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Acute Toxicity of Organophosphorus Insecticides to Marine Invertebrates

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Organophosphorus insecticides (OPs) are widely used in agriculture practice against a large variety of insect pests. Usually, they are known as nonpersistent agrochemicals and, in comparison with organochlorine insecticides, they have lower residual effects on terrestrial and aquatic ecosystems. The degradation process of organophosphorus insecticides is generally faster in respect to other organochlorines or carbamate agrochemicals (Sattar 1990). However, the phosphorodithioate category includes insecticides with different physic-chemical characteristics. For example, diazinon and phorate arethe most water soluble and can be easily mobilized in aquatic environments as demonstrated by GUS Index high values (Trevisan et al. 1991); on the contrary parathion and malathion are more persistent and they show a greater affinity to soil and sediments.

The environmental fate of the OPs may be different according to both the agricultural practice and the physic-chemical characteristics of the various compounds. Unfortunately, it is not possible to estimate the environmental risk associated to the agricultural practices on the basis of application dose because the data on the total amount of insecticides sold every year provided by ISTAT (National Institute of Statistics in Italy) (ISTAT 1990) are not detailed for each compounds. ISTAT data show a constant organophosphorous insecticide usage in the period 1984-1988. The mean Italian consumption of these agrochemical is about 20 tons per year as commercial formulations; they represent the highest amuont pesticides sold in Italy.

The environmental risk associated with OPs usage is correlated to their extremely high toxicity (Smart 1987). The biological action of OPs in mammals and arthropods consists in the attack to the neural transmission system which causes the interference with the nervous function of the target organisms. The acetylcholinesterase enzyme is necessary for impulse transmission through the nervous system and the OPs block the acetylcholinesterase enzyme by phosphorylation reactions. The effects of different phosphoric acids on acetylcholinesterase enzyme varies according to the OP structures. The chemical structures of esters influence both their intrinsic reactivity and the stereochemical configuration available for acetylcholinesterase enzyme phosphorylation.

As far as the freshwater environment, OPs toxicity to freshwater organisms is well known (Palawski et al. 1983; Strickman 1985; Zinki et al. 1987; Naqvi et al. 1988;); on the contrary relatively few studies have been reported for marine organisms (Sreenivasula et al. 1985; Suter et al. 1988). The lack of information in this toxicological area is particularly serious as the occurrence of OP residues in the marine environment is not so rare. The contamination of OPs usually regards river mouths, estuaries and lagoons. The largest input of OPs in the marine environment comes from transport of these compounds to the sea via surface waters (Baldi et al. 1986; Galassi S. 1991). A second source of pollution may arise from spraying of crops with OP pesticides on fields nearby the sea; as third source even industrial effluents with OP residues discharged directly into shallow waters through pipelines (Kjolholt 1985) has to be considered. Residual concentrations of the more persistant OPs (i.e. parathion and chlorpyrifos) were also

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found in marine sediments of lagoon areas (Readman et al. 1992): the concentrations of these compounds reached even high level of contamination (up to 8 ng/g).
The goal of the present investigation was to improve the knowledge on toxic effects of OPs by two tests with the marine species: *Artemia sp* and *Brachionus plicatis*.
The acute toxicity of the eleven OP agrochemical selected on the basis of major consumption in Italy, was carried out with Toxkits. Toxkits are cyst-based toxicity microbiotests developed by the Laboratory for Biological Research in Aquatic Pollution at the University of Ghent in Belgium (Vanhaecke et al. 1981; Snell et al 1989). The most attractive features of the microbiotests consists undoubtedly in the free maintenance of the cultures. The breeding of test organisms in sufficient numbers and in good physiological condition is considered as the major handicap for large scale toxicity testing and it is responsible for high costs of all conventional standard tests.
The use of Toxkits as screening bioassay is particularly suitable because of the easy transport and the availability of the kits, the cost-efficiency, the minimal training and equipment requirement.
An important advantage evidentiated by Toxkits is the chance of obtaining reproducible results. The testing procedure prescribes in fact to verify the cyst sensitivity with a reference substance, usually potassium dichromate, and to use same age organisms, only in similar physiological conditions at the beginning of the bioassay; these facts substantially contribute to a good repeatability of the cyst-based toxicity tests.

