Acute toxicity and histopathological alterations of Roundup® herbicide on "cachama blanca" (*Piaractus brachypomus*)[1]

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ABSTRACT.- Ramírez-Duarte W.F., Rondón-Barragán I.S. & Eslava-Mocha P.R. 2008. **Acute toxicity and histopathological alterations of Roundup® herbicide on "cachama blanca" (*Piaractus brachypomus*).** *Pesquisa Veterinária Brasileira 28(11):547-554.* Insti-tuto de Acuicultura, Universidad de los Llanos, Apartado Aéreo 110, Km 4 vía Puerto López, vereda Barcelona, Villavicencio, Meta, Colombia. E-mail: wramirezduarte@yahoo.com.mx

Acute toxicity of the glyphosate -N (phosphonomethyl) glycine- herbicide, Roundup®, in juveniles of cachama blanca, (*Piaractus brachypomus*), was evaluated and the histopathological lesions were assessed. The 96 h lethal concentration 50 was 97.47mg.L^{-1} (P<0.05). In the gill, necrotic and proliferative lesions were detected. In the liver, congestion, degenerative foci, hyaline droplets and lipidic vacuolization of the hepatocytes were observed. In the stomach mild hyperplasia of mucous cells was detected, which was also observed in the skin. In this latter tissue, a large increase in the thickness of the epidermis with necrotic lesions, infiltration of leukocytes and melanin pigment were observed. In the brain, degenerative foci of neuronal bodies in the telencephalon associated with gliosis and infiltration of eosinophilic granule cells/mast cells were shown. In conclusion, gills, liver, skin and brain are susceptible to Roundup®. Moreover, effects on the central nervous system could affect olfaction as well as individual and group behavior, the reproductive performance of the fish and hence have repercussions at the population level.

INDEX TERMS: Roundup®, glyphosate, acute toxicity, histopathology, *Piaractus brachypomus*.

RESUMO.- [**Toxicidade aguda e alterações histopatológicas induzidas pelo herbicida Roundup® em pirapitinga (*Piaractus brachypomus*).**] Este estudo avaliou a toxicidade aguda e as alterações histopatológicas induzidas pelo glifosato -N (fosfometil) glicina, na formulação Roundup® em juvenis de pirapitinga (*Piaractus brachypomus*) expostos durante 96 horas. A concentração letal 50 foi de 97.47mg.L^{-1} (P<0.05). Nas brânquias foram achadas lesões proliferativas e necróticas. No fígado foi observada congestão, processos degenerativos, gotas hialinas e presença de vacúolos lipídicos nos hepatócitos. No estomago e na pele foi detectada hiperplasia ligeira das células de moco. Nesta ultima, adicionalmente foi observado engrossamento da epidermes com lesões necróticas, infiltração de células leucocitárias e acumulação de melanina. No cérebro foram observados focos de degeneração de somas neurais na região do telencéfalo junto com gliose e infiltração de células granulais eosinofilicas/células mast. Concluindo, as brânquias, o fígado, a pele e o cérebro são órgãos suscetíveis ao Roundup®. Adicionalmente, os efeitos sobre o sistema nervoso central poderiam reduzir a olfação nos peixes, tanto como o comportamento grupal e individual, a performance reprodutivo e desta forma, repercutir no nível populacional.

TERMOS DE INDEXAÇÃO: Roundup®, glifosato, toxicidade aguda, histopatologia, *Piaractus brachypomus.*

INTRODUCTION

Agrochemical products use in agriculture is a very common worldwide practice that causes contamination of water bodies. One of the widely used agrochemical products is glyphosate the herbicide (GP) - N (phosphonomethyl)

[1] Received on November 19, 2007.
Accepted for publication on September 12, 2008.

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glycine - known commercially as Roundup®. Aquatic ecosystems can be contaminated with agrochemical products by leaching, run-off or direct or indirect spraying, this latter by action of the wind (WHO 1994).

