Bull. Environ. Contam. Toxicol. (2005) 74:785–792

DOI: 10.1007/s00128-005-0650-y

Bulletin of Environmental Contamination and Toxicology

Gill Modifications in the Freshwater Fish *Cyprinus carpio* after Subchronic Exposure to Simazine

A. L. Oropesa-Jiménez,[1] J. P. García-Cambero,[1] L. Gómez-Gordo,[2] V. Roncero-Cordero,[2] F. Soler-Rodríguez[1]

[1] Unit of Toxicology, Department of Animal Health and Medicine, Faculty of Veterinary Sciences, University of Extremadura, Avda. de la Universidad s/n, E-10071 Cáceres, Spain
[2] Unit of Histopathology, Department of Animal Health and Medicine, Faculty of Veterinary Sciences, University of Extremadura, Avda. de la Universidad s/n, E-10071 Cáceres, Spain

Received: 7 June 2004/Accepted: 19 January 2005

The extensive use of herbicides has increased the incidence of environmental pollution. Herbicides are among the most dangerous agents of water pollution, since they are applied to crops and in many cases directly into water for aquatic weed control. Among others, simazine is a selective pre- and post-emergence chloro-s-triazine herbicide which has widespread use in Extremadura (Spain) to control annual grasses and broadleaf weeds in the olive grove and vineyard. This systematic use of simazine caused discharges into natural waters after intense rainfalls at the end of 1997, reaching concentrations of 4.5 μg/L. Some of these waters were used for public supply and elevated simazine concentrations in tap water, higher than maximum legal limit (0.1 μg/L) (Council of the European Union (1998), were detected.

Histopathological studies in fish exposed to pollutants have shown that fish gills are efficient indicators of water quality. Fish gills are vulnerable to pollutants in water because of their large surface area and they are allways in close contact with the water. From a morphological as well as from a physiological point of view, the gill is a very complex organ involved in gas exchange, ion exchange, acid-base balance and nitrogenous waste excretion. The cellular responses of the gills to the toxicants indicate not only an impairment of the test organism, but also its adaptation to the pollution situation. Gills are therefore monitor organs potentially useful to reflect the health of aquatic organisms and water pollution (Evans 1987; Schramm et al. 1999).

As a consequence of the simazine contamination episode in Extremadura a research was initiated to study the effect of simazine in the fish living in contaminated waters by means of histopathological analysis. For this purpose, fish from contaminated waters were sampled and evaluated for histopathological lesions. In parallel, healthy fish were experimentally exposed to a 10-fold that of the higher concentration of simazine found in contaminated waters with the aim of determining if lesions observed in fish from contaminated waters were due to simazine exposure. Relevant histopathological findings were only found in experimentally exposed fish and this work shows the results of the laboratory exposure.

Correspondence to: F. Soler-Rodriguez

The purpose of this work was to investigate the toxic effects of a sublethal simazine concentration on the freshwater fish *Cyprinus carpio* (common carp), via structural study of gills. The established exposure concentration was comparable to that detected in the contamination episode occurred in Extremadura (Spain). Therefore it can further contribute to the knowledge of the toxic profile of simazine in concentrations comparable to real situations.

MATERIALS AND METHODS

The test material was simazine (2-chloro-4,6-bis(ethylamino)-s-triazine), white powder, purity of 95%, supplied by Probelte Laboratories (Murcia, Spain). A stock solution of simazine (1500 mg/L) was prepared in acetone.

Common carp (*Cyprinus carpio*) were supplied by the "Centro Nacional de Ciprinocultura Vegas del Guadiana" (Badajoz, Spain). They were 242.96 ± 82.21 g of body weight and 19.10 ± 2.27 cm of length on average. The size of the fish were similar to those sampled in the field study previously cited. Specimens were examined to determine the general health status and acceptability for study purposes. During the pre-study period, fish were acclimated to experimental conditions for two weeks. A 12-hour light/dark photoperiod was maintained for all animals during the experience. Fish were daily fed with commercially available carp chow (Dibaq-Diprotec, SA) at a rate of 2% of the mean body weight and feces and excess food particles were aspirated when required.

