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Bioconcentration of Chlorpyrifos, Chlorfenvinphos, and Methidathion in *Mytilus galloprovincialis*

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Recently, organophosphorus pesticides, carbamates, pyrethroids and triazines have largely replaced the organochlorine compounds in agricultural activities. The organophosphorus pesticides chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridil) phosphorothioate, CAS RN 2921-88-2, Dursban, Lorsban, Spannit], chlorfenvinphos [2-chlorophenyl ethenyl diethyl phosphate, CAS RN 470-90-6, Birlane, Supona] and methidathion [S-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl) O,O-dimethyl phosphoro-dithioate, CAS RN 950-37-8, Supracide, Ultracide] are widely used in the countries of the European Union (UNEP 1991) and have been detected at µg/L level in surface water of the Spanish Mediterranean coasts (Hernández et al. 1996). Recent studies proved the risks of these organophosphorus pesticides due to their short and long term effects on the survival and accumulation ability in the tissues of aquatic organisms (Serrano et al. 1995; *in press;* van den Brink et al. 1995).

The Bioconcentration Factor (BCF), defined as the ratio of the concentration of the chemical in the whole organism to that in the water, can be determined experimentally or deduced from physicochemical properties of the chemical, such as the n-octanol/water partition coefficient (K_{ow}) or water solubility. The BCF can be influenced by a variety of physiological and environmental conditions of the organisms (Huckle and Millburn 1990).

The aim of this study was to determine the accumulation ability of three organophosphorus pesticides in the Mediterranean mussel (*Mytilus galloprovincialis*), due to the potential risk presented by these compounds in the marine environment and their possible biotransformation. The BCF values were experimentally determined, and compared with theoretical considerations.

MATERIALS AND METHODS

Adult specimens of *M. galloprovincialis* (mean weight of soft tissue: 6.21 ± 2.50 g; mean size of major axis: 4.98±0.52 cm) were collected from Castellón coast (Spain). They were acclimatized in 150 L aquaria of sea water (salinity: 38 g/L; drawn up from 300 m offshore and filtered through a 1 µm net) for 30 days at temperature of 18±1°C with continuous aeration. Levels of dissolved oxygen and pH were measured twice daily. A daily diet of microalgae (composed mainly by *Tetraselmis suecica,* completed with *Skeletonema costatum* and *Chaetoceros sp.*)

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was supplied. The mortality of the stock organisms during the 30 days of acclimatization was less than 5%. Test organisms were selected from the acclimatized stock following recommendations given by FAO (Reish and Oshida 1986) for use in the experiments. Before acclimatization of the organisms, triplicate pools composed of 3 organisms were analyzed by capillary gas chromatography with a mass selective detector to exclude the possible presence of pesticides.

Three groups of 50 specimens of *M. galloprovincialis* in triplicate were kept in filtered sea water with 1 mg/L of chlorpyrifos, chlorfenvinphos and methidathion. 100 L of sea water were used for the experiments in tronchoconical experimental separate tanks each containing one replicate. Pesticide standards (purity 99%) for bioconcentration tests and analytical procedures were purchased from Dr. Ehrenstorfer Reference Materials (Germany). Pesticides were dissolved and delivered in 250 mL acetone. Two controls were performed: one control was maintained in clean sea water and another received acetone in equal amount to that delivered in the experimental tanks tested (250 mL). No differences were observed between the two control groups and no significant mortality was detected in either case.

It was pointed out (*Serrano et al. in press*) that 3.2 mg/L of chlorpyrifos provoke toxic effects after long term exposure, but not 1.0 mg/L. Therefore, the nominal concentration selected to study the bioconcentration for the pesticides was 1.0 mg/L. During the bioconcentration tests, organisms were exposed to the pesticides for 35 days, using the renewal test method (renewal of sea water solution every 4 days). The concentration of pesticides in sea water was determined before each renewal. Triplicate samples of 3 living organisms were collected from every treatment on days 1, 6, 8, 15, 20, 24 and 35 along the bioconcentration tests. Levels of pH, dissolved oxygen and temperature were controlled twice daily to maintain them at the optimum level. Animals were not fed during bioconcentration tests. During the experiments, the organisms were maintained in the natural photoperiod of January-February-March (0°, 40° N). Experimental aquaria were sited in isolated area, and organisms only were manipulated when it was neccessary for checking mortality and samplings avoiding stress as possible.