The Roundup® contains, in addition to GP, a cationic surfactant denominated polyoxyethylamine (POEA) that confers toxicological properties different from those of GP (Folmar et al. 1979). Previous studies have determined that the toxicity of Roundup® in fishes is given by the POEA and that its toxicity is affected by physical-chemical water parameters, such as pH, temperature and hardness (Folmar et al. 1979), as well as by the fish species with a LC_{50} (lethal concentration 50) for the Roundup® in teleosts ranging from 2.3 to 54.8mg L^{-1} (Folmar et al. 1979, Hildebrand et al. 1982, Jiraungkoorskul et al. 2002). Several authors have reported the development of necrotic and proliferative lesions, aneurysms and leukocyte infiltration in the gills (Nešković et al. 1996, Jiraungkoorskul et al. 2002, 2003), as well as degenerative changes, lipidic vacuolization and hyaline droplets in hepatocytes (Szarek et al. 2000, Jiraungkoorskul et al. 2003) in fishes exposed to commercial formulations of GP.

The aim of this study was to determine the acute toxicity of Roundup® in juveniles of cachama blanca (*Piaractus brachypomus*), as well as the histopathological lesions in the gills, liver, caudal kidney, skin, stomach, gut and brain after the exposure to different concentrations of the herbicide. Cachama blanca as a native species was chosen because it inhabits the basins of the Amazon and Orinoco rivers, areas that are affected by coca and poppy crops and in which fumigation with agrochemical products has become a common practice.

MATERIALS AND METHODS

Juveniles of cachama blanca of 44.1 ± 5.4g and 10.6 ± 0.9cm in length, clinically healthy, from the same spawning of the hatchery at the Instituto de Acuicultura de los Llanos (IALL) of the Universidad de los Llanos, (Villavicencio, Meta, Colombia) were used.

The commercial formulation of GP, Roundup® (Monsanto Co.) containing 48% GP, 15% POEA surfactant and 37% water, was obtained from a local commercial source.

The fish were acclimatized to the experimental conditions in the Bioassay Laboratory of IALL for ten days (altitude 422m, mean temperature 25°C) using underground water with the following characteristics: dissolved oxygen 7.2 ± 0.1mg L^{-1}, temperature 25.3 ± 0.3°C and pH 6.6. Before the acclimatization period, the fish were placed in a bath with 1% noniodized salt (NaCl) during one hour to eliminate ectoparasites. The animals were placed in constantly aerated, without filter- 64 L glass aquariums, fed twice daily (2% of the biomass of each group) with commercial fishfood and exposed to natural photoperiod. The fish were starved 24 h before the experimental phase and these were not fed during the experimental phase. A semi-static system with 50% exchange of water daily was used. The water parameters of quality, such as temperature, dissolved oxygen (Hach Portable LDO™ HQ10 Dissolved Oxygen Meter®, USA) and pH (Pinpoint pH Monitor®, American Marine Inc. USA) were measured twice daily. Additionally, calcium and magnesium concentrations at 24 and 96 h of the experimental phase were measured with a flame atomic absorption spectrometer (AAnalist 100 Atomic Absorption Spectrometer®, Perkin Elmer Instrument). To measure fish weight and length and to collect samples at the end of the experimental phase, the animals were tranquilized by immersion in cold (5°C) water.

The animals were distributed in six groups [Treatment (T) 0 (control, Roundup® free; *n*=21), T 1 (7.5mg L^{-1}, *n*=21), T 2 (15mg L^{-1}, *n*=21), T 3 (30mg L^{-1}, *n*=21), T 4 (60mg L^{-1}, *n*=21) and T 5 (120mg L^{-1}, *n*=14)] (*n*=7 in each aquarium). Three replications for T´s 0-4 and two replications for T 5 were made. The concentrations of T's were based in preliminary tests (Ramírez-Duarte et al. 2004) and these are shown as nominal concentrations. The Roundup® exposure was carried out for 96 h.

From the fish that had died during the experimental phase, and at 96 h from two surviving fish of each group desensitized by demedulation through a dorsal operculum cut behind the skull, a macrocospic examination was performed and samples of gill, liver, caudal kidney, stomach, anterior intestine, skin and brain were removed for histopathological examination. Necropsy was carried out following the protocol described by Reimschuessel et al. (1988). For branchial histopathological evaluation the second left branchial arc was used. Samples were fixed in 3.7% buffered formalin for histological processing. Tissue samples (4-5μm thick) were stained with traditional hematoxyline-eosin (HE). Histopathological evaluation was carried out in a Nikon ECLIPSE 80i (Nikon Corporation, Japan) optical microscope using a DS-5M camera (DS Camera Head + DS Camera Control Unit DS-L1, Nikon Corporation, Japan).