For the subchronic toxicity test (90-days), fish were randomly divided into two fibreglass tanks corresponding to a control (n=18) and to an exposed group (n=24). The tank dimensions were 140x100x58 cm and 195x80x60 cm, respectively. The tap water was prefiltered by means of activated charcoal in order to ensure a lack of contaminants and chlorine. A continuous-flow system was used that permitted the automatic and continuous renewal of the test solutions. The water flow was fitted to give a complete renewal of the volume of the test solutions in the tanks 2.5 times each day. The system was checked two times a day to ensure that the test solution was replaced properly and so the concentration of toxicant maintained as near as possible to the nominal value. Althought there are no standardized test for the assay here designed we followed the general conditions described in the Test Guideline 204 of the OECD (1984).

The addition of the stock solution (simazine in acetone) to the water was achieved by means of a precision peristaltic pump (Cole-Parmer®). The selected simazine dose (45 µg/L) was ten-fold that of the higher concentration detected in natural waters of Extremadura (4.5 µg/L), in order to assay the uncertain toxic effect in fish living in the contaminated waters during the simazine contamination episode. Controls only received the same volume of the vehicle (24 mg acetone/L of water). Dissolved oxygen, pH, temperature and total hardness in the aquaria water were daily monitored by specific controllers and measuring probes manufactured by B&C Electronics (Italy) allowing continuous readings. Residual chlorine levels

were evaluated using test strips for semi-quantitative determination (QUANTOFIX-chlorine, Macherey-Nagel, Germany).

Fish were observed for death, clinical signs or behaviour changes. Every fifteen days, 4 exposed and 3 control carps were anesthetized with a solution of tricaine methanesulfonate (MS-222; Aldrich Chemical Company) and euthanized by decapitation. Gills were macroscopically examined and immediately fixed in a solution of 5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 24-48 h pending further histopathological study.

Samples for light microscopy were routinely processed and embedded in paraffin. For the structural study, sections (5 μm thick) were stained with haematoxylin-eosin (HE). Morphometric analysis to determine the area occupied by cells of the interlamellar space was performed using a VIDS IV semi-automatic image analysis system (Analytical Measuring Systems, Cambridge, United Kingdom) on the interlamellar space of secondary lamellae, in order to determine the possible morphological variations in these structures.

A statistical package (SPSS for Windows, V. 11.5) was used to study the results. Differences between control and exposed groups were assessed according to the non parametric Mann-Whitney U-Test for independent samples with $p \leq 0.05$ set as statistical significance.

RESULTS AND DISCUSSION

During the assay the controlled parameters in water remained steady and the average values were: dissolved oxygen (9.5±1.1 mg/L); pH (7.81±0.26); temperature (21.93±2.08 °C), total hardness (182±21 mg/L as $CaCO_3$) and chlorine (negative). These values remained according to Test Guideline 204 of the OECD (1984).

There was no mortality, and fish did not show behaviour changes nor signs of poisoning. Carp experimentally exposed to simazine showed no macroscopic lesions excepting an elevated mucus production during the experiment, which is usually a consequence of an irritant action of a toxic.

There are four gill arches on each side of the teleost fish, each arch supporting many filaments arranged in two rows called hemibranchs (Alazemi et al. 1996). On the upper and lower surface of each filament there is a row of secondary lamellae. These contain three cell types: epithelial, endothelial and pilar cells. The interlamellar space, defined by the basal borders of two lamellae, contains four cell types: epithelial, chloride, mucous and granular cells (Gómez et al. 1998).

The frequency and severity of the gill findings in our study are tabulated in Table 1. All fish exposed to simazine developed severe hyperplasia in gills within the period 15-60 days. Slight necrosis was observed from the beginning, but turned out to be moderate between 45 to 75 days of exposure. Chloride cell proliferation

Table 1. Frequency and severity of the gill structural findings in fish exposed to simazine.

Exposure days	15		30		45		60		75		90	
Groups	C	E	C	E	C	E	C	E	C	E	C	E
Hyperplasia interlamellar space epithelium	0/0	++/***	0/0	+++/***	0/0	+++/***	0/0	++/***	+/*	++/**	+/*	++/**
Lamellar capillary aneurysms	0/0	0/0	0/0	0/0	0/0	0/0	0/0	+/**	0/0	0/0	0/0	0/0
Necrosis	0/0	+/*	0/0	+/*	0/0	+/**	0/0	+/**	0/0	+/**	0/0	+/*
Chloride cells prolif	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	+/*	0/0	+/*

Note. Frequency/severity. Frequency: 0 (absence), + (1-50% affected), ++ (51-99% affected), +++ (100% affected). Severity: 0 (absence), * (slight), ** (moderate), *** (severe). (C): control; (E): exposed fish.

was observed only at the end of simazine exposure and at a low quantity. Only occasional aneurysms were found at day 60.

