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Detecting Sublethal Effects of Organophosphates by Measuring Acetylcholinesterase Activity in *Gammarus*

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Organophosphorous insecticides (OPI, organophosphates) can contaminate surface waters through unintentional drift of aerial spraying for agricultural use, through watershed drainage or accidental spillage and in some countries even through intentional application. Freshwater species can thus be exposed to OPI concentrations which range from sublethal to lethal. The toxicity of OPI is basically caused by inhibition of acetylcholinesterase (AChE) activity in a great variety of target and non-target organisms, including aquatic invertebrates.

A major purpose for monitoring acetylcholinesterase activity is to detect sublethal responses to OPI exposure at the biochemical level before more serious physiological and population effects become manifest. Furthermore, the measurement of AChE activity is a sensitive parameter for testing water for the presence of OPI. Inhibition of AChE activity in fish from the environment had been used as a tool in diagnosting OPI pollution (Lockhart et al. 1985), but fish are able to detoxify OPI more easily than invertebrates. Thus significant AChE inhibition in fish can only be detected at higher OPI concentrations; they are therefore less suitable candidates for monitoring minute concentrations of OPI. Aquatic invertebrates tested so far for AChE inhibition have not proven sensitive enough for quantifying sublethal effects, but many turned out to be useful in cases where acute lethality is likely to occur (Day and Scott 1990). An adequate biomonitor should show a significant AChE reduction in low and sublethal concentrations proportional to ambient concentrations.

We studied sublethal as well as lethal effects of two widely used OPI on three species of the genus *Gammarus,* representing very sensitive aquatic invertebrates for a wide variety of chemicals (McCahon and Pascoe 1988). *Gammarus pulex* and *G. fossarum* are autochthonous European species; *G. tigrinus* is an immigrant species from North America. Gammarids are important freshwater detritivores, involved in the decomposition process of leaves and other decaying materials, and serve as an important food source for fish.

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MATERIALS AND METHODS

Gammarus pulex and *G. fossarum* were collected from small tributary rivers of the Rhine *(G. pulex* from the Magdloser Wasser / Vogelsberg, *G. fossarum* from the Kesselbach / Taunus; Central Germany). *G. tigrinus* was obtained from the Rhine river in the vicinity of Mainz (official km 499). Animals were acclimatized to laboratory conditions in tap water for 14 days prior to use in exposure studies.

All tests were performed in tap water (pH 7.3; 121 mg Ca^{2+}/L) at 15°C. The chemical purity of the compounds used was 98.5% for fenitrothion and 99.9% for parathion-methyl (both acquired commercially from Dr. Ehrenstorfer GmbH, Augsburg). For dissolution of the chemicals, we used acetone, which never exceeded more than 0.01% in the final test solution.

Acute toxicity tests were performed in static bioassay systems for fenitrothion and parathion-methyl. In each test, 20 individuals of *G. pulex* and *G. fossarum,* respectively, were exposed to five different organophosphate concentrations (0.625 - 10 μg/L) for 96 hr. In control tests with tap water containing 0.01% acetone no animals died. All tests were carried out in duplicate. LC_{50}(96 hr) values and their confidence intervals were calculated by logit transformation (Ashton 1972).

To perform sublethal experiments, the animals were exposed for 24 hr to various sublethal concentrations, or for various time periods in a single concentration (1 μg/L), after which treatment we measured AChE activity to quantify enzyme inhibition. A minimum of 20 individuals of different size, but equal average body mass, were used for each experiment. Homogenates of single animals were analyzed using the colorimetric reaction between the hydrolyzed acetylcholine (ACh) analogue, acetylthiocholine, and DNTB (2,2'-dinitro-5,5'-dithiobenzoic acid) according to Ellman et al. (1961). The original method was modified, by adding triton X-100 to the homogenization buffer in order to dissolve membrane-bound acetylcholinesterase (Meneely and Wyttenbach 1989; Thompson et al. 1991).

AChE activity was calculated as units of activity per g fresh mass (1 U/g = 1 μM of substrate hydrolyzed per min per g fresh mass). Statistical comparisons of AChE activities of exposed and unexposed animals were performed by Student's t test ($p < 0.05$).

Since *G. tigrinus* showed high mortality rates under laboratory conditions, only the short-time experiments (24 hr exposure to sublethal concentrations) was performed with this species.

RESULTS AND DISCUSSION

A summary on LC_{50} values for parathion-methyl and fenitrothion in *G. pulex* and *G. fossarum* is presented in Table 1. The two gammarid species tested belong to the most sensitive aquatic taxa among arthropods, as the comparison with various literature information shows: e.g., considering the two compounds, fenitrothion and parathion-methyl, LC_{50}(96 hr) values for various species of *Orconectes* were found in the range 10 - 269 µg/L (Johnson and Finley 1980; Poirier and Surgeoner 1987); even when compared to various insect species, the two *Gammarus* species turned out to be highly sensitive. They are ca. 1000 times more sensitive than many fish species tested (Johnson and Finley 1980).

