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Genotoxic and Teratogenic Effect of Pentachlorophenol, Pollutant Present in Continental Water Bodies in the South of Chile

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Pentachlorophenol (PCP) is used largely in agriculture and timber industries as a pesticide, bactericide, insecticide, herbicide and fungicide (Sato 1987; Quevedo et al. 1992). Its effectiveness as a biocide has been established, but there is great concern with regard to its distribution and persistency in the environment and to the possible danger to man, flora, and fauna exposed to this chemical agent and its related compounds (Wood et al. 1983; Uhl et al. 1986). PCP has been detected in river, well and lake water, in ponds, spring water, and in effluent water from sewage plants in different parts of the world. Numerous cases of intoxication of fish and mammals have been cited and it has been estimated that 85% of humans excrete PCP in their urine (Welsh et al. 1987). It has been reported that PCP occupied the second place among the pesticides most used in the USA and Canada with an annual production of 280 thousand tons (Cinelli 1978).

PCP is used in Chile as a fungicide, especially in the forestal region of the southern portion of the country where it is used as an export wood preservative. It has a wide range of toxicity for both aquatic and terrestrial organisms, included man. On the basis of information obtained at the Department of Chemistry of the University of Concepción, it can be assured that PCP can be detected in most river, brook, and creek waters of the Province of Concepción. On the other hand, several cases of intoxication and some deaths of forest workmen have been reported by hospitals of the Concepción area and by the Department of Toxicology of the University of Concepción.

The purpose of this paper is to study *in vivo* the genotoxic teratogenic effects of PCP in two different study models. We have used as a starting point the average annual concentration of this pollutant found in the lower part of the Bio Bio River.

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MATERIALS AND METHODS

In order to determine the genotoxic effect of PCP, the Micronucleus Test (MN) was carried out using premetamorphic larvae of the anuran amphibian *Caudiverbera caudiverbera* as a study model. For this purpose six treatment groups of 12 larvae each were set up. Four groups were placed in solutions containing 15 ppb[1] , 150 ppb, 300 ppb and 1.5 ppm of PCP. The fifth group was placed in filtered aquarium water and acted as a control. The sixth group was placed in a solution of 2-nitro-7-methoxinaphto [2,1-b] furan, best known as R-7000, which acted as a positive control group (Venegas et al. 1984; Lasne et al. 1987). All treatment units were connected to a system of constant aeration according to the methodology developed by Gavilán and Hermosilla (1984). After preliminary assays, the experiments were conducted for 6 days, after which the larvae were sacrified. Slides were made using blood obtained by heart puncture (Venegas et al. 1987; Venegas et al. 1990).

The same concentration doses of PCP were tested for the detection of the clastogenic effects of this chemical on plants. Each treatment used root tip meristems of 10 onion bulbs (uniform size of 20-30 gr) of *Allium cepa*. Root tips were stimulated to grow in cylindric containers placed in a treatment chamber in the dark, at a constant temperature of 20 ± 0.5 °C. Treatments were conducted during a 6 h period. One negative control group with distilled water and a positive control group exposed to 0.25% ethyl methanesulfonate (EMS) for 6 h were set up. After treatment, root tips were fixed, mounted on slides and stained with Feulgen according to the modified technique of Grant (1982). Histological preparations were examined to determine the number of MN per 1000 cells and the percentage of chromosome aberrations in anaphase and telophase (Venegas et al. 1985; Venegas et al. 1990).

Embryos of *C. caudiverbera* were used to determine the teratogenic effect of PCP. These were collected from the frog hatchery of the University of Concepción. The developmental stage of the embryos was determined according to the table proposed by Gosner (1960). The embryos selected presented an adequate gelatine consistency and pigmentation. The development of the embryos took place in culture and treatment units according to the method suggested by Gavilán and Hermosilla (1984).

Ten of these culture and treatment units were used, each contained 1 L of filtered pond water distributed as follows: two culture units which acted

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[1] The dose 15 ppb is the average annual concentration of PCP found in the mouth of the BioBio River. It was taken as the base for the selection of the other concentration doses. The BioBio River is the most important water resource of Chile, with a hydrographic basin available mainly for agriculture and forestry. In the lower part of the river, several industries (cellulose, iron, and petroleum) discharge their wastes directly into the river waters, causing deterioration of the quality of this hydric resource.

as control groups, eight experimental culture units containing PCP; the doses used were 15 ppb, 150 ppb, 300 ppb and 1.5 ppm.