MATERIALS AND METHODS

Eleven OPs, azinphos-ethyl, azinphos-methyl, chlorpyrifos, diazinon, dimethoate, fonofos, malathion, parathion-ethyl, parathion-methyl, phorate, omethoate organophosphorus insecticides were investigated in the present study. The OPs solutions used in the toxicity tests were prepared by dissolving in Milli Q water the appropriate amount of high purity standard (min. 95%) by Chem Service, West Chester, PA or Dr Ehrenstorfer GmbH, Augburg, D. Insecticide stock solutions were prepared by dissolving proper amount (always at concentration lower than the chemical water solubility) in Milli Q water. The insecticide concentrations of the stock solution were confirmed by NPD-GC analysis according to Baraldi et al. (1991). All the solutions were prepared immediately before each experiment and were stored at 4°C in the dark for no longer than a week.

Static acute toxicity tests were carried out with the Toxkits for marine waters; Toxkits, supplied by Creasel Ltd, Belgium, contain all the materials for the toxicity assays (6 plastic vials containing reference cysts of the organisms: connected salt solutions to make up one liter of standard seawater; 6 multiwell test plates; 6 parafilm strips; 6 polyethylene micropipettes; the Standard Operational Procedure Manual; 1 plastic vial containing 1 gram potassium dichromate ($K_2Cr_2O_7$) used as reference toxicant). 24h EC50 bioassays were performed in multiwell testplates using neonates obtained by hatching cysts of the estuarine rotifer *Brachionus plicatilis* (Rotoxkit M) and of the brine shrimp *Artemia sp.* (Artoxkit M kit).
Rotifer cyst hatching should start 28-30 hours before the toxicity test beginng and it has to be carried out in standard seawater (NaCl = 26.4 gr/l, KC1 = 0.84 gr/l, $CaCl_2.H_2O$ = 1.67 gr/l, $MgCl.6H_2O$ = 4,6 gr/l, $MgSO_4.7H_2O$ = 5.58 gr/l, $NaHCO_3$ = 0.17 gr/l, H_3BO_3 = 0.03 gr/l) (Snell and Persoone 1989). For each test the contents of one vial (100 mg of cysts per vial) was emptied into the hatching trough of the test plate and 2.5 ml of standard seawater were added, Each test plate was incubated at 25°C for 28 hours under direct lighting (3000 - 4000 lux). In a test plate there are 6 rows of 6 wells and 1 rinsing trough for each one, therefore a single plate can hold the control (first row) and 5 concentrations for each toxicant. After 28 hours of incubation, using a micropipette, approximately 50 rotifers were transferred from the hatching trough into the six rinsing troughs of the test plate. the first one tilled with 0.7 ml of standard seawater (control), and the other five filled with 0.7 ml of different toxicant dilutions.
Then about 5 neonates of *Brachionus plicatilis* were transferred from the rinsing troughs to each of the 6 wells of the same row (30 animals per concentration). The intermediate transfer of the rotifers from the hatching trough to the wells, via a rinsing trough, minimizes the dilution of the test solution in the actual test wells,

Artemia cyst hatching should begin 48 hours before the toxicity test (Persoone 1991). For each test, the contents of one vial with *Artemia* cysts (100 mg of cysts in each vial) was poured into a Petri dish (∅ 5 cm) with 12 ml of standard seawater.

Table 1. *Artemia* sp. acute toxicity (mg/l) for eleven organophosphorus insecticides.

	24h EC50					24h EC10			
Test chemicals	1st	2nd	3rd	Mean	V.C.	1st	2nd	3rd	Mean
Diazinon	20	17	n.d.	19	8.6	7.5	4.7	n.d.	6.1
Parathion - methyl	18	22	21	20	8.8	7.4	11	11	9.6
Parathion	-	-	-	> 25	-	8.5	4.7	n.d.	6.6
Phorate	-	-	-	> 50	-	22.5	n.d.	n.d.	22.5
Malathion	-	-	-	>140	-	-	-	-	>140
Azinphos - ethyl	3.6	3.1	3.2	3.3	8	1.1	1.5	n.d.	1.3
Azinphos - methyl	25	22	22	23	7.9	7	7.1	7	7
Dimethoate	305	308	297	303	1.8	165	167	158	163
Chlorpyrifos	1.9	2	2	2	2.9	0.8	0.8	0.9	0.8
Omethoate	250	248	265	254	3.6	80	88	88	85
Fonofos	9.1	9.1	9.5	9.2	2.5	4.9	4.6	6.9	5.5

n.d. = not determined

Each Petri dish was incubated at 25°C, under a light source (3000 - 4000 lux) for one hour and afterwards in darkness for 24 hours. 24 hours after the incubation step, the hatched larvae were transferred to fresh medium in a second Petri dish; 24 hours later a further incubation in darkness at 25°C the organisms were ready for the test beginning.

