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Use of Biomarkers to Evaluate Effects of Xenobiotic Compounds in the Biobio Basin (Central Chile)

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The aim of the present study was to make a preliminary evaluation, by means of biomarkers, of the toxicological effects of xenobiotic compounds on the biota of the basin of the River Biobio in Chile. A biomarker is defined as " ... a xenobiotically-induced variation in cellular or biochemical components or process, structures, or functions that is measurable in a biological system or samples" (NRC 1989). Such variations provide information on the amplitude of response of an organism in relation to the magnitude of chemical insult and on the relation between biological effects and environmental contamination. The study of biomarkers in bioindicator organisms, sampled in one or more areas suspected of pollution, and compared with those of organisms from a reference area, makes it possible to evaluate the potential danger to communities (McCarthy et al. 1989, McCarthy & Shugart 1990, Fossi & Leonzio 1994). Such an approach is appropriate in situations of high human impact, as in the Biobio River in Central Chile. It provides an "integrated" response to overall exposure, in spatial and temporal terms, comprehensive of the various toxicological and pharmacological interactions of the pollutant mixture to which the bioindicator organisms are exposed. The biomarkers chosen to evaluate environmental quality in the Biobio basin were the mixed function monooxygenases (MFO), indicators of chemical stress due to organochlorines, PAHs, dioxines and pentachlorophenols (Payne et al. 1987; Fossi and Leonzio 1994) and the esterases, indicators of chemical stress due to organophosphates, carbamates and neurotoxins (Thompson et al. 1988; Walker 1989).

The MFO system plays a determinant role in the initial stage of detoxification of xenobiotic lipophilic compounds. One of the basic features of this system is its substrate inducibility: xenobiotic compounds actively stimulate the synthesis of new functional proteins (Payne et al. 1987, De Matteis 1988). Induction is thus a quantitative or semi-quantitative signal of the present of xenobiotic substances. The inductive response is substrate-specific, that is, a given class of xenobiotics, for example polycyclic aromatic hydrocarbons, can specifically induce a single class of enzymes (the cytochrome P-448 family), whereas another class of contaminants, for example certain organochlorine insecticides (DDT and aldrin), induces a different family (cytochrome P-450). Because of its substrate specificity-inducibility, the MFO system is one of the most specific biomarkers, enabling the identification of various families of liposoluble chemicals responsible for induction. However, levels of MFOs are also induced by endogenous steroid hormones and may vary seasonally in fish.

The esterase family consists of two basic classes: type A esterases that detoxify organophosphates and type B esterases that are inhibited by them. Inhibition of type B esterases in the brain (acetylcholinesterase) and blood (butyrylcholinesterase and carboxylesterase) is a specific index of stress due to organophosphate and carbamate insecticides and neurotoxic xenobiotics (Ludke et al. 1975).

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MATERIALS AND METHODS

Three sampling areas with different human impact were chosen in the basin: 1) the Santa Barbara area (37° 40' 0" S, 72° 0' 56" O) upstream from the Biobio with reduced farming and industrial input; 2) the Nacimiento area (37° 30' 03" S, 72° 40' 08" O) (with industrial, farm and forestry pollution (timber treatment and pesticides); 3) the Concepcion area (36° 48' 49" S, 73° 03' 17" O) at the mouth of the Biobio with urban and industrial pollution (secondary products of timber, refineries, metallurgy, fish processing).

Potential bioindicators chosen among the fish groups were: a) fish of low mobility such as *Galaxias maculatus* ("Puye"), *Cheirodon galusdae* ("Pocha") and *Percilia irwini* ("Carmelita de Concepcion"), that give a site specific indication of pollution in the sampling area; b) highly mobile species such as *Percicthys trucha* ("Perca trucha"), *Basilichthys australis* ("Pejerrey"), *Salmo trutta* ("Trucha cafe"), *Mugil cephalus* ("Lisa") and *Eleginus maclovinus* ("Robalo"), that give a general picture of the pollution of the basin as a whole. Bioindicators chosen in the class of birds were: a) medium and low mobility species such as *Phalacrocorax olivaceus* ("Cormoran") and *Larus serranus* ("Gaviotin de cordilliera"), that give a site specific indication of pollution in the sampling area; b) mobile species such as *Larus dominicanus* ("Gaviota"), that give a general indication of pollution in the basin as a whole. Sampling was performed in October 1992, using the equipment of the EULA Centre. Electro-fishing and different types of nets were used to capture fish, and firearms to shoot birds. The specimens caught were quickly dissected and the liver and brain placed in liquid nitrogen.

