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Adverse Effects of Polluted Continental Water Bodies in Chile on Frog Adrenergic Synapse

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For many years health risks from chemical exposures have been examined by several programmes within the World Health Organization (Becking, 1992), including analysis of drinking waters derived from river basins exposed to industrial wastes. There is growing interest in an approach that evaluates susceptibility of the organism to pollutants by the use of biomarkers which may indicate the presence of contaminants in cells (McCarthy and Shugart, 1990; Winneke and Lilienthal, 1992). One of these markers is the isolated toad skin, which has been used extensively as a biological model to investigate the cellular effects of numerous drugs (Alarcon, 1995) which alter ion transport across the epithelium. Neurotoxic effects have occurred when chemicals interfere with nerve transmission because they enter the environment through the process of industrialization and pollution (Tilson, 1993). The adrenergic synapse between sympathetic nerve endings and skin mucous glands is a biomarker which has been studied by authors (Norris and Quevedo, 1993a) who showed that the response to nerve stimulation consisted of a rise in the potential difference (PD) and in the short-circuit current (SCC) across the skin. This response is due to an increase in active Cl- transport by the mucous glands (Thompson and Mills, 1981).

Effluents from cellulose and paper plants contain a mixture of chemical reagents used in digestion of wood, cellulose fibres and lignin, and of other chemical compounds including organochlorines derived from the bleaching process (Badinella, 1993). Several wood pulp and paper industries discharge their wastes into the Bio-Bio, a Chilean river 380 Km in length (Vlllth Region, 37.5° Lat., 73.5° Long.) causing pollution which affects urban centres and rural surroundings, and their effects on some biomarkers (Venegas et al.,1993) have been investigated. However, there are no studies on the neurotoxic effects of pollutants released in these effluents.

The aim of the present work is to examine the action of potentially toxic industrial wastes taken from different sources, on a) the responses of the frog nerve-skin preparation to electrical stimulation; b) the bioelectric

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parameters (PD and SCC) of the isolated toad skin; and c) the characteristics of frog sciatic nerve compound action potential.

A map of the location of the Bio-Bio river showing the sample sites is included (Fig. 1).

MATERIALS AND METHODS

Samples of industrial (forestry, paper and cellulose) effluents were taken directly from evacuation ducts into the Bio-Bio river. A sample from a non-industrial zone in the upper waters (Santa Barbara, uncontaminated by pesticides or other chemicals) was taken as a reference site The samples were passed through filter paper and then through a filter unit of 0.22 pore diameter (Millex-GV). They were kept at -25% until use.

Experiments were performed on frogs of the species *Caudiverbera caudiverbera* (180-350 g) collected from fresh water ponds in Concepción, Chile, during the spring and summer months, and on toads of the species *Pleurodema thaul* (10-20 g). Both frogs and toads were kept in tap water at room temperature (18-22%) at least 24 h prior to use, and fed on sow bugs (*Oniscus asellus*).

For the experiments on the neuroepithelial synapse, the amphibians were anaesthetised with 60 mg/kg i.v. pentobarbitone (Sigma Chem. Co.). The cutaneous branch of the tibial nerve (inferior cruris medialis) supplying part of the skin of the hindleg was isolated together with the attached piece of skin and mounted between perspex Ussing chambers as previously described in our laboratory (Quevedo et al.,1988). An area of 1.33 cm^2 was exposed to 3.5 ml phosphate buffered (pH 7.5) Ringers solution on both surfaces and gassed with a stream of air. The composition of the solution was (mM): NaCl 112, KCI 1.9, $CaCl_2$ 2.0, $NaHCO_3$ 2.3 and glucose 11. The PD across the skin was recorded on a Cole-Parmer two-channel recorder using calomel electrodes connected with the solutions bathing both surfaces of the skin through agar-Ringer bridges and to the first channel of the recorder. The SCC was monitored through Ag-AgCI electrodes connected to a voltage-clamp circuit (G. Metraux Electronique) and to the second channel of the recorder. The nerve was placed on a pair of Ag electrodes connected to the isolation unit of a Grass S44 stimulator. Square wave pulses of 4 ms duration at a rate of 10 Hz and 10 V intensity for 30 s were used. Preparations were stimulated at regular intervals (30 min.). For each preparation the control responses were stable.

