DIMETHOATE 40% ORGANOSPHOSPHOROUS PESTICIDE TOXICITY IN *Prochilodus lineatus* (PROCHILODONTIDAE, CHARACIFORMES) EGGS AND LARVAE

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ABSTRACT

Toxicity tests using early life stages of fish are of great importance in assessing risks to growth, reproduction and survival in polluted environments and are important tools for good environmental monitoring. However, a small number of standard bioassays of this type have been developed in Brazil. Curimbatá (*Prochilodus lineatus*) is an abundant South American characid fish of great commercial interest. It was chosen for testing different concentrations of 40% dimethoate, an organophosphate insecticide and acetylcholinesterase enzyme (AChE) inhibitor used widely in Brazil. The 48-h LC_{50} for eggs is higher than 16.0 $\mu g.L^{-1}$, whereas for recently hatched larvae it was found to be significantly lower (11.81 $\mu g.L^{-1}$, ranging between 10.23 $\mu g.L^{-1}$ - 13.65 $\mu g.L^{-1}$) and also significantl y lower than that for eggs by a Student t-test for independent samples ($p = 0.03$). The 96-h LC_{50} for 3-day old larvae was 10.44 $\mu g.L^{-1}$ (8.03 $\mu g.L^{-1}$ - 13.57 $\mu g.L^{-1}$), similar to that of recently-hatched larvae ($p = 0.76$). Larval mobility was also found to be reduced by this insecticide.

Keywords: pesticide, toxicity, fishes, bioassay, larvae.

RESUMO

Toxicidade do acaricida organofosforado dimetoato 40% aos ovos e larvas de *Prochilodus lineatus* (Prochilodontidae, Characiformes)

Os testes de toxicidade com os primeiros estágios de vida de peixes são de elevada importância no que se refere ao comprometimento das fases de crescimento, reprodução e sobrevivência dos organismos em ambientes poluídos, constituindo uma ferramenta importante para um adequado monitoramento ambiental. Entretanto, um pequeno número de bioensaios desta natureza tem sido desenvolvido no Brasil. O curimbatá (*Prochilodus linetaus*) é um peixe da ordem Characidae de grande interesse comercial e muito abundante na América do Sul. A espécie foi utilizada para testar diferentes concentrações de dimetoato 40%, um pesticida organofosforado inibidor da enzima acetilcolinesterase (AChE) utilizado em grande escala no Brasil. A CL50 (48h) para ovos é superior a 16,0 $\mu g.L^{-1}$; entretanto para larvas recém-eclodidas foi significativamente mais baixa (11,81 $\mu g.L^{-1}$, variando de 10.23 a 13,65) de acordo com o teste-t para amostras independentes ($p = 0.03$). A CL50 para larvas com 3 dias de vida foi de 10,44 $\mu g.L^{-1}$ (8,03-13,57), apresentando-se similar ao resultado encontrado para larvas recém-eclodidas ($p = 0.76$). A mobilidade das larvas foi reduzida na presença deste pesticida.

Palavras-chave: pesticida, toxicidade, peixes, bioensaios, larva.

INTRODUCTION

Humans have long interfered in nature by extracting natural resources and discarding residues into the environment. This impact has been intensified since the Industrial Revolution with many chemicals now being released into aquatic and terrestrial ecosystems as well as the atmosphere (Ochiai, 1995).

Chemical pollutants enter aquatic environments as agricultural pesticides in run-off water, urban drainage, and precipitation. Punctual-sources of pollution, including industrial discharges, which can contain harmful residues and domestic wastewater discharges also damage aquatic environments. Streams and rivers are generally the collecting environment for these pollutants (Rand *et al*., 1995).

Toxic effects can be sensed immediately after exposure to toxicants or when followed by a lag. These effects are determined by the toxicological characteristics of the substance and the ability of the organisms to metabolize it.

Some toxic effects in aquatic biota are reversible, whereas some others are not, leading the organisms to mortality. In many cases, toxic effects are reversible only if the organisms can escape the toxicant and migrate to an uncontaminated environment (Rand *et al*., 1995).

Toxicity tests with embryos and larvae are valuable for assessing potential impacts on growth, reproduction, and survival of organisms in polluted environments and are important tools for good environmental monitoring (Zagatto, 1999; Kristensen, 1994). Mckim (1977, 1985) states that in most cases the results obtained in an early life stage test reflect the effects that would be obtained in a life cycle test.