Necropsy and histopathological findings are described in agreement with the degree of severity of each lesion, comparing between T's.

LC_{50} was determined using the Trimmed Spearman-Karber (TSK) version 1.0 software. Water quality parameters were analyzed by one-way analysis of variance (ANOVA) with subsequent Tukey–Kramer test with GraphPad Instat® software (GraphPad Software, Inc.Version 3.06 for Windows).

RESULTS

Water quality parameters remained steady during the experimental phase (data not shown). Nevertheless, the pH gradually decreased in Roundup®-exposed T's, with significant difference between T 0 and T's 3-5 ($P<0.05$).

Lethal concentration 50 (LC_{50})

During the experimental phase 10 animals died from the 14 that composed T 5. Mortality analysis showed an LC_{50} value of 97.47 mg.L^{-1} of the Roundup® ($P<0.05$).

Necropsy findings

Animals of T 0 showed no alterations in necropsy examination. In T's 4 and 5 there appeared branchial congestion, erosion of branchial filaments and the presence of grayish material in the distal third of the filaments. In T 5 there was also a moderate increase of mucous production in the skin. In all T's, the wall of the stomach was congested and size of the liver increased with cleared edges and, additionally, the T's 4 and 5 presented a multifocal whitish colour on the surface of the liver.

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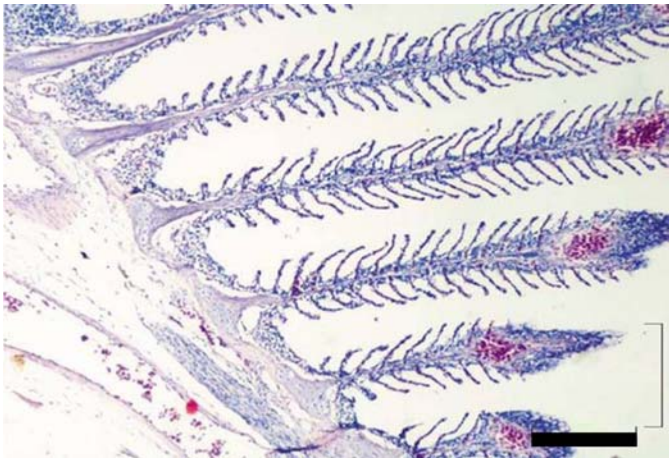

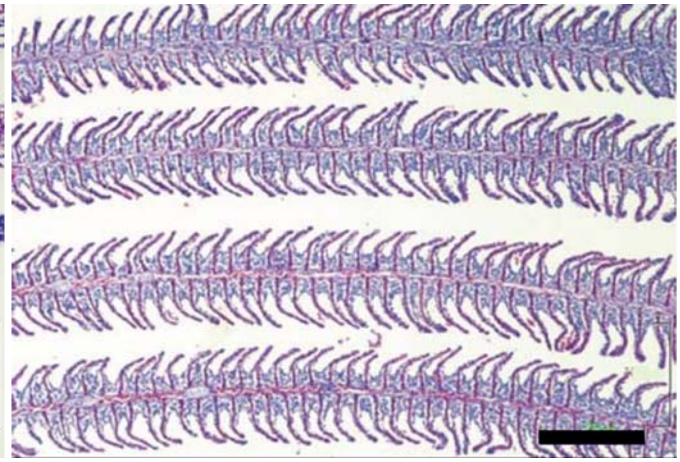

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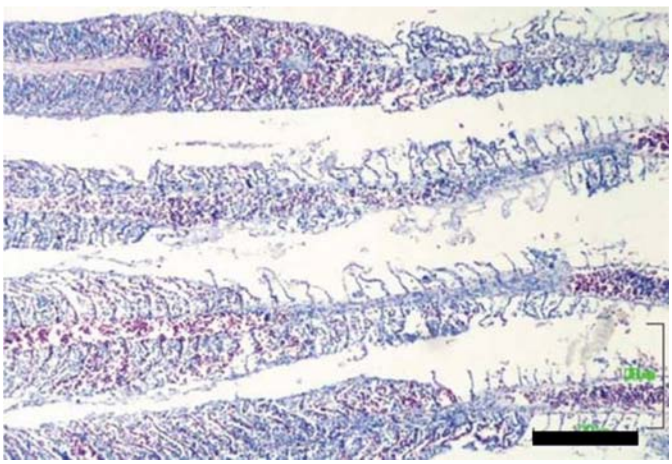

