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Bioavailability and Effects of Malathion in Artificial Sediments on *Simocephalus vetulus* (Cladocera: Daphniidae)

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The pollution of aquatic sediments is critical to the health of aquatic ecosystems. Sediment dwelling organisms are key links in food webs that can lead toxic materials to higher level consumers, such as fish and humans. Sediments in aquatic systems can be both sink and source for inorganic and organic contaminants. The assessment of sediment contamination by laboratory bioassays is essential since it measures the toxicity and the biological response associated with specific concentrations of chemical pollutants. Furthermore, these can indicate if degradation or binding could reduce the bioavailability of chemicals, and can also set the basis for the establishment of safe concentrations.

To carry out these tests, it is necessary to select an organism of ecological relevancy that is also readily available for testing. Cladocerans are an abundant group of ecological importance in aquatic ecosystems, since they act as the main link in the energy flow between the primary producers and higher trophic levels (Hanazato and Dodson 1995). The genus *Simocephalus* is cosmopolitan and can be found distributed in the bottom of the littoral zone in water bodies (Hann 1995), where the municipal and/or industrial discharges could exert pollution pressure on their populations. Willis et al. (1995) evaluated the sensitivity of *S. vetulus* to pentachlorophenol and determined that this organism has great potential to be used in toxicity evaluations.

Malathion (MA) is a wide spectrum insecticide. Its degradation rate depends on microbial activity, temperature and pH; when the pH range is 7.0-7.4, its $T_{½}$ is 10.5 days, however in natural river waters, with pH values *ca.* 8.2, the $T_{½}$ was 22 hours (Derosa and Stara 1988). This pesticide is used to control boll weevils and many biting and sucking insects that plague crops. Its toxic effect in insects is through the inhibition of the acetylcholinesterase enzyme (AChE) in the nervous system (Scaps et al. 1997).

The objective of the present study was to assess the sublethal effects of MA on AChE activity, lipid peroxidation level, and lipid and protein content in *Simocephalus vetulus*, and estimate the relationship between toxicity and pesticide bioavailability in artificial sediments.

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MATERIALS AND METHODS

Simocephalus vetulus was cultured in reconstituted semi-hard water (Weber 1993) and fed the microalga *Ankistrodesmus falcatus* in a concentration of $6.5X10^5$ cell mL^{-1}. Experimental conditions were: temperature=23.5°C ± 1.5°C, and photoperiod= 16:8 (light:dark). Artificial sediments were prepared by mixing 70 % of sand (particle size 0.05-0.2 mm), 25 % kaolin (particle size < 2 μm) and 5 % of dried cow manure (SETAC 1993). This mixture was autoclaved (15 min, 15 psi); sterilized sediments were stored in sealed plastic containers until its use.

Technical grade MA (Bayer™) was dissolved in acetone and spiked in the artificial sediments at five concentrations (1.0, 1.5, 2.3, 3.4, and 5.1 μg L^{-1}, final volume, including sediments and water). The sediment was covered with reconstituted semi-hard water (proportion1:4) and shaken during 48 hours. In order to determine equilibrium time, water and sediment were separated by centrifugation (4,000 rpm), and the MA concentration was measured at 0, 1, 2, 3, 4, 5, 6, 24 and 48 h, using a gas chromatograph.

For acute toxicity determination in the water system, ten newborn organisms (neonates, age less than 24-h old) were added to triplicated 100-mL glass jars, with 50 mL of test solution; five MA concentrations were tested in reconstituted semi-hard water: 1.0, 1.5, 2.3, 3.4, and 5.1 μg L^{-1}, plus the control. Acute toxicity was also determined in the water+spiked sediments system: ten neonates were added to triplicated glass vessels containing 10 g of sediment spiked with MA, plus 40 mL of reconstituted semi-hard water. Five MA concentrations were tested (1.0, 1.5, 2.3, 3.4, and 5.1 μg kg^{-1}, dry weight) plus a control group; equilibrium concentrations were achieved by mechanical shaking during 4 hours (equilibrium time), prior to the addition of the test organisms. The 48-h median lethal concentration (LC_{50}) was determined for both systems with the Probit method.

For the sublethal tests, twenty neonates were added to triplicated glass vessels which contained 150 g of sediment spiked with 1.0 μg kg^{-1} of MA, and 600 mL of reconstituted semi-hard water; this concentration corresponded to the LC_{10} determined for the sediment bioassay. Each group was exposed for: 0, 4, 6, 12, 24 and 48 h. After each time, all organisms were washed and then homogenized with 2 mL of buffer (tris pH 7.0). The homogenate was centrifuged at 7,000 g for 30 min at –5°C. The resulting pellet was evaluated for lipid concentration and the supernatant for AchE activity, lipid peroxidation level, and protein concentration.

