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Toxicity Assays of a Compound with Insecticide Properties: Chitosan Diethyl Phosphate

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Increases in world population have required development of higher efficiencies in the agricultural production of foods, promoting the ever-increasing use of fertilizers and pesticides. It is assumed that use of agricultural pesticides is responsible for 60% of the total production of foodstuffs in the U.S. (Kenawy et al.1992). In Chile, annual losses due to insect pests in agriculture are considerable. This is becoming complicated by accusations because of the lack of control in pesticide application, principally in fruit orchards.

One estimate suggested that 90% of conventional agrochemicals utilized never achieve the object for which they are applied, and worse, may produce undesirable biological responses in the environment (Kenawy et al. 1992; Kenawy, 1998). New technology for the production of pesticides, termed "slow - release formulations", consist designing a pesticide from which the active ingredient becomes available with duration and velocity determined for optimal biological activity (Kenawy, 1998). In this sense, the synthesis of slow - release pesticide/fertilizer combinations are desired. They may help to reduce the side-effects which often accompany the conventional application of agrochemicals. Furthemore, they may enhance slow - release formulation technology and, in particular, improve weight effectiveness by using polymers which may be beneficial to soil and crop when degraded (Kenawy et al. 1992).

One formulation of this type is the organophosphate polymer chitosan diethyl phosphate (ChDP) (Cardenas et al. 2002). Chitosan [poly-B-D-(1-4)-glucosamine] of this formulation was obtained from chitin isolated of the red lobster shells (*Pleuroncodes monodon*) abundantly available as a marine fisheries waste product in Chile's Biobío Region (36°- 38° Lat S, 73°- 74° Long W). Among its other interesting properties, chitosan is a biodegradable (Yamamoto and Amaike, 1997) and biocompatible (Koide, 1998) natural polysaccharide.

It's partial degradation products (oligomers) are advantageous because they act by inhibiting the growth of several phytopathogens (Hirano and Nagao, 1989), and at the same time stimulating natural defenses of the plants (Notsu et al. 1994). Given

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it's characteristics, a low environmental toxicity was expected, as well as it's complete biodegradation in the soil (Yamamoto and Amaike 1997). A low acute toxicity is expected from the ChDP due to two main factors: this compound releases the active product in a controlled manner and an environmentally-friendly polymer carrier was used.

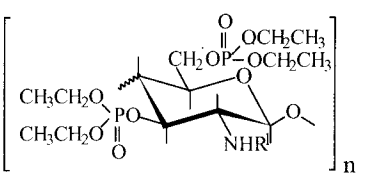
The objective of this study was to analyze the toxicity of ChDP in acute and chronic toxicity bioassays using extreme concentrations under the supposition that this substance, when used as an insecticide, could enter natural waters and have potentially deleterious effects on aquatic life. Methods also included determination of the effects of sublethal levels of ChDP on activities of the enzymes glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in the blood plasma of rainbow trout (*Oncorhyncus mykiss*), as well as effects of ChDP on the cellular microstructure in the liver of this species.

MATERIALS AND METHODS

In all assays ChDP, synthesized by Cardenas et al. (2002), was employed. The structure and chemical characterization of this product are presented in Table 1.

Table 1. Structure and some chemical properties of chitosan diethyl phosphate (after Cardenas et al. 2002).

Molecular weight *	19600 g/mol
Degree of substitution**	12%
Degree of acetylation**	2%



R^1 = -H, -$COCH_3$

* determined by gel permeation chromatography (GPC) and ** determined by proton nuclear magnetic resonance (^{1}H NMR).

Bacterial assays: Acute toxicity bioassays were performed using the MICROTOX® system, which uses the luminiscent bacteria *Photobacterium phosphoreum* to assess EC_{50} (Bulich 1979). The toxicity determinations were carried out on dilution samples in a range of 0 - 45 mg/L ChDP. The results were expressed as luminiscence inhibition (%), measured at 5 and 15 min (Bulich 1979; Ruiz et al. 1997).