Liver microsomal activity of the MFO system was assayed by spectrofluorimetric determination of resorufin (ethoxyresorufin (EROD), pentoxyresorufin (PROD) and benzyloxyresorufin (BROD)) dealkylation. The equipment of the Institutes of Biochemistry, Genetics and Pharmacology of Concepcion University was used. In the laboratory, aliquots of liver were weighed and suspended 1:5 (W:V) in 0.25 M sucrose buffer at pH 7.5. The tissue was homogenized with a Potter apparatus at 400 rpm and 4°C, working up and down the height of the beaker several times. The homogenate was centrifuged at 9,000 x g for 20 min at 4°C to remove the nuclear and mitochondrial fraction. The microsomal fraction was separated from the supernatant by ultracentrifuging at 100,000 x g for 60 min at 4°C. In order to obtain a pure microsomal fraction, the pellet was resuspended in 1.15% KCl buffer at pH 7.5. The supernatant was ultracentrifuged again under the same conditions, and discarded. The microsomal fraction was made up with 0.5 mL of 1.15% KCl buffer at pH 7.5 and the pellet frozen for 1 h at -20°C. The microsomal fraction, stored at -80°C until assay, was subsequently resuspended in 1.15% KCl buffer at pH 7.5, in the ratio 1 (g liver) : 2.6 (ml buffer). MFO activity was assayed in the liver microsomal fraction by resorufin dealkylation. These tests (Lubet et al. 1985) quantify the transformation of 7-ethoxyresorufin, pentoxyresorufin and benzyloxyresorufin into resorufin (mediated by cytochrome P-448 dealkylation, cytochrome P-450 dealkylation and P-448 and P-450 reactions combined, respectively). The reaction mixture (final volume 2.25 mL) consisted of 2.21 ml of 50 mM Tris-HCl, 25 mM HgCl2 at pH 7.5, 10 µl microsomal proteins and 10 µl NADPH. The substrate consisted of 10 ml of 0.41 mM 7-ethoxyresorufin, 1.76 mM pentoxyresorufin or 0.98 mM benzyloxyresorufin, according to the reaction tested. The mixture was placed in 1 x 1 x 4 cm quartz cuvettes and allowed to reach ambient temperature. The sample was read by fluorimetry at excitation and emission wavelengths of 522 nm and 586 nm respectively. The reaction was started by adding 10 ml NADPH to the blank. Activity was expressed in picomoles of resorufin/min/mg microsomal protein.

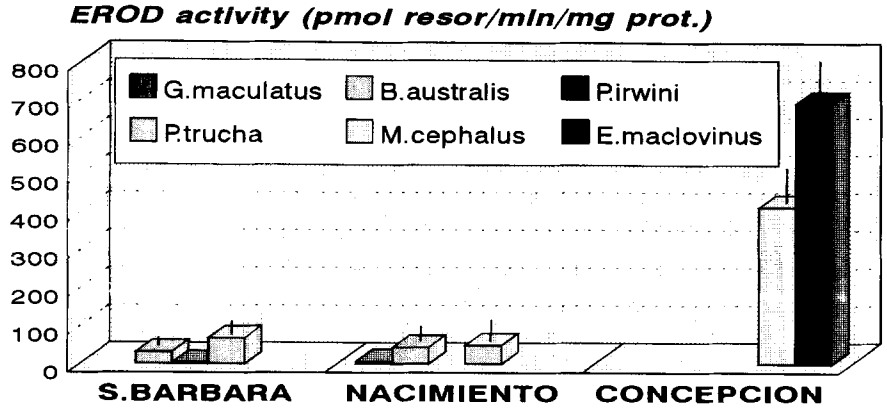

Figure 1 - Mixed function monooxygenase activity (EROD) in various species of fish from the Biobio River (means and SD).

Esterase activity was evaluated by spectrophotometric determination of cerebral acetylcholinesterase (AChE). The assays were performed with the equipment of EULA. Brain tissue was homogenized in 0.1% Triton x-100 and 25 mM Tris-HCl (pH 7.6), and centrifuged at 1000 x g for 10 min. The supernatant was tested immediately for AChE activity. Brain enzyme activity was evaluated spectrophotometrically by the method of Ellman et al. (1961), with acetyltiocholine as substrate.