Lipid concentration was determined by the method suggested by Postma-Ströes (1968). The pellet was mixed with 2.5 mL of concentrated sulfuric acid and placed in a boiling water bath for 10 min. Then, 50 μL was taken and 2.5 mL of phosphovanillin reagent added (solution of vanillin 9.02 mmol L^{-1} in phosphoric acid); samples were incubated for 10-min at 37°C. Olive oil was used as lipid standard. Absorbance was measured in a spectrophotometer at 530 nm.

The AchE activity was determined according to Hestrin (1949). Two mL of Tris

buffer (pH 7.0) and 1.0 mL of acetylcholine (Ach) standard (80 μmol) was added to one mL of supernatant; samples were incubated at 25°C for 35 min. The reaction was stopped by adding 2 mL of alkaline2M hydroxylamine, 1 mL of 4N HCl and 1 mL of 0.37M $FeCl_3$ in 0.1N HCl. The developed color was measured at 540 nm in a spectrophotometer. Enzyme activity was expressed as μmol of hydrolyzed Ach/mg protein/min.

Lipid peroxidation level (the oxidation of polyunsaturated fatty acids of the lipid constituents of cell membranes), was determined using the method of the thiobarbituric acid (TBA) (Beuge and Aust 1978); the 1,1,3,3-tetramethoxy-propane (Malondialdehyde bis, MDA) was used as malondialdehyde standard. Supernatant (500μL) were added to 2 mL of TBA; this mixture was incubated at 37°C for 30 min, then the sample was cooled for 15 min in an ice bath. The developed color was measured at 532 nm in a spectrophotometer. Lipid peroxidation level was expressed as nmol MDA/mg protein.

For the protein concentration, 2.5 mL of Bradford reagent (Coomassie brillant blue G-250) was added to 50 μL of supernatant (Bradford 1976); bovine albumin was used as a protein standard. The developed color was measured at 590 nm in a spectrophotometer (Varian DMS 90).

For bioaccumulation determination, malathion was mixed with sediments (1.0 μg kg^{-1}), and when equilibrium was reached (after 4 h), six groups of 10 individuals were exposed in 100 mL glass beakers, containing 10 g of MA-spiked sediments. After 0, 4, 6, 12, 24, and 48 h, one group of these organisms was separated, washed with distilled water and weighed; finally, the MA concentration was measured, both in sediments and in daphnids, by gas chromatography. MA concentrations were determined as follows: a sample of 0.05 g of sediment or individuals was homogenized with 40 g of anhydrous Na in a mortar. The extraction was made with 100 mL of a mixture containing chromatograph-degree hexane (50%) in methylene chloride, with mechanical agitation for 30 minutes. The extracts were cleaned for gas chromatography analysis in a Fluorisil column and evaporated to dryness in a N atmosphere (El Nabawi et al. 1987). Dried samples were reconstituted with 0.5 mL HPLC-grade hexane, and analyzed with a Varian chromatograph (Model 3400), using a 15-m column, 0.53 mm internal diameter and 1.5 μm film of methyl silicon. The injector temperature was 250°C, the detector temperature was 260°C, and helium was used as carrier gas (30 mL/min). The initial column temperature (120°C) was held for 1 min, then was raised to 150°C and held for 2 min, then raised to 205°C (at a rate of 10°C/min), then raised to 249°C (2°C/min), and held for 5 minutes.

The accumulation factor at steady state (expressed on a wet weight basis) was obtained as A= Co/Cs, where Co is the MA concentration in organisms, and Cs is the MA concentration in the sediment. Data were analyzed through analysis of variance (ANOVA). The significance of differences between groups was tested using the Duncan's mean test. The criterion for significance was $P<0.05$.

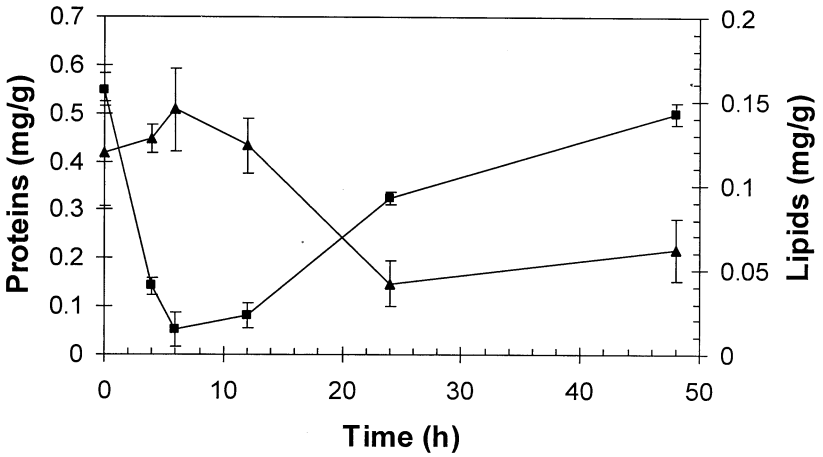

Figure 1. Toxic effect of malathion on the protein (■) and lipid (▲) concentrations of *Simocephalus vetulus*. Average value (n=3) ± confidence interval (P=0.05).