Fifty embryos with their normal gelatine in the initial stage of the neural plate were set in each unit. During the experiment (96 h), the water was not changed but was connected to a system of constant aeration at an environmental temperature (18 ± 2°C). Twenty four hours after the application of PCP, a random sample of 5 live embryos was taken daily and at regular intervals, from each culture unit (Dawson et al. 1985). Embryos were fixed in 10% formaline after anesthesia and examined morphologically. Embryos presenting typical morphological alterations due to the different concentrations of PCP were compared to the control group and photographed under magnifying glass and Scanning Electron Microscope.

The chemicals employed were purchased from E. Merck Darmstadt Germany. The compound R-7000 was provided and produced by Dr. Buisson, Institut Curie, Paris, France.

RESULTS AND DISCUSSION

Tables 1 and 2 present the results of the MN test carried out with erythrocytes of *C. caudiverbera* and root tips of *A. cepa*. In *C. caudiverbera* the four doses of PCP induced the formation of MN; 100% of the embryos survived after treatment and there were no deaths during the experiment, except at the dose 1.5 ppm: in which 75% of the larvae died between the third and fifth day.

Table 1. Frequency of MN in erythrocytes of *C. caudiverbera* larvae induced by PCP.

PCP DOSES	AVERAGE MN PER 1000 ± SE
Negative Control	0.167 ± 0.051
15 ppb	0.423 ± 0.062
150 ppb	0.716 ± 0.111
300 ppb	0.798 ± 0.023
1.5 ppm	0.153 ± 0.193
Positive Control	5.632 ± 0.463

Two histologic preparations were scored for each larvae, 2000 cells were scored per slide, therefore 4000 erythrocytes per larvae. Statistical analyses were made with Mann Withney U test ($p = 0.05$). Comparing results of the different doses with the negative control no significant differences were observed.

Table 2. Frequency of MN in root tips of *A. cepa*.

PCP	AVERAGE MN PER 1000 ± SE
Negative Control	0.111 ± 0.055
15 ppb	0.316 ± 0.071
150 ppb	0.524 ± 0.177
300 ppb	0.812 ± 0.314
1.5 ppm	0.195 ± 0.245
Positive Control	2.954 ± 0.751

Two histologic preparations were scored for each bulb, 2000 cells were scored per slide, therefore 4000 interphase cells per bulb. Statistical analyses were made with Mann Withney U test ($p = 0.05$). Comparing results of the different doses with the negative control no significant differences were observed.

Table 3. Type and frequency of anaphase-telophase (A-T) chromosome abnormalities induced in *A. cepa* by PCP.

PCP Doses	MI	Mean Number of A-T abnormalities (%)				Total A-T Abnormalities Mean (%)
		B	Ac	CL	R	
Control	17.3 ± 0.12	--	--	--	--	--
15 ppb	8.8 ± 0.19	--	0.5	--	--	0.50
150 ppb	6.4 ± 0.15	0.25	0.5	--	--	0.75
300 ppb	5.2 ± 0.03	0.25	0.75	--	--	1.00
1.5 ppm	3.1 ± 0.13	--	--	--	--	--

400 A-T cells per dose were counted. With the dose 1.5 ppm the low MI did not allow analysis. (MI=mitotic index; B=bridge; Ac=acentric fragment; CL=chromosome lagging; R=rings).

Table 3 presents the different types of chromosome aberrations induced by PCP in root tip cells of *A. cepa* detected in anaphase and telophase. These aberrations correspond to acentric fragments, simple and double bridges, chromosomes lagging and rings during anaphase. The mitotic index gradually decreased as the concentration of PCP increased.

The teratogenic effects of PCP on the embryos of *C. caudiverbera* can be described as follows:

- 1.5 ppm, the highest dose used, inhibits growth and 48 hours after its application 100% of the embryos die. The gelatine presents

alterations; it changes from normal and transparent to opaque. The embryos in neural fold show a clear depigmentation and an abnormal migration of pigment. Development of the anterior region is notoriously delayed.

- 300 ppb, the situation is similar to the ones previously described, but is less drastic. Growth is inhibited and 72 hours after its application, 46% of the embryos die. Embryos show inhibition of cephalization and present abdominal oedema.

- 150 ppb, the greater effect is observed after hatching because the swimming ability decreases. Together with growth inhibition, evident morphological alterations were found which appeared gradually as time elapsed: after 72 hours, dorsal and ventral alterations were manifested, such as occlusion of the neural tube and oedemas (Fig. 1). After 92 hours, 5% of the embryos die.

- 15 ppb, the swimming ability decreases less than in the previous case. After 96 hours, embryos only attained the formation of rudimentary gill buds, compared to controls which attained the stage of caudal circulation. An alteration in the axial morphogenesis was produced because xiphoses were seen in this group of treated embryos.