Daphnia assay: A second type of acute toxicity assay was carried out using laboratory cultured *Daphnia pulex* as the test organism. They were obtained from Bioassay Laboratory of University of Concepción, Concepción, Chile. Bioassays were carried out under the same conditions as those in which these organisms had

been cultured. Tests were carried out on one-day-old individuals, using five randomly selected test organisms per run. Laboratory conditions included ambient light with 10 h light/14 h dark photoperiod, and ambient temperature of 20 ± 2°C. Each assay was carried out in flasks with 10 ml of lake water which had been filtered, UV-treated to eliminate bacteria, and aerated to an oxygen saturation of 80%. Determination of toxicity in the assays included five nominal concentrations of ChDP from 0.1 to 22.5 mg/L A stock solution of the toxicant was prepared at the time of use by dissolving 22.5 mg in one liter of water. Four replicate assays were run at each concentration, as well as with untreated controls. The assay was static, and of 48 h duration. The $LC_{50\text{-}48h}$ was calculated using the Spearman-Karber method (Hamilton et al. 1977).

Trout assays: A chronic toxicity assay was carried out using juvenile *O. mykiss* (length 10 ± 2 cm, weight 6 ± 1 g) obtained from culture center in the Biobío Region of Chile. Test fishes were transferred to the laboratory under constant aeration in 120 L plastic vessels at a density of 120 individual/m^3. They were acclimated for 2 weeks in 120 L aquaria with 10 individuals/aquarium at 10 ± 2 °C, photoperiod of 10 h light/ 14 h dark, with aeration and daily feeding. Maintenance and test water were filtered to 25 µm, aerated and treated with germicidal UV light. The water was changed completely every 72 hr. Survival of 100 % was observed during the acclimation period, and over the test period. The assay was of the static type, with renovation of the water every 72 hr, and conditions maintained the same as those experienced by the test *O. mykiss* during the acclimation period. *O. mykiss* were exposed to 20 mg/L ChDP for 13 days. Sixty specimens selected at random were distributed evenly into six aquaria. Four of which received the ChDP treatment.

Ten individuals were sampled at three-day intervals after initiation of exposure to the ChDP, for a period of 13 d. The individuals were exanguinated using heparinized syringes and livers were removed for cytological assessment.

The blood was centrifuged at 3000 x g for 10 min at 4°C to obtain the plasma, which was then assayed for GOT, GPT, and total protein content. The GOT and GPT activity was determined using a Biosystems Co. # COD 11567 " *in vitro*" diagnostic test kit for kinetic assay of enzyme activity, which was read at 340 nm at 20°C in a Shimadzu UV-1601 spectrophotometer, following the methodology of Frankel et al. (1970). For the standard assay of GOT and GPT activity the reaction mixture, in a total volume of 1 mL, containing (for GOT) a concentration of 264 mmol/L of L-aspartate, 198 mmol/L of 2-oxoglutarate, malate deshydrogenase > 460 U/L, lactate deshydrogenase > 660 U/L, 0.2 mmol/L of NADH and 88 mmol/L of tris buffer (pH 7.8), was used. For GPT activity the reaction mixture, in a total volume of 1 mL, containing a concentration of 550 mmol/L of L-alanina, 198 mmol/L of 2-oxoglutarate, lactate deshydrogenase > 1350 U/L, 2.6 mmol/L of NADH and 110 mmol/L of tris buffer (pH 7.3), was used. The results were expressed as total activity of GOT and GPT in µmol/min/L. Total protein content of the plasma was determined using the

methods of Gornall et al. (1949), using a bovine serum albumin (BSA) standard. The results were expressed as mg/ml. All data (expressed as mean ± SE) were estimated by one-way analysis (ANOVA) followed by a LSD Post Hoc test. The difference was considered significant if the corresponding *p* value was less than 0.05, respect to control treatment.

The livers were sectioned, and fixed with 2.5% glutaraldehyde for observation by electron microscopy ; the instrument employed was a JEOL Corp. Model JEM-1200 EX II transmission electron microscope. Samples from treated and untreated controls were observed to the level of cellular organelles.

RESULTS AND DISCUSSION

Results obtained using the MICROTOX ® assay are shown in Table 2. It was observed after 15 min that the effective concentration was approximately four times lower than after 5 min. When compared with results of comparable assays conducted by Ruiz et al. (1997), the MICROTOX assay EC_{50} values obtained using *Photobacterium* showed ChDP to be more toxic than Diazinon and Malathion, less toxic than Tetraclorvinphos, and similar in toxicity to Methylchlorpyriphos (see Table 2).

In the *Daphnia* test, the average value for LC_{50} of ChDP was 20.9 mg/L, with a range of 15.7 to 27.9 mg/ l. This value was three orders of magnitude less toxic than values given for conventional pesticides with *D. pulex*, for example, Chlorpyriphos, which has an $LC_{50\text{-}48h}$ of 0.20 µg/l (Van Winjngaarden et al. 1993).