Pesticides, while of undeniable value to agriculture, are also significant agents of environmental impact. Frequently, organophosphorous contamination has been found in environments, elements of the food chain and humans (Rodrigues, 1994). These products enter water bodies as a consequence of rain and leaching from the soil (Kumar & Ansari, 1984), or because it is carelessly discharged directly into aquatic ecosystems. Therefore fish and other aquatic organisms may show its effects. In general, organophosphorous compounds cause adverse effects (Annes, 1978a; Ansari & Kumar, 1987; Rodrigues & Fanta, 1998), such as the inhibition of cholinesterase activity (Coppage, 1972; DuBois *et al*., 1949) in the brain, liver and muscle of some freshwater teleosts (*Channa gachua* and *Cirrhina mrigala*) (Verma *et al*., 1979).

The first effects of contaminants usually occur at the cellular or subcellular level (Pickering, 1981; Stephan & Mount, 1973), and consequently, cytopathological and histopathological alterations (Rudolph & Boje, 1987; Fanta, 1991). After the cellular changes, sequences of pathologies are observed in the fish, compromising survival and the structure of the population, affecting the ecosystem (Fry, 1971). The biochemical effects of dimethoate reported are vacuolation of the liver and a high degree of cytoplasmatic granulation (Anees, 1978a); reduced erythrocyte counts and haemoglobin concentration indicating that the insecticide exerts an effect similar to the production of anaemia (Aness, 1978b).

In this study, the native South American fish species curimbatá (*Prochilodus lineatus*), was chosen as an assay organism for acute toxicity tests. The genus *Prochilodus* includes more than thirty different species widely distributed throughout South America (Lowe-McConnel, 1987). The curimbatá is one of the species which is most caught in the Paraná river, including the Itaipú reservoir (Cordiviola & Campana, 1993; Agostinho, Julio & Petrere, 1994).

Fishing for curimbatá at Cachoeira de Emas, Mogi Guaçu river Paraná basin, SP, occurs from August to March during the migratory season (Godoy, 1975). Despite substantial environmental impacts, this river remains one of the most productive for migratory fish species in Sao Paulo state. However, in October 2002 (during the spawning season), deliberate use of pesticides in cultivated areas near the Mogi Guaçu river resulted in a loss of 30 fish t. At first, this was attributed to a combination of low dissolved oxygen levels and low water flow. However, after the fish were examined, symptoms of pesticides poisoned mainly by dimethoate, in addition to effects of industrial effluents were detected (Fig. 1) (Espíndola & Brigante 2003).

Dimethoate has been used worldwide, and many investigations on its toxicity to aquatic and terrestrial organisms have been carried out, in

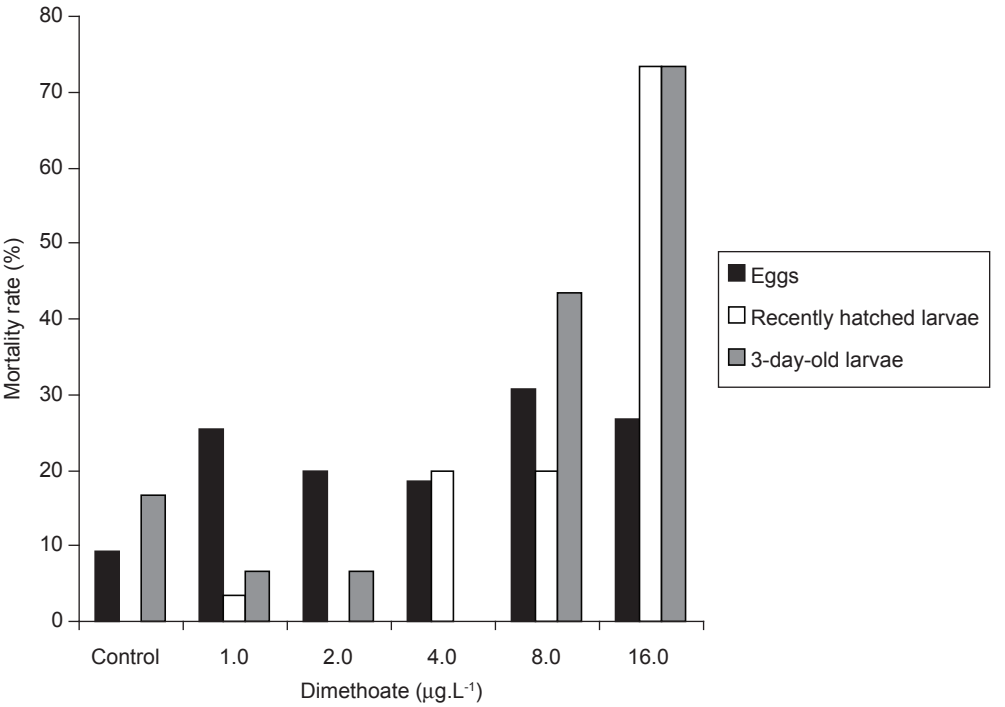

Fig. 1 — Comparison of acute toxicity of 40% dimethoate on recently hatched eggs and 3-day-old larvae of *P. lineatus*.