Since each experiment lasted eight to ten h, usually only one set of readings was made for each neuroepithelial synapse, that is to say, one preparation from one individual. In at least fifty percent of the synapses tested, preparations stored overnight at 0° C showed similar responses the next day: these second readings, however, have not been included in the statistics, as the SCC often decreased to half the original value.

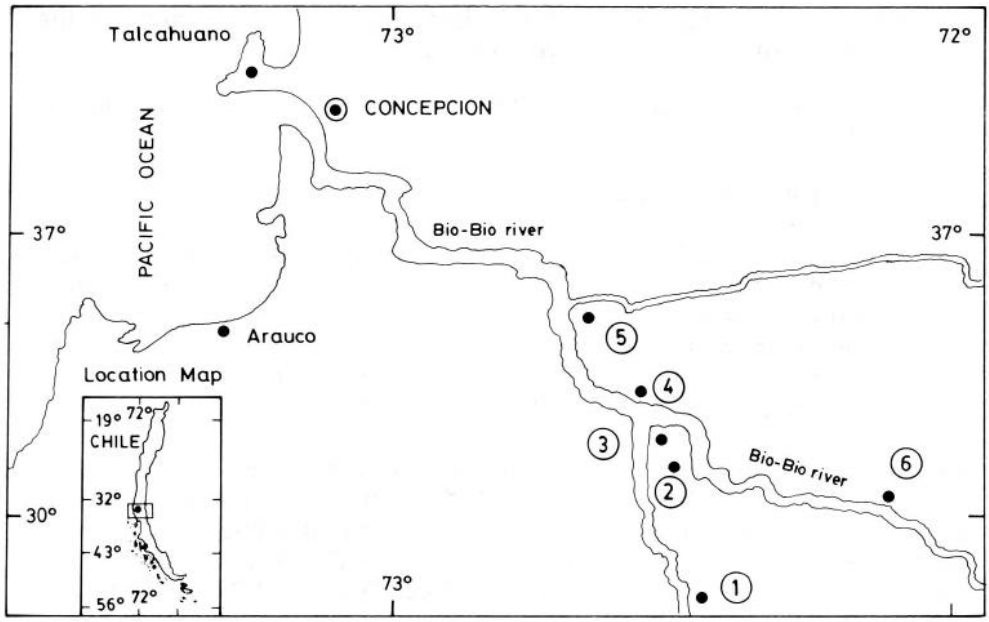

Figure 1. Location of sampling sites on the Bio-Bio river in studies of contamination effects. 1. Mininco, 2. Nacimiento, 3. Nacimiento, 4. Santa Fe, 5. Laja, 6. Santa Barbara. VIIIth Region, Chile (37.5° Lat., 73.5° Long.).

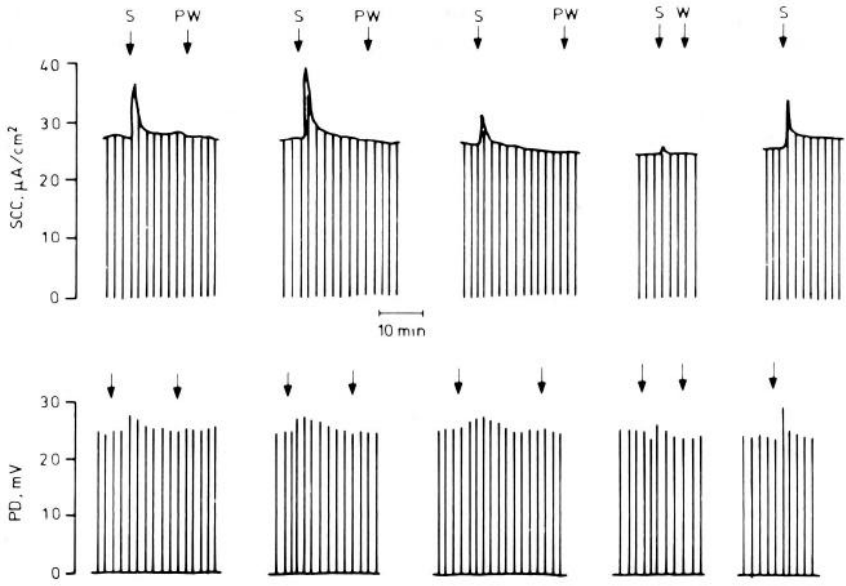

FIgure 2 Representative experiment Illustrating effect of cumulative doses of an Industrial effluent (inner and outer soluton) on the toad C Caudiverbera neuroepithellal response to electrical stImulation SCC= short -curcult current, PD=potential difference. W = washout At arrows marked PW, effect of 0 49ml, 0 82 ml and 1 15 ml polluted water sample 2 Note recovery of the response after washout

Samples of industrial effluents (pH 7.5, 300 mOsm) were added to the solutions bathing both surfaces of the skin to avoid artefacts due to volume changes. Preliminary experiments showed that a volume of 0.49 ml of the effluent samples induced a significant effect ($P<0.05$) and two subsequent increases of 0.33 ml each were followed by a dose-dependent decrease in the response provided that the samples were not washed out, thus reaching a cumulative dose of 1.15 ml in the bath.