A liquid-liquid extraction with dichloromethane (100+50+50 mL) was used to extract pesticides from sea water. Water residues in the extract were eliminated with anhydrous sodium sulfate (pesticide residue analysis quality, Baker). After pre-concentration with Kuderna Danish, the extract was dried under gentle nitrogen stream and dissolved in n-hexane for detection with Gas Chromatography (GC) (Hernandez et al. 1993). The recoveries of organophosphorus pesticides from sea water spiked at 1 mg/L ranged from 90 to 109% with relative standard deviations lower than 10%. Limits of detection were found to be 0.03, 0.08 and 0.03 µg/L, for chlorpyrifos, chlorfenvinphos and methidathion, respectively.

After collection, samples were frozen and later analyzed as wet weight soft tissue content of pesticide residues. They were thawed at room temperature, triturated, and mixed with anhydrous sodium sulfate. The extraction was carried out with acetonitrile:acetone (10:1, v/v) (pesticide residue analysis quality, Scharlau) by means of a blender set (Ultraturrax) (Greve and Heusinkveld 1983, Greve 1988). Cleanup of the extracts was carried out by means of an LC system based on normal phase-HPLC with a Silica Novapack column (Waters, Milford, MS, USA). n-hexane was used as mobile phase, and a mixture of hexane/ethyl acetate 99:1 (v/v) or ethyl acetate 100% was used for the elution of pesticides and metabolites (chlorpyrifos methyl-oxon and 3,5,6-trichloro-2-pyridinol) in fractions free of fat (*Serrano et al. in press*). The fat-free extracts were analyzed by GC (NPD/MSD). Recoveries of the procedure at 200 and 40 ng/g fortification levels were satisfactory. Limits of detection were found to be around 1 ng/g level for pesticides and 10-fold higher for metabolites.

GC analyses were performed on a Hewlett-Packard 5890 series II (Avondale, USA) with nitrogen-phosphorus detector, equipped with an HP 7673 autosampler. Splitless injections of 2 µL were performed on a fused silica HP Ultra 2 capillary column coated with cross linked 5% phenyl methyl-silicone with a length of 25 m x 0.25 mm I.D. and a film thickness of 0.33 µm. Helium was used as carrier gas at a flow of 0.5 mL/min as well as make up gas at a flow of 30 mL/min. The oven temperature was programmed as follows: 90°C during 1 min, 30°C/min to 180°C and 4°C/min to 270°C with a final hold for 20 min. Quantitation was carried out by means of external standard method. GC-MSD was performed with a Hewlett-Packard 5890 series II which was equipped with an HP 7673 autosampler and an MSD 5971 mass selective detector. Splitless injections of 2 µL were performed into a fused silica Ultra 2 capillary column coated with cross linked 5% phenyl methyl silicone with a length of 25 m x 0.25 mm ID and a film thickness of 0.33 µ m. Helium was applied as carrier gas at a flow of 0.7 mL/min. The oven temperature was programmed as follows: 90°C during 1 min, at 10°C/min to 140°C at 5°C/min to 270°C with a final hold for 10 min.

Lipid determinations were performed in samples containing 3-5 specimens by the colorimetric method of Zöllner and Kersch (1962). Values of lipid content of 1.5 % were used for bioconcentration and depuration data conversion of wet weight to lipid weight based concentration (pesticide concentrations in tissues are expressed as µg of pesticide per g of lipids in the sample).

Uptake and depuration rate constants of chlorpyrifos were calculated from the data obtained in the bioconcentration tests using a method based on the first order kinetic modeling (Spacie and Hamelink 1982; 1985). The following equation describes a first order kinetic,

$$\frac{dC_{org}}{dt} = k_1 \cdot C_w(t) - k_2 \cdot C_{org} \qquad (1)$$

where C_{org} (µg/g) and C_w (mg/L) denote the concentrations of pesticide in the mussels and sea water at any time (t) after the start of the experiment, and k_1 (L/(Kg•day)) and k_2 (l/day) represent the toxicokinetic uptake and elimination rate constants, respectively.