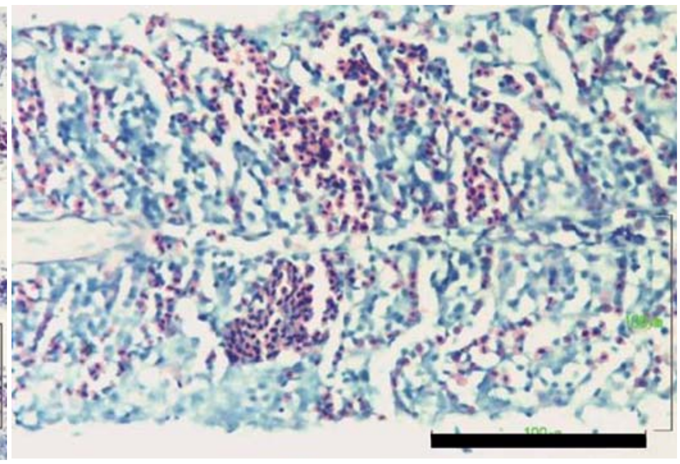

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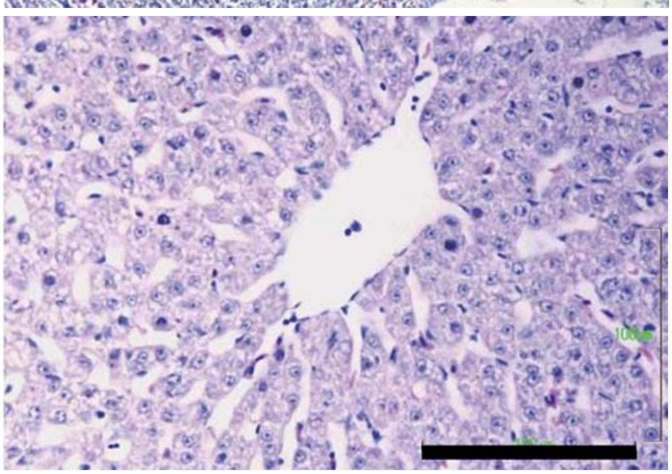

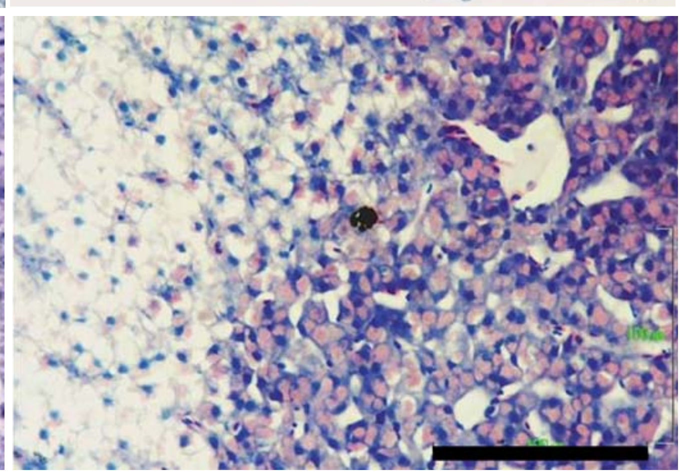

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Fig.1. Gill of T 0. They are present as long and thin lamellae, with thin lamellar and interlamellar epithelium. Scale bar 100µm. HE, obj.10x.

Fig.3. Gill of a T 5 fish that died during the course of the experimental phase. Visible are lamellar fusion in the distal half of the filaments and lamellar necrosis in the proximal half. Scale bar 100µm. HE, obj.10x.

Fig.5. Liver of T 0. Slight lipidic vacuolization of the hepatocytes is observed, preserving the homogeneity of its size. Scale bar 100µm. HE, obj.40x.

Fig.2. Gill of T 3. Generalized interlamellar hyperplasia from moderate to severe. Scale bar 100µm. HE, obj.10x.

Fig.4. Gill of a T 5 fish that died during the course of the experimental phase. Lamellar fusion and microhemorrhages are visible. Scale bar 100µm. HE, obj.40x.

Fig.6. Liver of T 5. A zone of cytoplasmic lipidic vacuolization with indistinguishable cell boundaries together with other zones of greater cytoplasmic density are shown. Hyaline droplets in the lipidic vacuolization area and in areas of greater cytoplasmic density are visible, and more frequent in the latter. MMC constitutes a fortuitous finding (MMC: melanomacrophage center). Scale bar 100µm. HE, obj.40x.