A 24-well plate was used for each acute toxicity test with *Artemia sp.* Every plate has 6 columns across and 4 rows down, and the organism transfer to the multiwell plate was accomplished in two steps: a) transfer of approximately 50 animals from the Petri dish into each of the six rinsing wells (first row); b) transfer of 10 organisms from the rinsing wells to each of the three actual test wells in the same column. Therefore a single plate can include the control (first column) and five concentration of each toxicant dilutions for a total of 30 animals per concentration. As described with rotifers the intermediate transfer of the brine shrimps through rinsing wells caused the reduction of the dilution for toxicant solutions in the test wells.

Once both *Brachionus plicatilis* and *Artemia sp.* organisms were distributed, each plate was placed in a 25°C incubator in the dark. Because of the short length of the tests (24 hr) the organisms were not fed and the medium was not renewed during the experiments.

Table 2. *Brachionus plicatilis* acute toxicity (mg/l) for eleven organophosphorus insecticides

Test chemicals	24h EC50 1st	2nd	3rd	Mean	V.C.	24h EC10 1st	2nd	3rd	Mean
Diazinon	30	27	27	28	6.2	21	19	19	20
Parathion - methyl	-	-	-	> 67	-	26	14	n.d.	20
Parathion	-	-	-	> 25	-	2.8	n.d.	n.d.	2.8
Phorate	-	-	-	> 50	-	24	n.d.	n.d.	24
Malathion	75	79	67	74	8.2	31	21	15	22
Azinphos - ethyl	-	-	-	> 5.2	-	2.5	2.5	n.d.	2.5
Azinphos - methyl	89	83	n.d.	85	5.5	20	28	n.d.	24
Dimethoate	218	251	264	244	9.8	39	48	52	46
Chlorpyrifos	1.4	1.9	1.7	1.7	15	0.4	0.5	0.5	0.5
Omethoate	301	292	291	295	1.8	157	108	101	122
Fonofos	8.8	8.9	8.7	8.8	1.1	2.8	3.2	3.8	3.3

n.d. = not determined

The number of living and dead organisms was recorded after 24 hours, Animals were considered dead if no movement was observed in 5 seconds. The EC50 and 95% confidence range were calculated by Probit Analysis Software (EPA 1981). The following procedure was followed for the detection of the EC50 values: a ranging test was performed with 1: 10 dilutions of the OPs stock solutions and subsequently a final test was undertaken according to the toxicity results obtained with five different toxicant concentrations plus a control. Each chemical was tested in two/three different experiments and potassium dichromate and sodium lauryl sulfate were used as positive control for both the Toxkits. Control survival was always 100% in all the experiments,

RESULTS AND DISCUSSION

Potassium dichromate and sodium lauryl sulfate (SLS) were used as positive controls for both the two marine invertebrates. The obtained results summarized as reported in Table 3.
The present results are in a good agreement with the data published by Persoone (Vanhaecke and Persoone 1984) during a ring test experiment with *Artemia sp.* and with the data supplied by Creasel Ltd for *Brachionus plicatilis.*

The 24h EC50 and EC10 values of both the marine invertebrates considered for the insecticides tested are reported in Tables 1 and 2. Comparisons of the EC50 values show that chlorpyrifos was the most toxic organophosphorus insecticide to both *Artemia sp.* and *Brachionus plicatilis.*

Table 3. *Artemia pp.* and *B. plicatilis* acute toxicity (mg/l) for potassium dichromate and SLS

	Potassium dichromate					SLS				
	I	II	III	Mean	V.C.	I	II	III	Mean	V.C.
Artemia salina	30	29.8	n.d.	29.9	0.4	30	24.6	n.d.	27.3	14
B. plicatilis	216	207	n.d.	211	3.1	15.3	16.5	16.4	15.9	3.8

In order of toxicity decreasing in both the marine organisms tested azinphos-ethyl was the second compound followed by fonofos while diazinon, parathion-methyl, azinphos-methyl showed a lower toxic response. The azinphos-ethyl tested on *Brachionus* let the only calculation of EC10 value; however the EC10 values were in a good agreement with EC50 values and allowed to assume an analogus toxicity classification of the different tested OPs. In the present investigation dimethoate and omethoate were the less toxic organophosphorus insecticides to the both marine organisms while it was not allowed to determine the EC50 values of parathion, phorate and malathion to *Artemia* and those of parathion-methyl, parathion and phorate to *Brachionus* because of low poor solubility in water. Comparing the toxicological responses of the two
species, it was evident that the sensitivity of the marine organisms was very similiar in testing diazinon, dimethoate, chlorpyrifos, omethoate and fonofos while *Artemia* resulted three times more sensitive than *Brachionus* to parathion-ethyl and azinphos-methyl and two times to azinphos-ethyl. On the contrary *Brachionus* was two times more sensitive than *Artemia* to malathion. The variation coefficients (V.C.) reported in Tables 1 and 2 are always lower than 10% with the only exception of chlorpyrifos tests with *B. plicatilis.* Therefore the results demontrated the good repeteability of Toxkit experiments. Besides the speed and ease of these toxicological analysis make the Toxkits an atractive alternative as screening procedure for organic micropollutants testing.