Table 1. LC_{50}(96 hr) values and 95% confidence intervals, calculated for *Gammarus pulex* and *G. fossarum* by logit transformation. Values are expressed in µg/L (= ppb).

Species	LC_{50}(96 hr) with 95% confidence interval	
	fenitrothion	**parathion-methyl**
Gammarus pulex	5.49 (4.6-6.6)	3.21 (2.7-3.8)
Gammarus fossarum	2.88 (2.4-3.4)	2.52 (2.2-2.8)

After a 24 hr exposure period to sublethal concentrations of fenitrothion and parathion-methyl, all three species showed a significant AChE inhibition, the degree depending on the concentration chosen. No mortality occurred during the experiments. The sensitivity of the three species tested was different, as shown in Figure 1. The most sensitive species was *G. pulex;* fenitrothion and parathion-methyl concentrations as low as 1 µg/L resulted in significant AChE inhibition (35 - 39% inhibition, corresponding to 61 - 65% activity) in *G. pulex* after 24 hr. The least sensitive *Gammarus* species tested was *G. tigrinus*. Significant enzyme inhibition could not be detected at fenitrothion concentrations lower than 3 µg/L (80% activity) and parathion-methyl concentrations lower than 5 µg/L (83% activity) in *G. tigrinus*.

The strong AChE inhibition in *Gammarus* species after a 24 hr exposure to very low concentrations of fenitrothion (1 - 6 µg/L) may be due to a metabolization process: In crustaceans, fenitrothion is oxidized to fenitrooxon (Reddy et al. 1990), which is retained in different members of this taxon (Takimoto et al. 1986). Fenitrooxon is known to be the more potent inhibitor of AChE activity (Schoor and Brausch 1980). A similar mechanism is assumed for parathion-methyl (Reddy et al. 1990).

For *G. pulex* and *G. fossarum* we observed a continuous reduction of AChE activity with increasing time (Figure 2). Within the first 2 days, the enzyme inhibition caused by the two compounds tested was nearly the same. After 3 days and longer exposure times, fenitrothion turned out to be the more potent inhibitor. The lowest level of AChE activity was measured for *G. pulex* after a

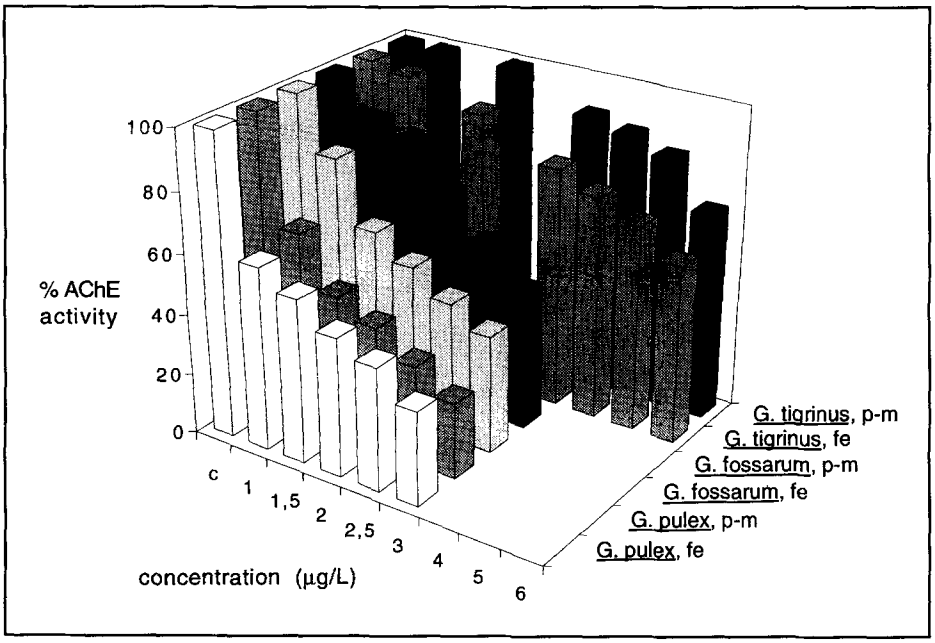

Figure 1. Relationship between percent reduction of AChE activity after 24 hr at different OPI concentrations for the two compounds and the three species tested. c: control group. p-m: parathion-methyl, fe: fenitrothion.

5 days contamination period with fenitrothion. Although the activity was only 27% of the control, no single test animal died within this time period.

