. Controls received methanol only. Preliminary experiments indicated that this concentration of methanol had no effect on *Daphnia* and *Chironomus* cholinesterases.

Effects of parathion and 3,4-dichloroaniline on *Chironomus* biotransformation metabolism were investigated in 4-day static exposures, using a similar design as in the experiments
on cholinesterase inhibition. In each replicate, 70 fourth-instar larvae were exposed in beakers containing 0.5 liter testing solution and 80 g quartz sand. Chemicals were added in 0.5 ml DMSO. Water and DMSO controls were included. Testing concentrations were 50 µg/liter 3,4-dichloroaniline, 0.5 µg/liter parathion and 0.1 mg/liter naphthalene (Sigma).

Biochemical Analyses

Homogenization and subsequent storage of enzyme extracts were conducted on ice. Cholinesterase extracts were prepared by homogenization of 50 Daphnia with 400 µl of Tris-HCl (0.1 M, pH 7.5) or 5 Chironomus larvae with 1 ml of Tris-HCl. Homogenates were centrifuged for 10 min at 10,000g at 4°C. The homogenization medium used for the extraction of Chironomus biotransformation enzymes (Lee and Scott, 1989) contained protection agents to prevent breakdown of enzymes (0.1 M sodium phosphate, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM 1-phenyl-2-thiourea (from a 100 mM stock in ethylene glycol monomethyl ether), 1 mM phenylmethylsulfonyl fluoride thiourea (from a 100 mM stock in ethylene glycol monomethyl ether)). Seventy fourth-instar larvae were ground with 2 ml medium. The homogenate was spun for 20 min at 4°C at 10,000g. The resulting supernatant was used for the determination of enzyme activities.

Cholinesterase activity was determined by the method of Ellman et al. (1961) using a SLT 340 ATTC microplate photometer with a 414 nm filter. Final concentrations were DTNB 0.32 mM, ATC 0.5 mM (Daphnia) or 0.87 mM (Chironomus), and 0.1 M Tris–HCl, pH 7.5. The reaction was monitored at 25°C. Activities of MROD and EROD were assayed by modifications of the methods of Mayer et al. (1977) and Burke and Mayer (1974), respectively. The reaction was started by addition of NADPH solution and followed by recording the fluorescence in a Dynatech microplate fluorometer using 530-nm excitation and 590-nm emission filters. Final concentrations were 2.5 µM alkoxyresoruﬁns and 250 µM NADPH. ECOD activity was assayed by the method of Aitio (1978). One hundred micro liters of enzyme extract were incubated at 25°C in the presence of 5 µM 7-ethoxycoumarin and 250 µM NADPH. After 20 min, 10% trichloroacetic acid was added to stop the reaction and to precipitate protein. The product of the reaction, umbelliferone, was fluorometrically measured at pH 10.4 in a Hitachi F 2000 spectroﬂuorometer set to an excitation wavelength of 375 nm and an emission wavelength of 455 nm. GST was measured by the method of Habig et al. (1974) with a microplate photometer (see above) and a 340-nm filter using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction was started by addition of reduced glutathione (GSH) and monitored as the change in absorbance at 340 nm. Final concentrations were 0.1 mM CDNB and 8 mM GSH. Protein was measured by the method of Bradford (1976) using bovine serum albumin, fraction V (Sigma), as standard.

Statistical Analyses

Immotility EC₅₀ values were calculated by probit analysis using a computer program (Peltier and Weber, 1985). The observed concentration dependence of ChE inhibition was fit by logit analysis [minimum logit χ² method (Ashton, 1972)]. IC₅₀ and IC₂₀ values and an estimate for the ChE inhibition theoretically caused by the immotility EC₅₀ were derived from the fitted logit model. The response of biotransformation enzymes in chemical treatments was compared with that in controls by the nonparametric Mann–Whitney test (InStat, GraphPad Software).

RESULTS

Cholinesterase Inhibition

In 24-h acute bioassays with Chironomus and Daphnia, anti-ChE compounds (parathion, dichlorvos, and aldicarb) decreased ChE in a dose-dependent manner (Fig. 1).
### TABLE 1
Comparison of Acute Toxicity (Immotility) and Cholinesterase (ChE) Inhibition Caused by Parathion, Dichlorvos, and Aldicarb in *Daphnia* and *Chironomus* in 24-h Bioassays