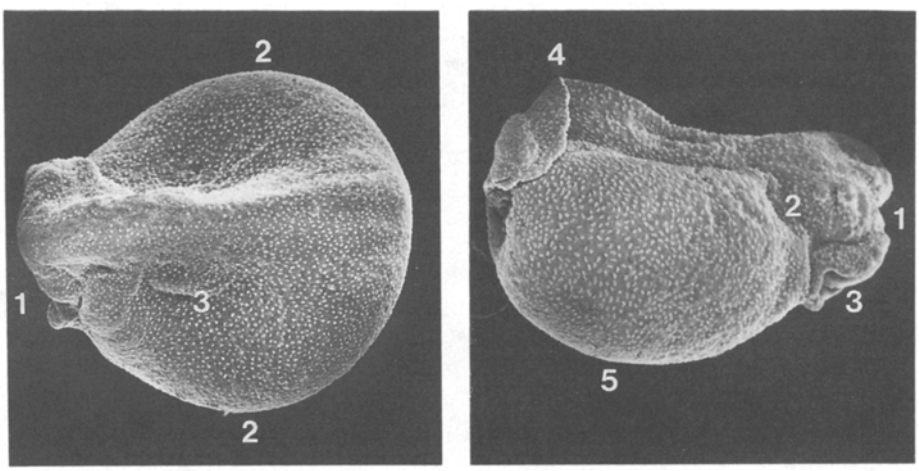

Figure 1. *C. caudiverbera* embryos developed in a solution of 150 ppb.

A. Neural tube presenting microcephalia (1), oedema and blocked vitellolysis (2). A profound alteration at cephalic organs' level and an asymmetry of the pronephros (3) are observed.
B. Caudal bud presenting microcephalia(1), abnormal development at gill (2) and adhesive organ level (3), deviation of the axial axis especially at the caudal end (4), blocked yolk lysis and therefore oedema (5).

The MN and chromosomal aberration tests in anaphase and telophase were used to evaluate the genotoxic effects of PCP. An analysis of results indicate that even if there is an increase of MN and that aberrant forms in different stages of cell division are observed (Table 1, 2 and 3), there is not a significant dose-response relationship; therefore we have to conclude that no clear mutagenic effects occur in the models studied. The highest dose used had a drastic effect on the cellular cycle of both models. This toxicity was evidenced in a decrease of the mitotic index (Table 3), thus, the found number of MN was very low.

In relation to teratogenic effects, inhibition and delays in development of normal growth of embryos and early larvae of *C. caudiverbera* is evident.

From an extraembryonic point of view, high doses within the first 24 hours produce alterations on the jelly enclosing the embryos which changes from normal and translucid to opaque. This jelly acts as a natural barrier against potential toxic compounds that might be absorbed by the embryos; however it might also favor the absorption of substances into the vital space of the embryo.

During the experiment, behaviour alterations related to muscular response and caudal circulation were observed in the embryos. These produced an early eclosion of embryos due to rapid and repetitive movements. This hyperactivation was reported in relation to embryos and larvae of the American frog *Rana catesbeiana* (Cooke 1972) treated with 2 and 4 ppm of another insecticide, DDT, and to embryos of *C. caudiverbera* treated with the same substance (Gavilán et al. 1988). It is important to point out that bivalent cations such as zinc, lead and cadmium, and other acrydine-related compounds can induce similar effects on embryos of *Xenopus laevis* (Dumpert and Zeitz 1984).

Thus, it could be concluded that the teratogenic action of different chemical contaminants induce similar malformations. This has been observed in amphibians and other aquatic animals as a consequence of possible multiple and non-specific effects that chemical contaminants could produce on biological systems. Incidently, cadmium and DDT produce less availability of energy molecules due to inhibition of oxidative phosphorylation in amphibians (Pérez-Coll et al. 1985). This same phenomenon occurs with PCP. Recent investigations have shown inhibition of sodium transport by PCP in toad skin (Quevedo et al. 1992). This could indicate that dynamic molecular phenomena occurring at the plasma membrane level and inside the cell might be responsible for the alteration of the complex phenomenon known as normal program of development of embryos and larvae in amphibians. Further investigations are necessary at this level, for while PCP is forbidden in industrialized countries, it is used widely in underdeveloped countries, especially in those which are great wood exporters.

Finally we can say that *C. caudiverbera* constitutes an adequate model of genetic toxicological and teratogenic studies because of its low

number of large chromosomes (2n = 26) and because the amount of eggs delivered by an adult female in each spawning (ca. 10000 to 12000). This constitutes a bulk of homogeneous and synchronous material that can be used for research purposes. On the other hand, due to the natural aquatic environment of the Chilean frog, tadpoles can be kept in direct contact with chemical agents to be assayed.

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