The main histopathological findings in the gills of carp exposed to simazine can be observed in Figure 1.

The most remarkable cellular change in the gills of exposed fish was hyperplasia of epithelial cells of the secondary lamellae and consequently an increase of the interlamellar area occupied by cells (Fig. 1a). This could be explained as a consequence of the direct contact between the epithelia and the herbicide simazine. Hyperplasia can reduce the absorption of oxygen by the gills. However, hyperplasia produces an increase of the distance between blood and water in which pollutans are dissolved, lowering toxic agent penetration (Morgan and Tovell 1973; Mallat 1985). These findings have also been reported by Nešković et al. (1993), who exposed carp to different atrazine concentrations (1.5, 3.0 and 6.0 mg/L) for 14 days. Gills of control fish were normal during the experience and only a slight hyperplasia of epithelial cells in the interlamellar space, and not in all fish, was observed between day 75 to 90. We consider this finding in control fish was not relevant.

The use of morphometric procedures to quantify such lesions was recommended by Hinton et al. (1992). Therefore, in the present work, a morphometric analysis of the hyperplasic phenomena was performed to quantify the degree of alteration in exposed and non-exposed individuals. The results were related with the histopathological findings, since the area occupied by epithelial cells was increased as was the severity of the hyperplasia of the interlamellar space epithelium. The evolution of the hyperplasic processes and the comparison of the average surface area occupied by epithelial cells in the interlamellar space of the secondary lamellae between exposed and control fish is showed in Figure 2.