<table>
<thead>
<tr>
<th>Species</th>
<th>Chemical</th>
<th>EC₅₀ (immotility)</th>
<th>ChE IC₅₀ (±SE)</th>
<th>ChE IC₂₀ (±SE)</th>
<th>IC₅₀/EC₅₀ ratio</th>
<th>Predicted ChE inhibition at the immotility EC₅₀</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(95% confidence limits)</td>
<td></td>
<td></td>
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<tr>
<td><em>Daphnia</em></td>
<td></td>
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<tr>
<td>Parathion</td>
<td>2.35 (2.25, 2.47)</td>
<td>0.61 ± 0.14</td>
<td>0.40 ± 0.18</td>
<td>0.26</td>
<td>98.9%</td>
<td></td>
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<tr>
<td>Dichlorvos</td>
<td>0.233 (0.225, 0.242)</td>
<td>0.17 ± 0.04</td>
<td>0.11 ± 0.03</td>
<td>0.73</td>
<td>75.5%</td>
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<tr>
<td>Aldicarb</td>
<td>227.6 (212.9, 243.4)</td>
<td>95.0 ± 44.8</td>
<td>35.0 ± 27.2</td>
<td>0.42</td>
<td>77.9%</td>
<td></td>
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<tr>
<td><em>Chironomus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parathion</td>
<td>7.2 (6.6, 7.8)</td>
<td>2.9 ± 1.0</td>
<td>1.3 ± 1.0</td>
<td>0.40</td>
<td>84%</td>
<td></td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>10 &lt; EC₅₀ &lt; 20</td>
<td>6.2 ± 3.1</td>
<td>2.2 ± 2.0</td>
<td>0.3 &lt; ratio &lt; 0.6</td>
<td>77% &lt; inhibition &lt; 83%</td>
<td></td>
</tr>
<tr>
<td>Aldicarb</td>
<td>23 (22, 25)</td>
<td>27 ± 13</td>
<td>5.9 ± 3.5</td>
<td>1.2</td>
<td>53%</td>
<td></td>
</tr>
</tbody>
</table>

* ChE activity was measured in at least three samples (pools of 50 *Daphnia* neonates or 5 *Chironomus* fourth-instar larvae) per concentration.
* EC₅₀, IC₅₀, and IC₂₀ values are expressed as µg/liter.

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contrast, 3,4-DCA had no effect on ChE even at lethal concentrations (not provided). This pattern of response was expected since ChE represents the target site of anti-ChE, whereas there is no evidence of anticholinesterase action by 3,4-DCA. The concentration–response relationship of ChE inhibition by anti-ChE compounds was mathematically described by the logit model (Ashton, 1972) (Fig. 1).

Table 1 compares the acute toxicity of anti-ChEs (immotility EC₅₀ values) with their effects on ChE (IC₅₀ and IC₂₀) for *Daphnia* and *Chironomus* recorded in the same experiment. Ratios of the ChE IC₅₀ to the respective EC₅₀ are in the range 0.26 to 1.2. A ChE inhibition of 20% is often regarded as the threshold above which decrease of ChE indicates exposure to organophosphates or carbamates. Therefore, IC₂₀ values were also estimated from the fitted logit models (Table 1).

### Biotransformation Enzymes

Cytochrome P450-dependent enzymes were measured as three different O-dealkylations (EROD, MROD, and ECOD). Enzymatic O-dealkylation could not be detected in *Daphnia*, but was present in control *Chironomus* at appreciable rates (Fig. 2). 3,4-DCA significantly inhibited cytochrome P450-dependent enzyme (EROD, MROD, and ECOD) activities to about 30% of control values (Fig. 2). Parathion had no clear effect on cytochrome P450-dependent enzymes (Fig. 2). Treatment with the model inducer naphthalene increased cytochrome P450-dependent enzyme activities to about 125% of control values (Fig. 2). There was no apparent difference in the response of the three MOs (EROD, MROD, and ECOD), except that the MROD increase induced by naphthalene was statistically not significant. None of the chemicals investigated had an effect on GST activity, which was 63.1 ± 7.2 pmol min⁻¹ mg⁻¹ protein in controls (not presented).

### DISCUSSION

#### Cholinesterase Inhibition

To describe the relationship between ChE inhibition and immotility observed in acute tests with *Daphnia* or *Chironomus*, ratios of the ChE IC₅₀ to the corresponding immotility EC₅₀ were calculated (Table 1). These ratios ranged from 0.26 to 1.2 (Table 1). Assuming that the toxic action of anti-ChEs is based on ChE inhibition, a toxic response as immotility should occur at a fixed, species-specific level of ChE inhibition. Variability among ratios of ChE IC₅₀ to immotility EC₅₀ may, on one hand, reflect...
ChE inhibition in an acute test does not allow the prediction of the life-threatening situation (Ludke et al., 1975). In studies with aquatic invertebrates, ChE inhibition after exposure to lethal concentrations of anti-ChEs was usually in the range 70–100% (Abdullah et al., 1994; Bocquene et al., 1991; Detra and Collins, 1991). In some cases, however, lethal effects of anti-ChEs may occur at a ChE inhibition of 40% or below (Bocquene et al., 1991; Escartín and Porte, 1996).