addition to studies on occupational risks it presents to humans. In Canada, nearly 100,000 kg of this substance is used every year (Environment Canada, 1987), and in the 1990s fish mortality in many rivers on Prince Edward Island was an issue of concern. From 1994 to 2000, sixteen events of fish mortality were registered and attributed to pesticide contamination, with 12 incidents occurring from 1999 to 2000 (DFO, 2000)

The aim of this study was to test the acute toxicity of the organophosphorous pesticide 40% dimethoate ($C_5H_{12}NO_3PS_2$) in early life stages of *Prochilodus lineatus* and compare it to the sensitivity of other fish species.

MATERIAL AND METHODS

Acute toxicity tests were assayed with eggs of *P. lineatus*, recently hatched larvae, and 3-day old larvae. The experiments were carried out at the Aquaculture Research and Training Center (CEPTA-IBAMA) in Pirassununga, São Paulo, Brazil. (21° 56' S e 47° 22' W).

Sexually mature *P. lineatus* were caught in the Mogi-Guaçu river, Pirassununga and transported to adjacent hatchery facilities at CEPTA. In the laboratory, the specimen received hormonal doses to induce spawn. Fertilized eggs, collected from the spawning tank, were transferred to a 60 l funnel incubator with a continuous water flow of 29 °C, pH 6.5, and 5.67 $\mu g.L^{-1}$ dissolved oxygen (DO). The toxicity tests were initiated at the blastula stage, five hours after fertilization.

All the bioassays (eggs, recently hatched larvae, and 3-day-old larvae) were carried out in 1 L glass beakers lined with a plastic bag to avoid cross-contamination with toxic residues. The assay vessels were aerated, but no water was changed during the tests. Concentrations of 0.0, 1.0, 2.0, 4.0, 6.0, 8.0 and 16.0 $\mu g.L^{-1}$ of 40% dimethoate were tested in duplicate in the assays, using water from the incubator to prepare the solutions. Tests were carried out using controlled photoperiod (12:12 h light/dark) and constant aeration, temperature, dissolved oxygen and pH. The mortality was measured at the end of the tests.

Twenty-five *P. lineatus* eggs were used in each experiment. Dead eggs (with opaque and motionless embryos) were counted every hour until they hatched (8 h 20 min from the beginning of the experiment), at which point the tests were terminated.

Ten recently hatched and ten 3-day-old larvae were used in each experiment. Considering that the larvae were endogenously nourished by their own yolk for approximately 10 days after hatching, no food was offered. For the recently hatched larvae, tests were conducted until the opening of the mouth, a period of 48 h, whereas tests with 3-day old larvae were carried out for 96 h.

The LC_{50} values were calculated with Spearman-Karber Software (Hamilton *et al*., 1977) using a statistical analysis procedure developed by the Environmental Sanitation and Technology Agency of the state of São Paulo (CETESB, 1990). Mortality levels were compared with Student t-tests for independent samples using the BioStat 2.0 Software.

RESULTS

Mortality controls of the eggs, observed at regular intervals of one hour was low, while for the 40% dimethoate solutions the values were high, especially at the end of the tests (Table 1). However, with an increase of pesticide concentration no trend concerning mortality was observed. A low mortality rate (25%) was found in the highest organophosphorous concentration (Table 2) avoiding the calculation of LC_{50}. However, this is likely higher than16.0 $\mu g.L^{-1}$. Hatching began 8 h 20 min after the beginning of the tests for all concentrations.

The 48-h LC_{50} for recently hatched larvae was calculated to be 11.81 $\mu g.L^{-1}$ (10.23 $\mu g.L^{-1}$-13.65 $\mu g.L^{-1}$), whereas the 96-h LC_{50} for 3-day old larvae was found to be 10.44 μgL^{-1} (8.03 $\mu g.L^{-1}$-3.57 $\mu g.L^{-1}$). The highest mortality rate for recently hatched and 3-day-old larvae (73% and 43.33%, respectively) occurred in the 16.0 μgL^{-1} and 8.0 $\mu g.L^{-1}$ concentrations (Tables 3 and 4, respe-

TABLE 1
Mortality per hour of *P. lineatus* eggs exposed to dimethoate.