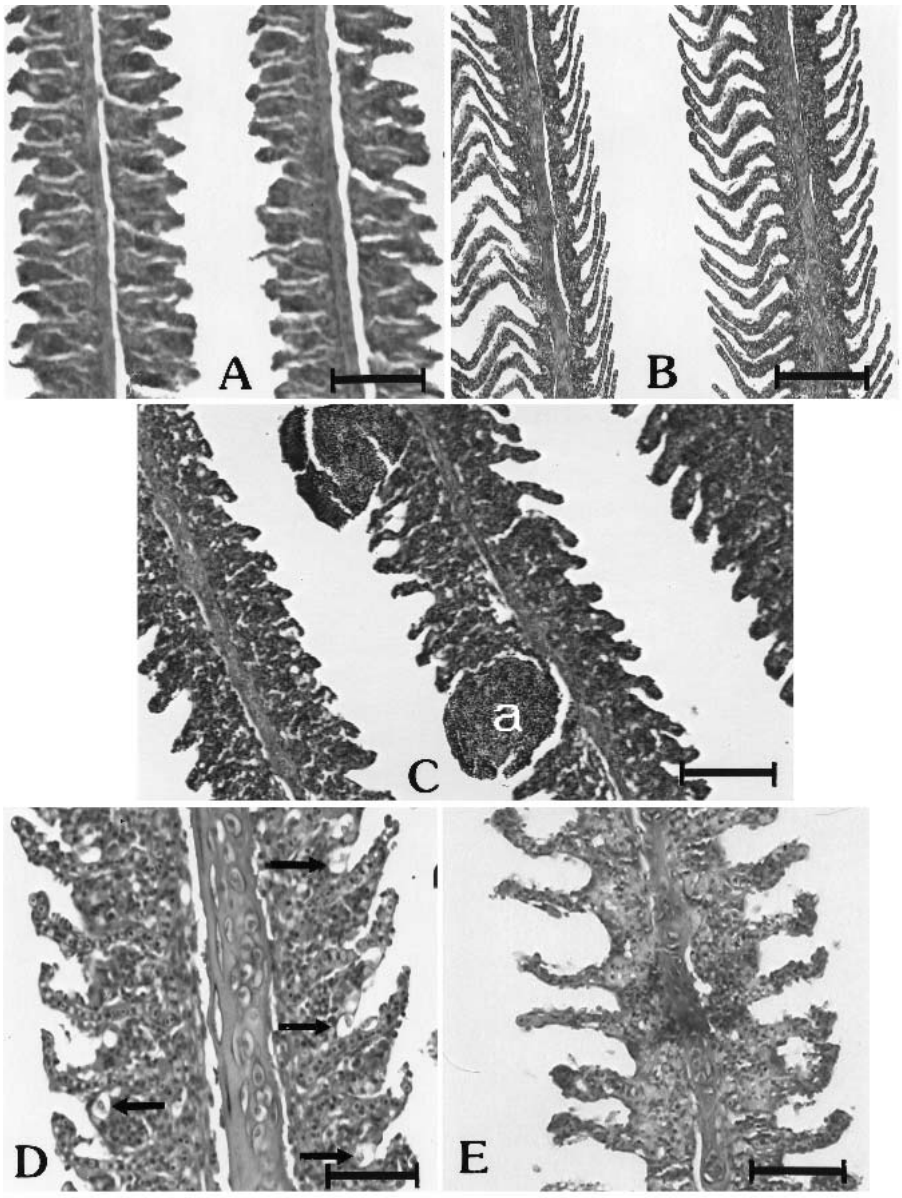

Figure 1. Histopathological findings in the gills of carp exposed to simazine. **(A)** Epithelial hyperplasia with lamellar fusion at day 45 of simazine exposure. Scale bar: 50 μm. **(B)** A control carp gill at day 45. Scale bar: 50 μm. **(C)** Aneurysm (a) in secondary lamellae at day 60 of simazine exposure. Scale bar: 50 μm. **(D)** Focal necrosis (arrow) and epithelial hyperplasia at day 60 of simazine exposure. Scale bar: 25 μm. **(E)** A control carp gill at day 60. Scale bar: 25 μm.

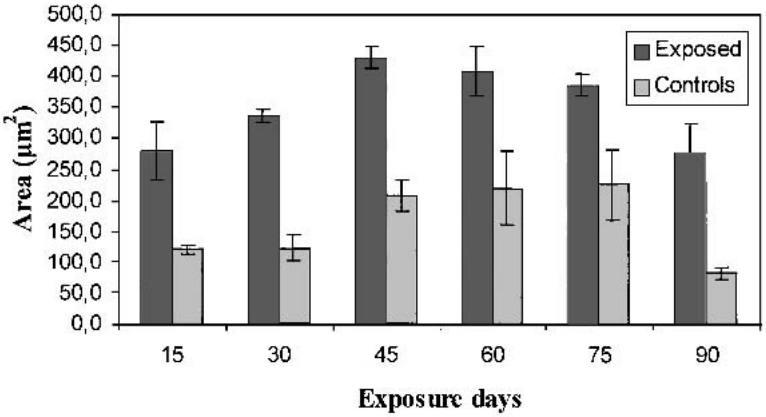

Figure 2. Evolution of the area in the interlamellar space of the secondary lamellae occupied by epithelial cells. At each sampling day there was significative differences ($p \leq 0.05$) between control and exposed fish.

Another finding was proliferation of chloride cells observed in the gills of exposed fish during the last third of the assay, from days 75 to 90. These cells are involved in the regulation of the acid-base balance and detoxification function of the gill apparatus (Rojik et al. 1983; Mallat 1985; Laurent and Perry 1995). Our results suggest that simazine exposure could affect the ion exchange in the fish gill or alternatively chloride cells might play a role in the detoxification mechanism of simazine.

The lamellar capillary aneurysms found in the gills of exposed fish (Fig. 1c) at day 60 of simazine exposure were isolated lesions, and it can be due to the loss of volume regulatory control in the respiratory epithelium as Teh et al. (1997) suggested.

Regarding the focal necrosis of the interlamellar space (Fig. 1d), it was apparent throughout the experimental exposure and became more marked at days 45, 60 and 75 (Table 1). This finding agrees with that reported by Meyers and Hendricks (1985) in fish exposed to 15 µg/L of atrazine. The epithelial hyperplasia of the interlamellar space may also be the result of compensation for cell necrosis (Gómez et al. 1998).

Fish gill pathologies are common symptoms of toxic effects of a wide variety of aquatic pollutants, including organochlorines, petroleum compounds, organophosphates, carbamates, miscellaneous herbicides, acidificants, nitrogenous compounds, heavy metals, salts, and chemotherapeutic agents. The morphological anomalies commonly include "hyperplasia with lamellar fusion, epithelial hypertrophy, telangiectasia, edema with epithelial separation from

basement membranes, general necrosis, and/or epithelial desquamation" (Meyer and Hendricks 1985; Gómez et al. 1998). Gross morphological anomalies in the gill epithelium of yearling coho salmon (*Oncorhynchus kisutch*) exposed to the herbicide atrazine (15 μg/L for 114 h) included necrosis, desquamation, hypertrophy and hyperplasia, and telangiectasia (Meyer and Hendricks 1985).