Histopathological findings

Gills. Histopathological examination of gills revealed morphological differences between T 0 and T's 1-5. In T 0 (Fig.1) lamellae were long and thin, with a slight lifting of lamellar epithelium, conserving the epithelial cells normal. Slight multifocal processes of interlamellar hyperplasia and infiltration of mononuclear cells in lamellae were rare.

Branchial injuries of greater severity were observed at higher Roundup® concentrations. In all Roundup®-exposed T's, generalized proliferative branchitis appeared (Fig.2), accompanied by lifting of lamellar epithelium, hypertrophy of epithelial cells and generalized congestion, as well as a reduction of the length and folding of the lamellae. In T 2, the foci of necrosis of the lamellar epithelium were accentuated with greater severity at higher concentrations (Fig.3) and lamellar denudation or the formation of pseudoepithelium that joins the distal ends of lamellae, mainly in the proximal half of the filaments; in distal half, lamellar fusion, microhemorrhages (Fig.3 and 4) and aneurisms were observed. Additionally, in T's 2-5 hyperplasia and hypertrophy of chloride-compatible cells and of mucous cells were present in interlamellar spaces and in the pharyngeal region, accompanied by severe infiltration of mononuclear cells.

Liver. In T 0 (Fig.5) the liver maintained its normal morphology, except for slight congestion and lipidic vacuolization of the hepatocytes. The hepatocytes appeared homogeneous both in size and cytoplasm density.

Congestion and the lipidic vacuolization were common to all T's. Between T 1 and T 0 there were not differences in severity. In T's 2-5, congestion and lipidic vacuolization were accentuated and more severe at higher concentrations, presenting areas of lipidic vacuolization

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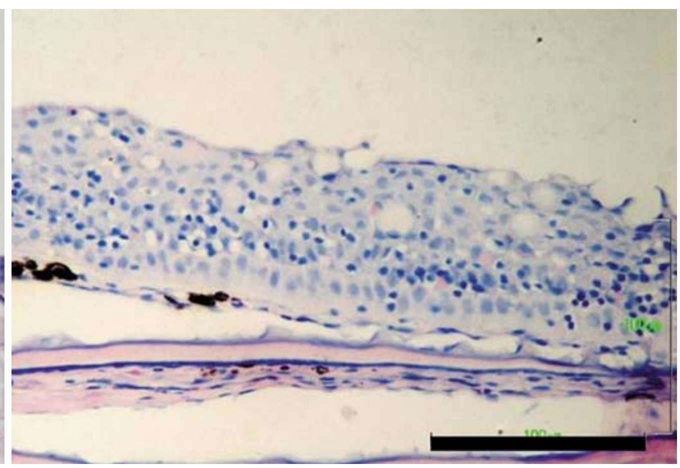
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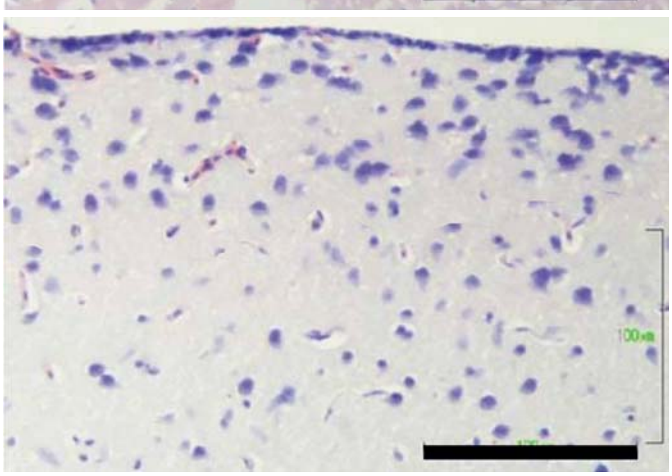