RESULTS AND DISCUSSION

EROD activity was found to be highly induced in fish (*Mugil cephalus, Eleginus maclovinus*) from the mouth of the Biobio River (Fig.1). Values were 10-20 times higher than in fish (*Percicthys trucha, Basilichthys australis, Percilia irwini, Galaxias maculatus*) sampled in the Nacimiento and Santa Barbara areas. This may be due to 3-MC type inducers such as probably PAH, dioxines, certain PCB congeners and pentachlorophenols, in the water. The latter are strong inducers of this specific isoform of cytochrome P-448 (Shull et al. 1986). On the other hand, induction of BROD activity (cytochrome P-450/P-448 dependent) was almost completely absent. Liver EROD and BROD activities were found to be induced in birds sampled in the Concepcion area (Fig.2). The bioaccumulator species, *Phalacrocorax olivaceus*, sampled in this station had much higher monooxygenase activities than the same species sampled in the Santa Barbara area. *Larus dominicanus* sampled at Santa Barbara and Concepcion generally had similar values that were much lower than those of the fish-eating *Phalacrocorax olivaceus*. The high mobility of *Larus dominicanus* explains the similarity in values obtained in these two areas, whereas the strong induction observed in the *Phalacrocorax olivaceus*, almost totally absent in the gull, must be due to its different feeding habits (the former fish-eating, the latter omnivorous) and the metabolic and biochemical peculiarities of the genus *Larus* (Fossi et al. 1991, Brouwer et al. 1990). The only specimen of *Larus serranus* analyzed had much lower monooxygenase activities than the other species, at least an order of magnitude lower than in gulls of the Mediterranean (Fossi et al. 1986, Fossi et al. 1989, Fossi et al. 1991). These data confirm the restricted chemical stress, due to liposoluble

xenobiotics, in the Santa Barbara area. The induction found in *Phalacrocorax olivaceus* is probably due to biomagnification of "3-MC type inducers" along the food chain.

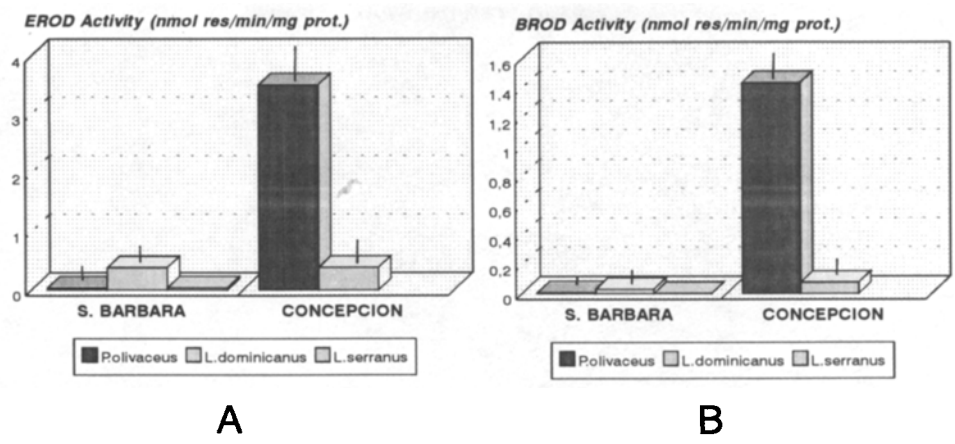

Figure 2 - Mixed function monooxygenase activity, A) EROD, and B) BROD, in various bird species in the Biobio basin (means and SD).

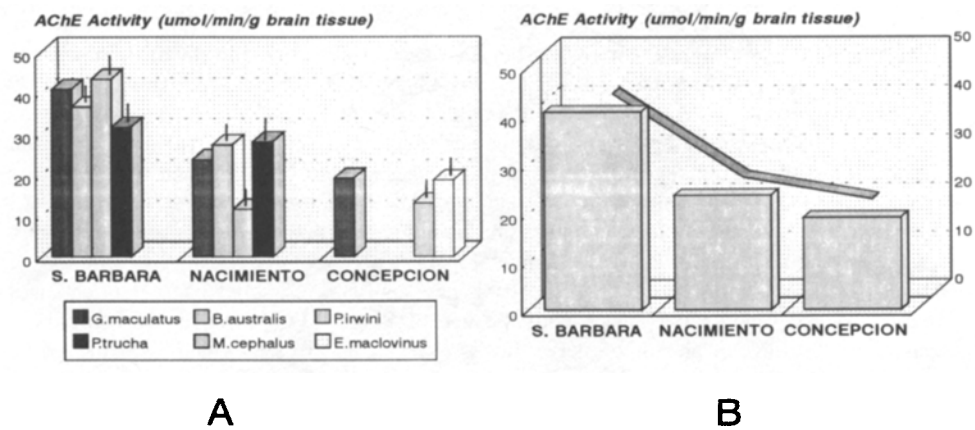

Figure 3 -A) Brain acetylcholinesterase (AChE) activity in various species of fish from the Biobio River (means and SD). B) Brain acetylcholinesterase (AChE) activity in pool of *Galaxias maculatus* sampled in the Biobio River.

In the various fish sampled in the Nacimiento area (*Percicthys trucha, Basilichthys australis, Percilia irwini, Galaxias maculatus*), especially those caught at the mouth of the Biobio (*Galaxias maculatus, Mugil cephalus, Eleginus maclovinus*), marked inhibition of brain AChE was found with respect to fish in the Santa Barbara area (Fig.3 A). The data for *Galaxias maculatus* sampled in the three areas were particularly significant (Fig. 3B). Brain AChE activity was inhibited 42% and 54% in the Nacimiento and Concepcion areas, respectively. This may be due to substances with anticholinesterase activity, such as organophosphate and carbamate insecticides (Ludke 1975, Thompson et al. 1988), and to neurotoxins such as pentachlorophenols (Villena et al. 1992, Montoya & Quevedo 1990), in the river water. These substances are heavily used in agriculture, forestry and timber processing in the basin (Barra 1992).