For the study of *Pleurodema thaul* skin the mounting procedure and measurement of bioelectric parameters were similar.

Isolated sciatic nerves of the frog *C. caudiverbera* were used to analyse the compound action potential. Briefly, the experiments were performed at room temperature (18-22°C) and the potentials were displayed in a 5115 Tektronix storage oscilloscope (Quevedo et al., 1979)

Values throughout the text refer to means±SEM for each nerve-skin preparation and for each isolated toad skin. Statistical analysis was performed using Student's t test for paired samples.

RESULTS AND DISCUSSION

Stimulation of the neuroepithelial preparation was followed immediately by a transient increase in the bioelectric parameters of the skin. The increase in SCC usually consisted of two main components. The first component was a rapid rise in current from 42.8±3.3 uA/cm^2 to 53.1±5.2 µA/cm^2 (n=15). The second component consisted of a slow rise when the rapid component was declining; the peak was very variable, usually smaller than that of the first rise, and was sometimes followed by a third and even slower component. The profile of the rise in PD was similar although always smaller in magnitude than that of the rise in SCC; a rapid initial component rose from 36.2±3.4 mV to 40.0±4.1 mV (n=15). Since the slow component was often nearly continuous with the rapid component, and very difficult to measure, it was not further analysed. The values throughout the work refer to the initial rapid rise in SCC and in PD.

In 15 nerve-skin preparations, stimulation of the nerve every 30 min. for a period of eight to ten h induced repetitive responses which did not decline significantly in magnitude. The addition of 0.49 and 1.15 ml of a sample of non-polluted water to both surfaces of the preparation had no effect on the responses to nerve stimulation. Cumulative doses (0.49, 0.82 and 1.15 ml) did not alter the responses in ten experiments.

Fig. 2 illustrates the action of cumulative doses of a sample of polluted river water (inner and outer solution) on the frog C. caudiverbera neuroepithelial response to electrical stimulation.

Table 1. Effect of cumulative doses of five industrial effluents on frog nerve-skin preparations after electrical stimulation, and on the bioelectric parameters of isolated toad skins.

Effluent sample site and dose	Nerve-skin preparation % decrease in PD	Nerve-skin preparation % decrease in SCC	n	Isolated skin % decrease in PD	Isolated skin % decrease in SCC	n'
Site 1						
0.49 ml	22.8±2.9^{+}	20.3±2.8^{+}	20	1.4±0.3	1.2±0.2	10
0.82 ml	32.2±3.8^{+++}	34.8±3.2^{++}		1.9±0.3	1.3±0.4	
1.15 ml	52.5±4.2^{+++}	51.4±2.5^{+++}		2.7±0.4^{NS}	2.1±0.6^{NS}	
Site 2						
0.49 ml	23.0±3.8^{+}	27.8±3.9^{+}	9	1.1±0.4	0.9±0.2	9
0.82 ml	35.2±4.8^{++}	45.5±4.9^{++}		2.3±0.6	2.4±0.5	
1.15 ml	52.2±5.5^{+++}	68.9±6.1^{+++}		3.3±0.7^{NS}	3.4±0.4^{NS}	
Site 3						
0.49 ml	18.6±1.3^{+}	24.4±3.4^{+}	9	1.5±0.5	1.7±0.4	9
0.82 ml	34.2±3.8^{++}	44.4±3.4^{+++}		2.0±0.5	1.7±0.6	
1.15 ml	60.0±5.5^{+++}	58.9±5.1^{+++}		2.6±0.3^{NS}	2.2±0.4^{NS}	
Site 4						
0.49 ml	28.5±2.5^{+}	32.2±2.8^{+}	9	1.3±0.4	1.8±0.6	9
0.82 ml	41.4±2.9^{++}	46.1±4.2^{++}		1.9±0.3	2.3±0.4	
1.15 ml	65.7±5.7^{+++}	72.6±5.2^{+++}		2.8±0.6^{NS}	3.0±0.7^{NS}	
Site 5						
0.49 ml	16.7±1.3^{+}	16.4±1.1^{+}	9	1.2±0.2	1.3±0.3	9
0.82 ml	33.4±3.2^{++}	52.0±4.5^{++}		1.9±0.6	2.2±0.5	
1.15 ml	71.5±5.4^{+++}	69.4±5.1^{+++}		2.9±0.4^{NS}	3.1±0.6^{NS}	