By comparing the results reached with the toxicological response of freshwater organims (Table 4). it is evident that *Brachionus p.* and *Artemia* sensitive was lower than those of the other aquatic species, i.e. *Daphnia magna* and *D. pulex, Gammarus pulex, G. lacustris, G. fasciatus, Simocephalus sp., Asellus aquaticus.*
The considered marine organims, *Artemia sp* and *B. plicatilis,* are euryhaline species, so they may have a greater osmoregulation capacity which contributes to a greater resistance to toxic effects of OPs. Besides the toxicity of a micropollutant depends on the medium composition. Salinity, hardness and pH of the medium can in fact greatly influence the results of these ecotoxicological tests. Generally the toxicants are less effective in medium with highest salinity (Inman and Lockwood 1977; Stephenson 1983).
Freshwater *Brachionus rubens* and *B. calyciflorus* demostrated a more similiar toxicological response in diazinon and parathion-methyl testing, while *B. rubens* and *calyciflorus* is more sensitive than the marine invertebrates to malathion and on the contrary *Artemia sp* and *B. plicatilis* to chlorpyrifos.

The *Artemia sp* and *B. plicatilis* 24h EC50 values for OPs resulted to vary from about 1 mg/l to more than 300 mg/l. The toxicological effect classification in *Artemia sp.* of the OPs can be summed up in the following order: chlorpyrifos > azinphos-methyl > fonofos > diazinon > parathion-methyl > azinphos-methyl > omethoate > dimethoate and in this one for *B. plicatilis:* chlorpyrifos > fonofos > diazinon > malathion > azinphos-methyl > dimethoate > omethoate.

The two marine organism results had the same sensitivity magnitude order to OPs - values similar to other freshwater rotifers - but they showed lower sensitivity than those of the most common used freshwater organisms *(Daphnia* and *Gammarus).*

Table 4. Freshwater and marine organisms acute toxicity (mg/l) for organophosphorus insecticides - a)Sanders ans Cope (1966) b) Sannders at al. (1969) c) Van Wijgaarden et al. (1993), d) Snell and Persoone (1989) e) Johnson and Finley (1980) f) Meier et al. (1976), g) Fernandez- Casalderry et al.(1992), h) Persoone (1996) i) Ferrando and Andreu-Moliner (1991).

Test chemicals	*Daphnia magna*			*Daphnia pulex*	*Gammarus pulex*	*Gammarus lacustris*
	24h LC50	48h LC50	96h LC50	48h LC50	48h LC50	96h LC50
Diazinon	-	-	0.002 (f)	0.9 (a)	-	200 (b)
Parathion - methyl	-	-	-	-	-	-
Parathion	0.002 (h)	0.00014 (e) 0.0008 (a)	-	0.6 (a)	-	0.6 (b)
Phorate	-	-	-	-	-	-
Malathion	0.001 (e)	0.0009 (a)	-	1.8 (a) - 2 (e)	-	1.8(b)
Azinphos - methyl	-	-	-	3 (a)	-	-
Chlorpyrifos	-	-	-	-	0.00008 (c)	-

	Gammarus fasciatus			*Simocephalus sp.*	*Brachionus rubens*	*B. calyciflorus*	*Asellus aquaticus*
	24h LC50	48h LC50	96h LC50	48h LC50	24h LC50	24h LC50	48h LC50
Diazinon	0.2 (e)	-	-	-	-	29.22 (g)	-
Parathion - methyl	-	-	-	-	-	29.19 (g)	-
Parathion	-	-	0.0038 (e)	0.0035 (a)	-	-	-
Phorate	-	-	-	-	-	-	-
Malathion	-	0.00076 (e)	-	0.003 (a)	35.3 (d)	33.72 (g)	-
Azinphos - methyl	-	-	-	-	-		-
Chlorpyrifos				0.0008 (c)	-	11.85 (i)	0.0043 (c)

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