RESULTS AND DISCUSSION

The 48-h LC_{50} for malathion in water was 2.9 μg L^{-1} (95% confidence limits 2.4 to 3.6), and for MA spiked in sediment was 3.8 μg kg^{-1} (95% confidence limits 2.1 to 4.4).

Lipid concentrations at 4 h, 6 h and 12 h of exposure to the pesticide were not significantly different from the control group, but at 24 h and 48 h, lipids were significantly reduced (P=0.05) in 64.7 and 47.9 %, respectively (Fig 1). Soluble protein concentration (mg/g of tissue, wet weight) decreased with time during the first 12 hours, but at 48 h the concentration was not significantly different from the control group (Fig 1). A gradual increase in lipid peroxidation level was observed with respect to time, reaching its maximum value at 48 h of exposure. This value was 665.2 % higher than for the control group (P=0.05) (Fig. 2). *S. vetulus* in the control group produced 1.3±0.1 μmol/mg protein/min of hydrolyzed Ach, in average. When tests organisms were exposed to MA, AchE activity decreased 18.9, 35.2, and 48.0 % at 4, 6, and 12 h, respectively with respect to the control. On the other hand, when exposure time increased (24 and 48 h), the enzyme activity rose, but these values were significantly lower than the control (P=0.05) (Fig. 2).

The accumulation of MA in *S vetulus* is shown in Fig. 3. A short initial phase (0-4 h), with an accelerated increase in insecticide uptake, was observed; then, an intermediate phase (4-12 h), during which the MA concentration remained more or less constant (5.6 ng g^{-1} tissue, wet weight), and a third phase during which pesticide concentration decreased slowly (4.1 ng g^{-1} wet tissue). The MA bioconcentration factor was 2.1. Fig. 3 also shows the MA elimination from sediments; insecticide concentration diminished with respect to time but did not disappear, and

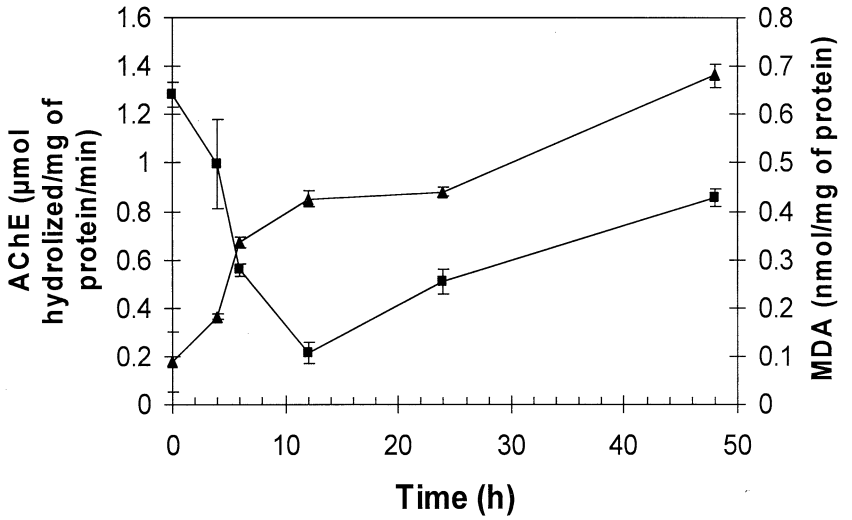

Figure 2. Toxic effect of malathion on the acetylcholin-esterase activity (■) and lipid peroxidation level (▲) of *Simocephalus vetulus*. Average value (n=3) ± confidence interval (P=0.05).

we recorded 1.2 ng MA g^{-1} sediment at 48 h. A one-compartment model for MA elimination from the exposed population was determined. The elimination constant (k_e) was obtained by linear regression analysis (k_e=6.3 ng h^{-1}; r^2=0.999) and the $T_{½}$ determined was 108.4±0.004 h. For the sediments, the changes in MA concentration with respect to time (Cst) was fitted to the following equation: $Cst=0.144\ e^{-0.03t}$. The k_e was 31.4 ng h^{-1} and the $T_{½}$ was 22.04 h.

According to the 48-h LC_{50} value for malathion to *S. vetulus* in water and in sediments, this pesticide can be classified as highly toxic (Metelev et al. 1983); these values are similar to those obtained for *S. serrulatus* in water (3.5 µg L^{-1}) (Sanders and Cope 1966).