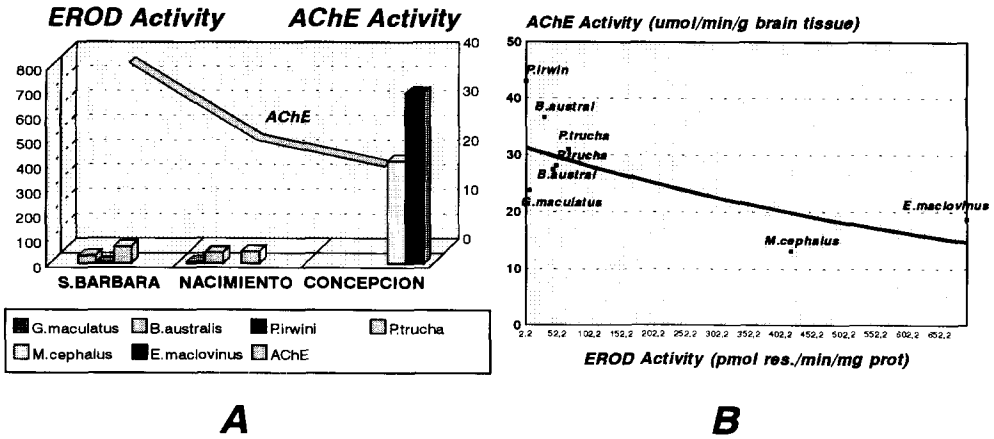

Figure 4 - A) Mixed function monooxygenase (EROD) and brain acetylcholine (AChE) activities (mean values) in different fish species in the Biobio river; B) Inverse exponential correlation between mixed function monooxygenase (EROD) and brain acetylcholine (AChE) activities ($r = 0.6998$, $p < 0.005$).

The existence of an inverse correlation between EROD induction and AChE inhibition in fish in the various sampling areas (Fig.4) is likely to be due to compounds such as pentochlorophenols (PCPs), widely used in the basin. PCPs and their salts are used as algicides, bactericides, fungicides, herbicides and insecticides in various industrial applications, especially for the seasoning and conservation of timber, and in agriculture (Williams 1982). Mean annual world production was estimated to be 90,000 tons in 1983 (IRPTC 1983). Recently the production and use of these compounds has been drastically diminished, due to their high environmental persistence and the possible production of dioxins during processing. Experimental trials have associated a series of toxic effects and metabolic and functional changes with the use of PCPs. PCPs are highly neurotoxic, damaging the myelin sheath of nerve fibers (Villena et al. 1992) and blocking nerve transmission at neuromuscular junctions (Montoya & Quevedo 1990). They also cause histological changes in the liver and kidney (Villena et al. 1992). PCPs are strong inducers of the P-448 isoform of the MFO system (3-MC type inducer) in the liver (Shull et al. 1986) and form covalent bonds with DNA (Ommen et al. 1986). An annual input of 500 tons of PCPs has been estimated for the study area (EULA). Due to their low BCF, they could be the main cause of the strong induction encountered in fish. These compounds also biomagnify to a limited degree, and hence induce MFO activities in organisms such as birds, at the top of the food chain. In the area of Concepcion, compounds such as chlorinated hydrocarbons, found in samples analyzed by gas chromatography (Focardi, personal comunication), and dioxins, potentially produced in paper bleaching and present as impurities in PCP-based insecticides, could combine to induce monooxygenase activity in fish and birds. In fact, it is rare to find a single dose-effect relationship caused by a single pollutant in practice; more often we have a family of dose-effect relationships corresponding to the different interactions of the mixture of contaminants to which the organisms are exposed (McCarthy & Shugart 1990). The large-scale use of organophosphate and carbamate insecticides in forestry areas is another certain cause of anticholinesterase activity.

In conclusion, the results of this preliminary investigation reveal a situation of heavy chemical stress in the study area, especially at the mouth of the Biobio, due to "3-MC type inducers" which are probably neurotoxic. EROD induction in *Mugil cephalus* and *Eleginius maclovinus* was particularly alarming, being about 7 times higher than that found in strongly induced Mediterranean fish (*Zoosterisessor ophiocephalus*) (Fossi et al. 1992). This warning makes it mandatory to identify the dose-effect relation between biomarker responses (especially EROD and AChE) and increasing concentrations of PCPs. This will require laboratory research and field studies throughout the basin, with a larger number of sentinel species.

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