When the pesticide concentration in water remains constant, as in the present case, and $C_{org}=0$ at t=0, the solution to equation (1) is:

$$C_{org} = \frac{k_1 \cdot C_w}{k_2} \left(1 - e^{-k_2 \cdot t}\right) \qquad (2)$$

The kinetic parameters were calculated by fitting equation (2) to the data using non-linear least-squares method (Ahsanullah and Williams 1989; *Serrano et al. in press*). Goodness of the fit was determinated by ANOVA and F-test ($\alpha<0.05$) (Zar 1974; Sokal and Rohlf 1979; Draper and Smith 1981). Differences among BCF values were assessed by means of T-test ($\alpha<0.05$) (Sokal and Rohlf 1979). Excel 5.0 and Statgraphics 7.0 were used to carry out the statistical analysis.

RESULTS AND DISCUSSION

The pesticide concentrations in the sea water of the experimental aquaria were in reasonable agreement with the nominal concentrations, allowing us to consider the pesticide concentration in the sea water as constant for toxicokinetic modeling (Table 1).

Table 1. Actual pesticide concentration in experimental tanks during the bioconcentration tests.

	Pesticide concentration (mg/L)		
Day of experiment	Chlorpyrifos	Chlorfenvinphos	Methidathion
0	1.1	0.9	1.1
4	0.8	1	0.7
8	0.7	0.9	0.8
12	1.2	1.1	0.9
16	0.9	1	0.7
20	0.9	1.2	1.1
24	0.8	0.8	1.2
28	0.8	0.9	0.9
32	1.1	0.7	0.9

Sea water samplings were carried out before each renewal of the water solution. Days of renewal: 4, 8, 12, 16, 20, 24, 28, 32.

Analysis of physical and chemical water quality parameters for the experimental aquaria showed that conditions remained stable during the experiments, maintaining optimum levels (Table 2).

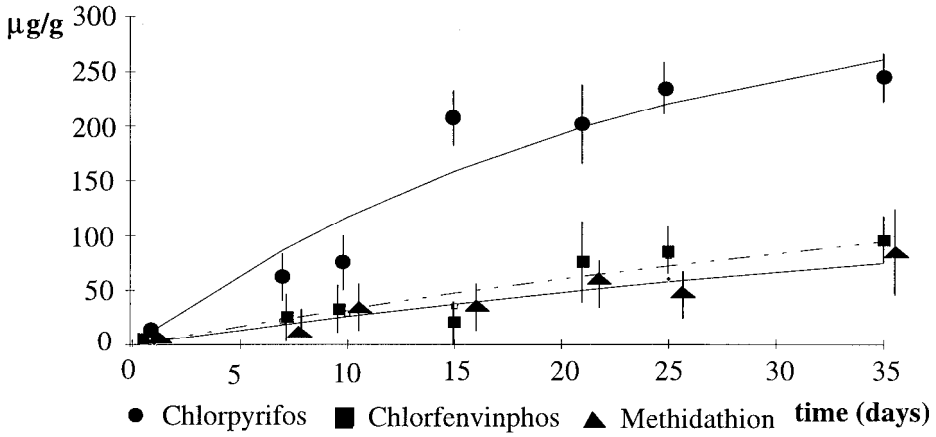

Figure 1. Lipid based pesticide concentration in mussel soft tissue along the bioconcentration tests. Vertical bars represent Standard Deviation.

Table 2. Physicochemical characteristics of sea water used in the experiments (mean ± std, n=3)

	Chlorpyrifos	Chlorfenvinphos	Methidathion
Temperature (°C)	18.3±0.3	18.3±0.3	18.5±0.2
Oxygen (mg/L)[a]	7.9±0.4	7.9±0.5	7.8±0.4
pH	8.4±0.2	8.3±0.2	8.3±0.2
Salinity (g/L)	38±1	38±1	38±1
NH_4^+ (mg/L)	nd	nd	nd

[a]8.4 mg/L= 100% water saturation, nd= not detected

Figure 1 shows the lipid based concentrations of pesticides measured during the bioconcentration experiments and the fit of the first order kinetic function (equation 2). As can be seen, chlorpyrifos is accumulated to higher concentrations than methidathion and chlorfenvinphos. The lipid based BCF values calculated from these functions (α<0.05) are shown in Table 3, together with the range of log Kow determined by different methods (Noble, 1993) and the water solubility of each pesticide studied. A clear relationship between the concentration of pesticide in the tissues and their physicochemical characteristic was found. The calculated BCF values are higher when the log Kow increases and the water solubility of pesticides decreases, as it could be expected (Miyamoto et al. 1990). The BCF value obtained for chlorpyrifos in *Mytilus galloprovincialis* is in significant agreement (α<0.05) with the value calculated by *Serrano et al* (*in press*) (385 ± 166 L/Kg).