Protein concentration has been used to evaluate the effects of environmental stressors on aquatic organisms (Nacimiento et al. 1998). In the present study, the organisms exposed to a sediment-water system showed a reduction in this parameter at 12 h; after this time, the protein content tended to increase, reaching its maximum value at 48 h (100%) (P=0.05). Under these conditions, MA concentrations were not lethal and organisms were able to recuperate after the initial damage.

It was shown that MA exposed sediments produced a decrease on AchE activity at all the exposure times, and reached its minimum value at 12 h (87.1%, with respect to the initial time, Fig. 2). In this respect, Scaps et al. (1997) found that MA inhibits the AChE activity in *Nereis diversicolor*. An additional effect was the incremental in AChE activity of *S. vetulus* in the sediment-water system at 24

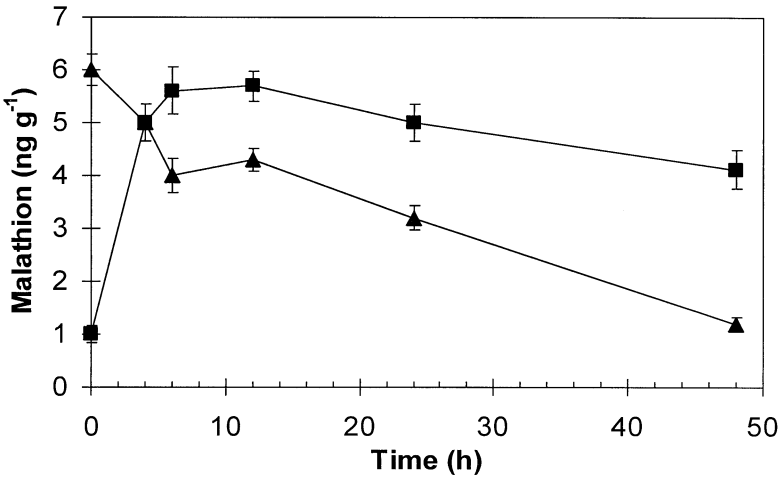

Figure 3. Malathion concentration in *Simocephalus vetulus* (■) and MA elimination course from sediments (▲), in MA spiked artificial sediments. Average value (n=3) ± confidence interval. (P=0.05).

and 48 h, but these values were lower than the control; one explanation is that the daphnids were able to eliminate the MA at that time.

Lipid peroxidation has been associated with cellular damage and death; additionally, this process can produce loss of lipids (Hoving et al. 1992). The products of lipid peroxidation include lipid epoxides, hydroperoxides, epoxy alcohol, ethane, pentane, 4-hydroxy-alkenals and MDA (Hoving et al. 1992). In the present study we observed an increase in the MDA concentration with respect to time. Parkinson (1995) demonstrated that the main metabolite of MA (malaxon) is capable of producing free radicals; this may explain the increase in lipid peroxidation activity as well as the decrease in lipid concentration in *S. vetulus*.

The reduction in MA concentration with respect to time was related to a rapid absorption of MA in the cladoceran. The three-step bioconcentration kinetics here described has also been observed in other aquatic invertebrates exposed to pesticides (Streit 1979). The first step represents a period of MA transfer from sediment to organisms; the plateau denoted a steady-state stage in which the uptake and expulsion of the agent were in equilibrium. In the last phase, the organisms eliminate MA. Malathion was rapidly bioconcentrated by *S. vetulus*, since the time between the maximal absorbed concentration and the beginning of depuration was long; it can be suggested that the elimination of this xenobiotic was slow, as can be concluded from the $T_{1/2}$ (108.6 h).

We determined a linear relationship between lipid peroxidation activity and bioconcentration when organisms were exposed to MA, but AchE activity did not follow a similar trend. These differences can be explained considering that lipid peroxidation is mediated by free radicals, as a primary mechanism of cell mem-

brane destruction and cell damage (irreversible effect) (Plaa and Witschi 1976), while AchE activity in the tissue is concentration-dependent as well as time-dependent (Anasari and Kumar 1984). The main aim of toxicokinetic studies with aquatic organisms is to estimate the toxicant concentration causing minimum biological effects on a population, in this study we demonstrated that MA produce toxic effects on *S. vetulus* and can be bioconcentrated at levels even lower than the threshold limit for the drinking water value established in México (0.19 mg/L). Also, we suggest the use of a model based on toxicokinetics, which is fundamental for the extrapolation of toxicological data from one species to others. It could also be used for monitoring bioavailability in water and sediment samples, and as a standard method for the regulation and control of cholinesterase inhibitors, such as MA.

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