Table 2: Parameter toxicology of MICROTOX® assay: EC_{50} (Effective concentration), LI % (luminescence inhibition) and TU (toxicity units) for ChDP.

	5 min			15 min		
Product	**EC_{50}**	**LI %**	**TU**	**EC_{50}**	**LI %**	**TU**
ChDP (mg/L)	13.80	11.01	9.10	4.00	3.30	30.30

Figure 1A shows that exposure of the rainbow trout juveniles to sublethal levels of ChDP which increased the activities of both GOT and GPT in the plasma. The basal level of GOT 77.79 ± 58.33 µmol/min/L increased by the ninth day of exposure to 1469.03 ± 1446.42 µmol/min/L, followed by a decrease to the 13th day to 704.88 ± 261.95 µmol/min/L. The GPT at a basal level of 123.87 ± 20.82 µmol/min/L showed a tendency to rise in activity to the ninth day of exposure to 571.77 ± 105.76 µmol/min/L (Fig.1A). The increase in GOT may be explained as the manifestation of an adaptive response of the fish to the unfavorable condition produced by the pesticide. This process may be related to the function played by these enzymes in processes which involve the utilization of amino acids for oxidation and/or gluconogenesis (Begum and Vijayaraghavan, 1996).

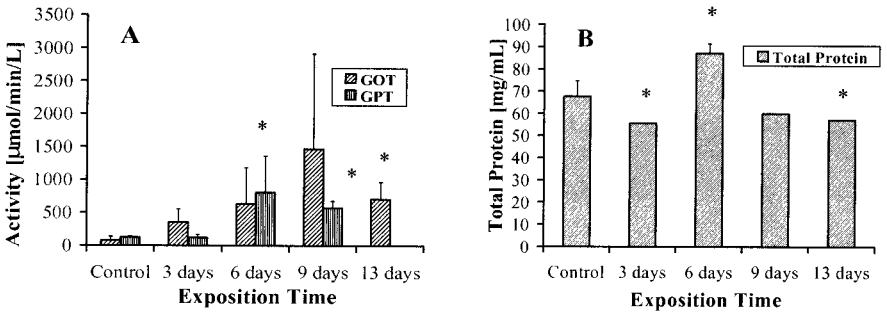


Figure 1. A. Average activity transaminases GOT and GPT of plasma (±sd) and B. Average protein total of plasma (±sd) of *Oncorhyncus mykiss* in controls and over different time periods of exposure to 20 mg/L ChDP (n=60). * $p<0.05$.

The high levels of these enzymes in the plasma of fishes exposed to 20 mg/L ChDP may also be attributed to transformations in the hepatocyte cellular structure, where disorganization and/or breakage of cellular organelles may have occurred.

Measurements of total plasma protein content in ChDP-exposed *O. mykiss* showed that this declined compared with the control at three days, and then recovered to a value above that of controls at six days (Fig. 1B). The decrease in plasma proteins at day 3 in the experimental group (Fig. 1B) may be attributable to injury produced by the insecticide thus producing a decline in albumin and/or other plasma proteins originating in the liver. This may be due to decline in the synthesis, and/or to a greater utilization, of these proteins in energy metabolism of the organism as part of it's metabolic reaction to the pesticide presence (Reyes 1994; Guilhermino et al. 1998). This would be due to the stimulation of proteolytic processes where amino acids were utilized as a metabolic energy source (Begum and Vijayaraghavan 1996). Similar effects on plasma protein concentration have been observed in *Anguilla anguilla* after 48 h of sublethal exposure to the insecticide Phenithrothion (Sancho et al. 1997).

The elevated total protein content of the plasma of *O. mykiss* observed after six days of exposure to the pesticide (Fig.1B) may indicate that the organisms assayed were able to generate a type of adaptive homeostatic response, or hypertrophy of the liver as a response to stress produced by the active group of the insecticide (Stebbing 1981). This response could be translated into an increase in hepatic synthesis of proteins in the acute phase, the function of which would be to protect the organism from the diverse injuries arising from the defensive response (Alberts et al. 1990). The tendency observed, where the proteins recovered to initial values from the proteinemia, may indicate that due to the slow-release of the active group from the insecticide that the fishes had begun to adapt to the presence of the pesticide. Long term studies are required to evaluate such a possible adaptation.