40% Dimethoate Concentrations ($\mu g.L^{-1}$)	Dead Eggs				
	t_i = 0 h	t_i =3 h 35 min	t_i = 5 h 15 min	t_i = 6 h 15 min	t_f = 8 h 20 min
Control	0	3	1	1	2
1.0	0	3	1	1	15
2.0	0	0	2	3	12
4.0	0	1	6	4	3
8.0	0	3	1	2	17
16.0	0	3	2	3	11

TABLE 2
Dimethoate toxicity tests for *P. lineatus* eggs.

Dimethoate Concentrations ($\mu g.L^{-1}$)	Eggs		Mortality rate (%)	Final parameters		
	t_i = 0 h	t_f = 8 h 20 min (dead eggs)		DO ($\mu g.L^{-1}$)	T (°C)	pH
Control	75	7	9	6.00	26.37	7.0
1.0	75	19	25	6.15	26.07	7.0
2.0	75	15	20	6.12	26.13	7.0
4.0	75	14	18.66	5.83	26.17	7.0
8.0	75	23	30	5.93	26.23	7.0
16.0	75	19	25	5.91	26.50	7.0

TABLE 3
Mortality of recently hatched larvae of *P. lineatus* exposed to 40% dimethoate.

Dimethoate 40% Concentration ($\mu g.L^{-1}$)	Recently hatched larvae		Mortality rate (%)	Final water quality		
	t_i = 0 h	t_f = 48 h (mortality)		DO ($\mu g.L^{-1}$)	T (°C)	pH
Control	30	0	0	5.84	27.97	7.0
1.0	30	1	3.33	5.82	27.97	7.0
2.0	30	0	0	5.84	27.93	7.0
4.0	30	6	20.00	5.77	28.13	7.0
8.0	30	6	20.00	5.70	28.23	7.0
16.0	30	22	73.33	5.58	28.30	7.0

48-h LC_{50} = 11.81 $\mu g.L^{-1}$ (10.23-13.65).

TABLE 4
Dimethoate toxicity for 3-day-old larvae of *P. lineatus*.

Dimethoate Concentrations ($\mu g.L^{-1}$)	3-day-old larvae		Mortality rate (%)	Final water quality		
	t_i = 0 h	t_f = 96 h (mortality)		DO ($\mu g.L^{-1}$)	T (°C)	pH
Control	30	5	16.66	5.77	28.10	7.0
1.0	30	2	6.66	5.71	28.10	7.0
2.0	30	2	6.66	5.70	28.00	7.0
4.0	30	0	0	5.69	28.00	7.0
6.0	30	10	33.33	5.66	27.90	7.0
8.0	30	13	43.33	5.73	27.97	7.0

96-h LC_{50} = 10.44 $\mu g.L^{-1}$ (8.03-13.57).

ctively). These results are not statistically different (p = 0.76).

Eggs from *P. lineatus* were more resistant to 40% dimethoate than were the larvae. However, this difference was significant only when compared to recently hatched larvae (p = 0.03). For 3-day old larvae the difference was not significant (p = 0.059). Larval mortality was observed to increase with higher concentrations of 40% dimethoate, while egg mortality remained relatively constant between 20% and 30% in all test concentrations (Fig. 1).

In addition to acute toxicity, we observed that the mobility of larvae exposed to dimethoate 40% solutions was lower compared to the control.

Recently hatched larvae were only capable of vertical movements, while 3-day old larvae were also capable of horizontal movements. With higher concentrations of 40% dimetoate, the movements of recently hatched larvae and 3-day old larvae were slower than the control.

DISCUSSION

Some LC_{50} values for organophosphate compounds in freshwater fish are reported in the literature (Menzie, 1969; Verma *et al.*, 1979; Verma *et al*., 1982; Karim *et al*., 1985; Rodrigues, 1994). Exemplifying, 96-h LC_{50} value of 4.57 $\mu g.L^{-1}$ was found for *Saccobrachus fossilis* exposed to dimethoate (Verma *et al*., 1979). A dimethoate compound showed a 48-h LC_{50} value of 0.14 $\mu g.L^{-1}$ for *Pteronarcys california* (Menzie, 1969). The dimethoate Rogor 40 presents LC_{50} values of 58 $\mu g.L^{-1}$ for *Salmo gairdnerii* and is classified as an intermediary toxicity chemical (Verma *et al*., 1982). However, studies carried out in Sudan (Africa) concluded that concentrations of dimethoate lower than 80 $\mu g.L^{-1}$ cause neither mortality nor toxicity to *Oreochromis niloticus* (tilapia) and *Gambusia affinis* (mosquitofish) (Karim *et al*., 1985). Therefore, the range of

organophosphorous toxicity is very large among fish, and this fact depends on the nature of the pesticide, different formulations, species of fish, physiological and biological conditions of individuals, like: size, age and sex of individual organism and environmental factors (Rand & Petrocelli, 1985).