Beyers and Sikoski (1994) suggested that the measurement of ChE inhibition after acute exposures could be used to conservatively predict no-adverse-effect levels for chronic exposures in fish. No-observed-effect concentrations (NOECs) for ChE inhibition following acute exposures of Colorado squawfish to carbaryl and malathion were 15 and 5 times lower, respectively, than NOECs for survival and growth determined in a 32-day early life stage test with the same species (Beyers and Sikoski, 1994). The ChE inhibition at the NOECs for ChE inhibition in that study was 13.3 and 23.8% for carbaryl and malathion, respectively (recalculated from Beyers and Sikoski, 1994). Because pooling was necessary for ChE analysis in Daphnia and Chironomus, the number of replicates per concentration was limited in this study. Due to the low power of an ANOVA under these conditions, NOECs for ChE inhibition were in a range similar to that of the IC50 values (not provided) and obviously were not a reasonable estimate of a threshold for effects on ChE. As an alternative, IC20 values of ChE inhibition were estimated (Table 1). IC20 values ranged from about one-sixth of the immotility EC50 (most cases) to one-half of the EC50 (Daphnia/dichlorvos) (Table 1). Published chronic toxicity data were available only for Daphnia and parathion. For Daphnia, Kühn et al. (1989) report an immotility EC50 of 2.0 μg/liter and a NOEC for reproduction of 0.002 μg/liter (21-day test). The chronic NOEC for reproduction was approximately 200 times more sensitive than the acute ChE IC20 found in this study. From these data on parathion, it appears that the determination of ChE inhibition by anti-ChEs in an acute test does not allow the prediction of the threshold concentration of sublethal effects in Daphnia.

Biotransformation Enzymes

The 1.2- to 1.3-fold induction of cytochrome P450-dependent enzymes by the model inducer naphthalene is in the lower range of values reported for Musca domestica imagines. With naphthalene or phenobarbital as inducer and naphthalene, bayon, or aminopyrine as substrate Capdevilla et al. (1973) found apparent induction rates of 1.2–4.3, depending on substrate. Lee and Scott (1989) report differential induction of M. domestica MOs by phenobarbital of 2.6-fold (MROD), 2.9-fold (EROD), and 10.0-fold (ECOD), which is in contrast to our observation of no apparent difference in the response of O-dealkylation enzyme reactions (MROD, EROD and ECOD) in Chironomus.

Parathion can react with mammalian cytochrome P450 in a suicidal fashion, which results in inhibition of activity (Butler and Murray, 1993). No inhibition was observed at a sublethal concentration in this study, however, preliminary range finding data indicated inhibition of EROD in the acutely toxic range (not provided). 3,4-DCA is a weak phenobarbital-type inducer of cytochrome P450 in rat (McMillan et al., 1990). Although phenobarbital-type inducers are partly effective in insects (Ronis and Hodgson, 1989), 3,4-DCA did not act as an inducer in these experiments, but inhibited cytochrome P450-dependent enzyme activities. It was beyond the scope of this study to address the mechanism of the inhibition.

Cytochrome P450-dependent MO activities in insects have been studied mainly because of their relevance for target species susceptibility to pesticides. Among other mechanisms, target species resistance and cross-resistance can be based on the elevation of MO activities involved in the detoxification of insecticides (Kotze, 1993; Lee and Scott, 1989). Consequently, cytochrome P450 inhibitors such as piperonyl butoxide are included in some pesticide formulations to overcome MO-based resistance or to generally increase the formulation’s efficiency. MOs are, on the other hand, responsible for the activation of phosphorothioates to their toxic oxon analogues (Fukuto, 1987). Coadministration of the cytochrome P450 inhibitor piperonyl butoxide may therefore mitigate phosphorothioate toxicity on Chironomus (Ankley and Collyard, 1995). The responsiveness of the MO system in Chironomus presented in this report and the indirect evidence for its relevance to the toxicity of xenobiotics presented by Ankley and Collyard (1995) may encourage further studies. The prediction of effects of chemicals to aquatic insects requires a better understanding of, first, which environmentally relevant compounds are able to modulate the MO system and, second, which xenobiotics will vary in their toxicity to aquatic insects depending on the induction status of the MO system. In conjunction with other parameters, MOs may be useful variables in toxicity studies with aquatic insects. The application of MOs in routine testing with Chironomus, however, is limited because of the small range of the induction response.
CONCLUSIONS

For most anticholinesterase compounds investigated with Daphnia and Chironomus in acute tests, the IC₅₀ of cholinesterase inhibition was only slightly more sensitive (up to three times) than the conventional criterion immotility. At least in acute toxicity testing of anti-ChE compounds, the measurement of ChE inhibition does not appear to provide additional insights, except if there is doubt about the involvement of ChE inhibition. Cytochrome P450-dependent monooxygenase activities in Chironomus were both induced and inhibited by sublethal levels of environmental chemicals. They may be useful variables in toxicity studies.

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REFERENCES