Transferring exposed animals into toxicant-free water resulted in a progressive recovery of AChE activity (cf. Fig. 2). The rate of recovery was lower than the rate of increasing inhibition during the contamination period. Starting from a reduced AChE activity in the range of 27 - 34% (fenitrothion) and 50 - 63% (parathion-methyl), the enzyme activity reached 75 - 85% of the level of non-exposed animals at day 21 (i.e., 16 days decontamination period). During the first week in toxicant-free water, the enzyme activity increased rapidly, but slowly and steadily afterwards.

In contrast to the fast inhibition rate, a long time period was required to recover AChE activity to nearly normal levels. This trend has been observed in different other species with a variety of OPI (e.g., van der Wel and Welling 1989; Lockhart et al. 1985). It is generally known that even moderately lipophilic substances, such as the two compounds tested (n-octanol/water partition coefficient log

K_{ow} = 3.6 and 1.9 for fenitrothion and parathion-methyl, respectively), can rapidly penetrate membrane structures of aquatic animals (e.g., Streit and Siré 1993) and thereafter start reaction at suitable receptor sites. In contrast, the process of recovering the AChE activity is not only attributed to the elimination rate (e.g., Streit 1992), but to dephosphorylation of OPI and resyntheses of fresh enzyme (Reddy et al. 1990). In view of this there is a need to space OPI applications at time intervals sufficient to protect non-target organisms.

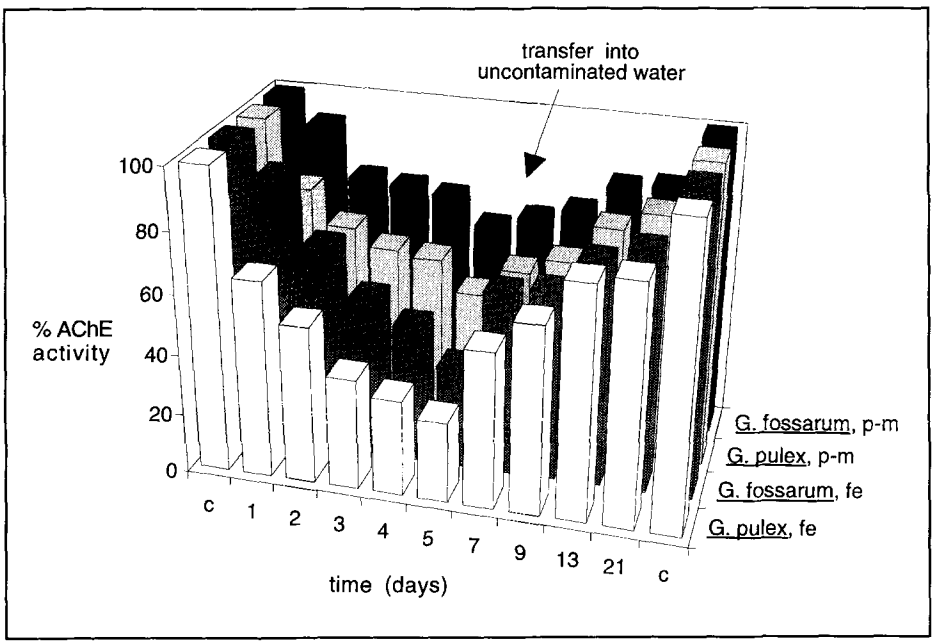

Figure 2. Relationship between the reduction of AChE activity during a 1 µg/L exposure period (days 1 - 5) and a later recovery period (days 7 - 21). c: control group. p-m: parathion-methyl, fe: fenitrothion.

It should be pointed out that the term "sublethal" as used throughout this paper exclusively refers to laboratory conditions. Pesticide concentrations sublethal under laboratory conditions could well lead to the death of individuals within the natural environment. This may result from a change in behavior, which in a lotic environment would lead to a drifting and/or might facilitate predators to catch them. An increase in drift rates under the influence of organophosphates has been described by Everts et al. (1983), Poirier and Surgeoner (1987), Kreutzweiser and Sibley (1991). Reduced escape responses, enabling predators to feed on crustaceans easily, was observed by Ward and Busch (1976). Further "sublethal" effects of *Gammarus* individuals in the laboratory, which would directly influence population fitness rates, might include behavioral changes in reproduction, disrupting precopula pairs, as found through the action of various environmental stress factors (Davis 1978; Poulton and Pascoe 1990).

We conclude that minute sublethal concentrations of fenitrothion or parathion-methyl (in the range of those found in freshwater habitats, e.g., resulting from operational spraying of OPI in adjacent areas) does result in significant reductions of AChE activity in three species of the amphipod genus *Gammarus*. *G. pulex* in particular has the characteristics of a suitable bioindicator for lowest levels of organophosphate contamination. We could demonstrate its utility by quantifying AChE inhibition as a parameter of toxicity.

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