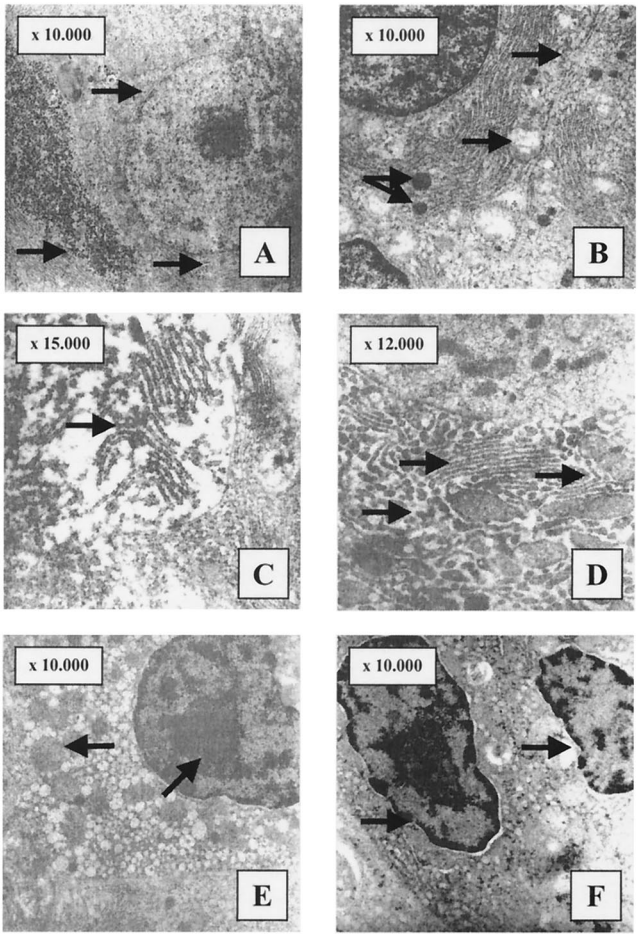


Figure 2. Electron micrographs of *O. mykiss* hepatocytes from fishes exposed to 20 mg/L of chitosan diethylphosphate (ChDP) for periods of days indicated. **A** = control, unexposed; **B** = 3 days; **C** and **D** = 6 days; **E** = 9 days; **F** = 13 days (n=60).

Livers of all *O. mykiss* exposed to ChDP showed hepatocytes with altered cellular structures (Fig 2A). By the third day of exposure a decrease was observed in the glycogen content; lysosomes appeared, and there was proliferation of smooth endoplasmic reticulum (Fig. 2B). It was also observed that the mitochondria underwent changes in turgidity, appearing swollen and disorganized. This was confirmed by observing the clear spaces, which these occupied in the cytoplasm (Fig. 2B). The development observed in the smooth endoplasmic reticulum was probably due to it's typical role in detoxification processes in the cell, where it produces enzymatic complexes responsible for elimination of toxic materials (Geneser 1994).

After six days of exposure, hepatocytes may be observed in the liver which show complete disorganization of the cytoplasm, cellular destruction, and necrosis (Fig. 2C). Also observed was the development of Golgi apparatus with appearance of dictyosomes dispersed in the cytoplasm and dilation of distal lateral portions of the cisterns with an electrodense material (Fig. 2D). Figure 2E shows hepatocytes after nine days exposure to ChDP, demonstrating a progressive increase in the number of alterations, *i.e.,* swollen mitochondria and nuclei chromatin with high level of condensation. At 13 days, hepatocytes were observed with pleomorphic nuclei (Fig. 2F). The breakage of mitochondria and cellular necrosis seen in some hepatocytes confirmed the high levels of GOT and GPT observed after six days of exposure. There was a positive correlation between plasma levels of GOT and GPT and the ultrastructural disorganization observed in the hepatocytes. These results indicate the importance of conducting pathological assessment of the liver and activity of some damage indicator enzymes as for example, EROD activity (Khan and Payne 2002).

From the results of this study it is concluded that ChPD, although of comparatively intermediate toxicity when compared to conventional pesticides in the MICROTOX assay, was of relatively low toxicity to *Daphnia.* Although alterations were observed in the rainbow trout at both the biochemical and cytological levels, the use of 20 mg/L should be considered a high test level, and it was remarkable that it caused no mortality during the assays. At the high concentration (20 mg/L) used, this pesticide indeed caused sublethal alterations in the rainbow trout, either due to action of the entire molecule, or to the (slow) release of active groups from the polymeric matrix. Although there appeared to be some biochemical adaptation to the effects of this pesticide, the hepatic alterations observed could be expected to lead to long term debilitation or death of the trout. Future research is required to determine the effects of this pesticide on aquatic species at lower concentrations after it's release and dilution in natural waters resulting from typical agricultural applications.

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