In this study, values of 10.44 $\mu g.L^{-1}$ for 48-h LC_{50} and 11.81 $\mu g.L^{-1}$ for 96-h LC_{50} were found for recently hatched and 3-day old larvae, respectively. The difference between these results and those reported in the literature are likely due to different commercial formulations of dimethoate (*e.g.*, the use of different solvents), species-specific differences, or different sensitivity at the many life stages.

Toxicity tests in the first stages of a fish life cycle (embryos, larvae, and juveniles) are commonly used to predict environmental damages. Those life stages are more sensitive to environmental impacts than the subsequent ones (Kristensen, 1994).

Organophosphorous compounds inhibit the acetylcholinesterase enzyme (AChE), which hydrolyzes the neurotransmitter acetylcholine. Modeling the acute toxicity of these compounds based on simple structure prediction is rather difficult because of the high specific activity of AChE and the specificity of the metabolic processes of each species (De Bruijin & Hermens, 1993; Legierse *et al*., 1999).

A significant decrease of AChE activity in *Danio rerio* adult specimens occurred in chronic tests using 0.27 $\mu g.L^{-1}$ of Paration solution, within a Non Observed Effect Level (NOEL) of 0.12 $\mu g.L^{-1}$ (Jarvinen *et al*., 1983).

Roex *et al*., (2002) testing the toxicity of Paration in the first life stages of *Danio rerio* found a LC_{50} of 1.9 $\mu g.L^{-1}$ and a NOEL of 0.0043 $\mu g.L^{-1}$. After 250 days of exposure to a 0.9 $\mu g.L^{-1}$ solution, the AChE activity in *D. rerio* adult specimen was reduced and the NOEL found was 0.2 $\mu g.L^{-1}$. Roex *et al*., (2002) found that *D. rerio* adults are 2 orders of magnitude more susceptible to AChE activity inhibition by organophosphorous compounds than juveniles. Thus, organisms within the same species have different sensitivities to AChE inhibitor substances.

In the present study distinct sensitivities to 40% dimethoate were observed within the early life phases of *P. lineatus*.

Characidae and Siluridae fish eggs are protected by a complex extra-cellular double-layered matrix (Bazzoli & Rizzo, 1990; Bazzoli, 1992). These two layers provide the embryos more protection against abnormal conditions in the environment than those observed for larvae. This fact is probably responsible for reducing the *P. lineatus* susceptibility to 40% dimethoate.

Some studies report that hatching can be affected by exposure to chemicals (Strmac & Brauunbeck, 1999; Villalobos *et al*., 2000). The hatching of teleosts eggs is initiated with the release of the chorionase enzyme around the embryo. It is likely that some chemicals inhibit the release of this enzyme, thus enlarging the hatching span (von Westernhagen, 1988). Therefore, delay in hatching can be an adaptive response to avoid exposure to potentially noxious environments, since one of the roles of chorium is to protect the embryo (Blaxter, 1988).

Hatching of *P. lineatus* eggs was not altered in acute toxicity tests with low concentrations of 40% dimethoate. However, in higher concentrations it could occur.

Fish mobility is generally affected after exposure to pesticides, probably as a consequence of AChE inhibition (Battacharya, 1993; Richmonds & Dutta, 1992; Saglio *et al*., 1996; Bretaud *et al*., 2000). Studies on endossulfan, reported that this substance decreases the glycoside absorption in the intestine (Rao *et al*., 1988) as well as oxygen consumption (Naqvi & Vaishnavi, 1993). This should be also the likely cause of reduced mobility.

We observed a decrease in larvae mobility at high concentrations of 40% dimethoate. The most important consequences for such an effect are the increased vulnerability to predators, reduced ability to take food and decrease in energy available for growth (Little & Finger, 1990; Little *et al*., 1993; Carlson *et al*., 1998). Dikshith & Raizada, 1981; Dikshith, 1986) reported that the signs of toxicity of dimethoate in fish (*Channa punctatus*) included jumping, erratic movement, imbalance and death.

P. lineatus eggs are more resistant to 40% dimethoate than larvae. Larval mortality increased for higher 40% dimethoate concentrations, while

egg mortality was between 20% and 30% for all concentrations tested. The highest mortality rate (73%) occurred at the 16.0 $\mu g.L^{-1}$ concentration for both recently hatched and 3-day old larvae.

At higher concentrations of 40% dimethoate recently hatched larvae and 3-day old larvae motilities were lower than the control organisms. The hatching spans of *P. lineatus* eggs were not altered for the concentrations of 40% dimethoate tested.

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