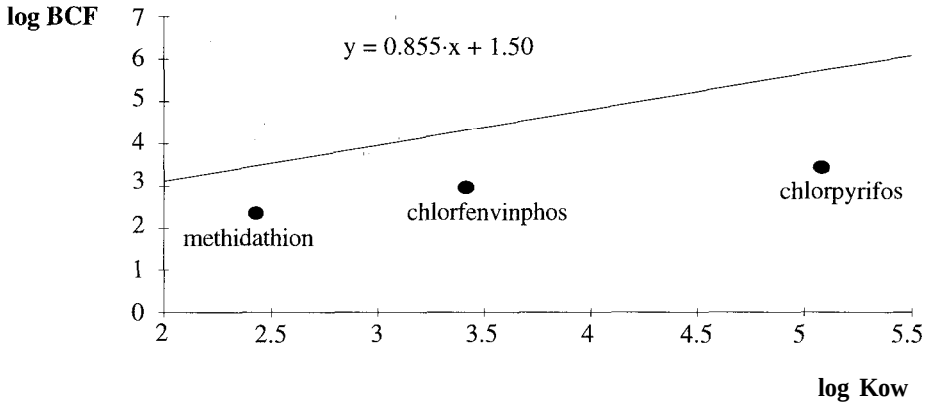

Figure 2. Graphic representation of log BCF versus log K_{ow}. Predicted equation by Connell (1988) for molluscs (line) and experimental results (solid circles) for methidathion, chlorfenvinphos and chlorpyrifos

Figure 2 shows the calculated log BCF value and the theoretical equation for molluscs proposed for *Mytillus edulis* by Connell (1988) (log BCF = 0.855 log K_{ow} + b (reflection of fat content)). As seen in Fig 2, our calculated BCF values are lower than expected from Connell's equation. Theoretically, the bioconcentration of xenobiotics with molecular weight between 100-600 take place by passive diffusion. Afterwards, the substances reach the circulatory fluids and are stored in fatty tissues and organs. Therefore, the values of log K_{ow} and log BCF should be similar. In our case, a positive non-linear correlation between BCF and K_{ow} was found in accordance with Connell's equation. Nevertheless, log BCF values are lower than log K_{ow} (Table 3). Probably, the metabolic biotransformations that the parent organophosphorus pesticides suffer in the aquatic organisms, as well as physiological and environmental factors could provoke these differences. The presence of metabolites of chlorpyrifos was investigated in mussels exposed over 35 days to 1 mg/L of chlorpyrifos. In these samples the metabolite 3,5,6-trichloro-2-pyridinol, together with the parent pesticide, was confirmed by mass selective detection (*Serrano et al. in press*).

Metabolites from organophosphorus pesticides were not detected in short term exposures (4 days) at exposure concentrations of pesticides ranging from 1 to 56 mg/L. In long term exposures (35 days) at 1 mg/L of chlorpyrifos, neither toxic effects nor chlorpyrifos-oxon were detected (Serrano et al. 1996 a, 1996 b). Nevertheless, the metabolic derivative 3,5,6-trichloro-2-pyridinol was detected at concentration of 0.10 ± 0.01 µg/g. In this case, the bioactive oxon derivative from chlorpyrifos would be deactivated to the inactive diester and finally hydrolyzed to 3,5,6-trichloro-2-pyridinol (Hutson and Roberts 1985).

Table 3. Calculated BCF values and physicochemical properties of the pesticides studied

	BCF (L/Kg)	log BCF	Water Solubility (mg/L)	log K_{ow}
Chlorpyrifos	400 ± 119	2.6	2 (25°C)	4.96-5.20
Chlorfenvinphos	255 ± 78	2.4	145 (23°C)	3.10-3.82
Methidathion	193 ± 170	2.3	250 (20°C)	2.42

It can be concluded that the bioaccumulation ability of chlorpyrifos, chlorfenvinphos and methidathion in living tissues represents a potential environmental risk to marine organisms and humans. Theoretical predictions of behaviour of this kind of compounds in the biota are imprecise, they have to take biotransformation, physiological and environmental factors into account. More studies on the environmental behaviour of organophosphorus pesticides must be carried out in order to know and foresee the actual risk presented by these compounds in marine environments.

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