Results are expressed as percent decrease±SEM: for the frog *C. caudiverbera* in the skin response of the potential difference (PD) and of the short-circuit current (SCC) over the basal values of the non-stimulated skin; n = number of neuroepithelial synapses tested: and for the frog skin, of both electrical parameters; n' = number of toad skins tested.
Significantly different from the response to nerve stimulation in the absence of effluent: $^{+}p<0.05$; $^{++}<0.01$; $^{+++}<0.001$. Response of isolated toad skin: NS = not significant. (Student's paired t test).

Table 1 summarizes the inhibitory effect of increasing doses (0.49, 0.82, and 1.15 ml) of five industrial effluents on the bioelectric responses to nerve stimulation. Significant dose-dependent reductions in the response were found in the preparations following exposure to each sample: the average decrease was 60.3±3.7% for the PD and 64.4±3.9% for the SCC in the five experimental groups and this decrease was reached after 142.7±20.3 min. These effects were usually reversible after removal of the effluent by a threefold washout.

The table also shows that the bioelectric parameters of P. thaul skin were not significantly altered by any of the contaminated effluents.

Table 2. Microcontaminant concentration in the effluent from a cellulose industry (site 1, 1993)

Compound	Summer	Autumn	Winter	Spring
Phenol (ppb)	2.203	ND	0.561	1.058
2 chlorophenol (ppb)	0.130	ND	0.100	1.020
2.4 dichlorophenol (ppb)	0.429	0.122	0.436	ND
4 Cl 3 methylphenol (ppb)	0.112	0.093	ND	0.781
2.4.6 trichlorophenol (ppb)	0.410	0.326	1.141	0.920
2.3.6 trichlorophenol (ppb)	0.067	ND	0.020	0.022
Pentachlorophenol (ppb)	0.870	0.360	3.920	ND
Total organic halogens (ppm)	12.000	ND	7.450	9.000

ppb = µg/l = mg/m^3; ppm = mg/l; ND = not done.

The bioelectric parameters of *P. thaul* isolated skin (PD and SCC) and the latency, magnitude and conduction velocity of *C. caudiverbera* sciatic nerve compound action potential (n =10) were not altered on application of samples of polluted water.

The industrial effluents used in this study induced significant impairment in transmission at the neuroepithelial synapse of the frog *C. caudiverbera* but had no effect on the sciatic nerve compound action potential or on the ion transporting skin of the toad *P. thaul.* The major pollutants in these effluent samples are organochlorines (Venegas et al.,1993); high values of total phenols (0.571 to 6.562 µg/l) were found in effluents from cellulose industries (Faranda and Parra, 1993) in the river system from the towns of Mininco and Nacimiento (sample sites 1, 2 and 3) towards the mouth of the river (sample sites 4 and 5). The values found by these authors are above the figures accepted by the Water Pollution Research Reports (Rivera and Angeletti, 1988) which are 0.1 µg/l for individual pesticides and 0.5 µg/l for total pesticides.

Table 2 contains a summary of the results of the analysis of a water sample collected from a cellulose bleaching plant located on site 1. Pentachlorophenol (PCP) has far higher toxicity than other organochlorines present in these effluents: Badinella (1993) found that the LD_{50} dose at 96 h in fishes was of the order of 40 nM and that the annual concentration range in contaminated effluents was 3-30 nM. Apparently these concentrations had no effect on the sciatic nerve action potential (Montoya et al.,1988) or on active Na^+ transport in the isolated skin (Quevedo et al.,1992); however, the adrenergic synapse was found to be susceptible either to these low PCP concentrations, or to the combined effect of different chemical contaminants (Montoya et al., 1990). The final concentrations of PCP used in our work on the corneal epithelium (Norris and Quevedo, 1993b) ranged from about 8.0 to 28.0

nM; these figures confirm a potentially toxic level of the average annual concentration of the chemical found by Badinella (1993) in Bio-Bio water, since ion transport was irreversibly blocked in this tissue. Both the frog corneal epithelial transport (Wiederholt, 1988) and the adrenergic synapse response to nerve stimulation (Thompson and Mills, 1981) are due principally to active Cl^- transport. Our results are in agreement with an inhibitory effect of PCP on Cl- transport in both biomarkers.