The structural changes found in our carp gill study, such as epithelial hyperplasia and multifocal necrosis, are chronic alterations not specifically assigned to any particular agent and can be induced by a range of different stressors including pesticides, heavy metals or low oxygen content (Mallat 1985; Hinton and Laurén 1990). In our opinion, exposed carp developed an adaptive process to the new conditions created by the simazine in water, evidenced by chloride cells proliferation along with the progressive remission of hyperplastic and necrotic phenomena.

Since control carp did not show structural alterations, we can conclude that the histological analysis revealed that all the lesions found in the gill of carp, continuously exposed to 45 μg simazine/L water for 90 days, could be considered as moderate and non-specific pathological responses.

Although the purpose of this study was achieved we consider that it would be convenient to perform new exposure studies using higher concentrations of simazine or longer exposure periods, in order to gain a better understanding of the toxic effect of simazine in fish.

Acknowledgments. We thank Ms. Pilar Parras for her technical assistance and Centro Nacional de Ciprinocultura "Vegas del Guadiana" for the supply of carps. This study was financed by the Comisión Interministerial de Ciencia y Tecnología (CICYT, Spain) and the European Commision (Ref: 1FD1997-CO3-03).

REFERENCES

Alazemi BM, Lewis JW, Andrews EB (1996) Gill damage in the freshwater fish *Gnathonemus petersii* (Family: *Mormyridae*) exposed to selected pollutants: an ultrastructural study. Environ Technol 17:225-238

Council of the European Union (1998) Council Directive 98/83/CE of 3 November 1998 on the quality of water intended for human consumption. Official Journal L 330, 05/12/1998, p 32-54

Evans DH (1987) The fish gill: site of action and model for toxic effects of environmental pollutants. Environ Health Perspect 71:47-58

Gómez L, Masot J, Soler F, Duran E, Roncero V (1998) Structural and ultrastructural study of the gills of tench (*Tinca tinca* L.) after experimental poisoning with copper sulphate. Revue Méd Vét 149:387-394

Hinton DE, Lauren DJ (1990) Integrative histopathological approaches to detecting effects of environmental stressors on fishes. In: Adams SM (ed)

American Fisheries Society Symposium, 8. Biological Indicators of Stress in Fish. American Fisheries Society, Bethesda, p 51-66

Hinton DE, Baumann PC, Gardner GR, Hawkins RA, Hendricks JD, Murchelano RA, Okihiro MS (1992) Histopathologic biomarkers. In: Hugget RJ, Kimerle RA, Mehrle PM, Bergman Jr HL (eds) Biomarkers, Biochemical, Physiological and Histological Markers of Anthropogenic Stress. SETAC Publication, Lewis Publishers, p 155

Laurent P, Perry SF (1995) Morphological basis of acid-base and ionic regulation in fish. Adv Comp Environ Physiol 22:91-118

Mallat J (1985) Fish gill structural changes induced by toxicants and other irritants: A statistical review. Can J Fish Aquat Sci 42: 630-648

Meyers TR, Hendricks JD (1985) Histopathology. In: GM Rand, SR Petrocelli (eds), Fundamentals of Aquatic Toxicology. Methods and Applications, Hemisphere Publishing Corp, Washington, DC, p 283

Morgan M, Tovell PWA (1973) The structure of the gill of the trout, *Salmo gairdneri* (Richardson). Z Zellforsch Mikrosk Anat 142:147-162

Nešković N, Elezovic I, Karan V, Poleksic V, Budimir M (1993) Acute and subacute toxicity of atrazine to carp (*Cyprinus carpio* L). Ecotoxicol Environ Saf 25:173-182

Organisation for Economic Co-operation and Development (1984) OECD Guidelines for Testing of Chemicals. Test Guideline 204. Fish, Prolonged Toxicity Test: 14-day Study (adopted 4 April 1984). OECD Publications, Paris.

Rojik J, Nemcsok J, Borross L (1983) Morphological and biochemical studies on liver, kidney and gill of fish affected by pesticides. Acta Biol Hungarica 34:81-92

Schramm MA, Behrens A, Braunbeck T, Eckwet H, Köhler HR, Konradt J, Müller E, Pawert M, Schawaiger J, Segner M, Triebskorn R (1999) Cellular, histological and biochemical biomarkers. Environ Sci Forum 96: 33-64

Teh SJ, Adams SM, Hinton DE (1997) Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquat Toxicol 37:51-70