The similarity in the decrease of the neuroepithelial response to nerve stimulation in the five experimental groups could be due to the fact that the effluents contain groups of similar compounds (Hellou, 1993).

Our results show that samples of uncontaminated river water had no effect on the response of the neuroepithelial synapse of the frog *C. caudiverbera* to nerve stimulation, whereas all the samples of industrial effluents examined induced a significant and usually reversible decrease in the response. The characteristics of the sciatic nerve compound action potential and the bioelectric parameters of *P. thaul* skin were not significantly altered by any of the polluted effluents; the lack of effect on the toad skin probably reflects a species difference.

To conclude, the neuroepithelial synapse of the frog *C. caudiverbera* is a highly sensitive tool for the detection of potential neurotoxicity in polluted water.

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REFERENCES

Alarcón JM, Quevedo L, REYES P (1995) Inhibitory action of hydrogen peroxide on a high-resistance epithelium. Pharmacology 50: 111-118

Badinella MT (1993) Estimación del riesgo de contaminación, derivado de las descargas de compuestos organoclorados de origen industrial en el río Bio-Bio, Chile (Tesis Doctorado Ciencias Ambientales). Proj. EULA (Europe-Latinamerica Proj.), Concepcion, Chile, pp. 120-133

Becking GC (1992) Methodology in neurotoxicity activities within the World Health Organization and International Programme on Chemical Safety. Toxicol Lett 64-65 Spec N° 209-215

Faranda F, Parra O (1993) *In:* Vallutazione de la qualitá delle acque et ecologia del sistema limetico e fluviale del fiume Bio-Bio. EULA, Programma interuniversitario di ricerca scientifica aplicata e formazione. Monografie scientifiche 12, pp. 15-161

Hellou J, Warren WG, PAYNE JF (1993) Organochlorines including polychlorinated biphenyls in muscle, liver and ovaries of cod, *Gadus morhua.* Arch Environ Contam Toxicol 25:497-505

McCarthy J, Shugart LR (1990) Biomarkers of environmental contamination. In: Biomarkers of environmental contamination. Lewis Publishers, Boca Raton, Florida, pp. 3-14

Montoya G, Quevedo L (1990) The effects of pentachlorophenol (PCP) on the toad neuromuscular function. Comp Biochem Physiol 996:193-197

Norris B, Quevedo L (1993a) Pentachlorophenol (PCP) inhibits ion transport in the isolated toad cornea. Gen Pharmacol 24: 867-872

Norris B, Concha J, Contreras G, Contreras E ((1993b) Calcium channel blockers apparently decrease noradrenaline release from nerve-skin terminals in Caudiverbera caudiverbera. Gen Pharmacol 24: 971-976

Quevedo L, Neumann V, Schmidt E, Cárdenas H (1988) Action of lycorine on noradrenergic response of a nerve-skin preparation. Cell Molec Biol 34: 295-302

Quevedo L, Montoya G, Ferraris R, Venegas W (1992) Inhibition of the sodium transport by pentachlorophenol (PCP) in toad skin (*Pleurodema thaul*). Comp Biochem Physiol 101C: 365-369

Rivera J, Angeletti G (1988) *In:* Water Pollution Research Report 9: Organic Micropollutants in Drinking Water. E. Guyot SA, Brussels, p. 1 and 15

Thompson IG, Mills JW (1981) Isoproterenol-induced current changes in glands of frog skin. Am J Physiol 241 (Cell Physiol. 10): C250-C257

Tilson HA (1993) Neurobehavioural methods used in neurotoxicological research. Toxicol Lett 68 (1-2): 231-240

Venegas W, Hermosilla I, Quevedo L, Montoya G (1993) Genotoxic and teratogenic effect of pentachlorophenol, pollutant present in continental water bodies in the South of Chile. Bull Environ Contam Toxicol 51: 107-114

Wiederholt M (1988) Ion transport by the cornea. NIPS 3: 97-99

Winneke G, Lilienthal H 1992) Extrapolation from animals to humans; scientific and regulatory aspects. Toxicol Lett 64-65 Spec N°239-246