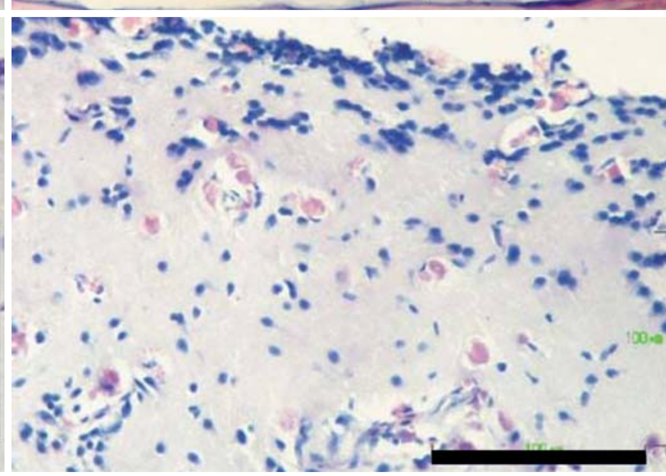
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Fig.7. Skin of T 0. Normal morphology of the epidermis, that appears thin with a regular surface and the dermis. Scale bar 100µm. HE, obj.40x.

Fig.9. Brain of T 0. The normal structure of the brain and meninges is presented. Scale bar 100µm. HE, obj.40x.

Fig.8. Skin of a T 5 fish that died during the course of the experimental phase. Increased epidermal thickness, festooned pattern of the epidermal surface, hyperplasia and hypertrophy of mucus cells, leukocyte infiltration into the epidermis and subepidermal levels accumulation of melanin are visible. Scale bar 100µm. HE, obj.40x.

Fig.10. Brain of T 1. Infiltrated EGC/MC in meninges and telencephalon cortex can be seen (EGC/MC: eosinophilic granule cells/mast cells). Scale bar 100µm. HE, obj.40x.

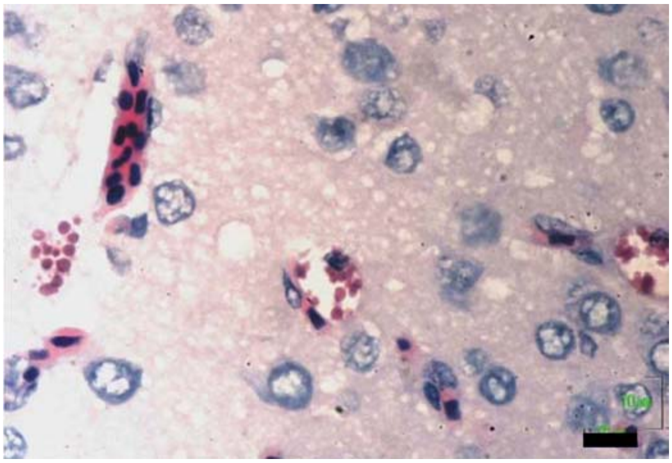

Fig.11. Brain of a T 5 fish that died during the course of the experimental phase. Visible are the degenerative processes of neuronal somas with apoptotic neuronal body-associated EGC/MC and EGC/MC with cytoplasmic hyaline droplets (EGC/MC: eosinophilic granule cells/mast cells). Scale bar 10µm. HE, obj.100x.

and areas of greater cytoplasm density in which the cytoplasm is occupied by strongly-eosinophilic, dense reticular material and for hyaline droplets (Fig.6). Additionally in T's 2-5, anisocytosis and multifocal necrotic processes were observed.

Skin. In T 0 (Fig.7), the skin presented a normal morphology with a thin epidermis and a regular epidermal surface. Small subepidermal accumulations of pigment were also visible.

In all Roundup®-exposed T´s the epidermis was thicker due to mild hyperplasia of pavimentous and mucous cells. Additionally, T's 3-5 presented a mild infiltration of mononuclear cells and spongiosis with necrosis of pavimentous cells (Fig.8). Although pigment accumulation was also visible in T 0, in T's 1-5 it was more generalized and more frequent.

Kidney. T 0 showed a normal morphology. In all T's melanomacrophage centers (MMC) were detected; they were more frequent in Roundup®-exposed T's. In fish of T 5, congestion, multifocal hemorrhages in organ periphery and multifocal necrosis of the tubular epithelium were observed.

Intestine. There were no morphological differences between T 0 and Roundup®-exposed T's.

Stomach. T 0 presented a normal morphology. In T's 3-5, mild hyperplasia of mucous cells was visible.

Brain. In brain, considerable differences between T 0 (Fig.9) and T's 1-5 were observed. In T's 1-5 and one of the animals of T 0 there was infiltration of eosinophilic granule cells/mast cells (EGC/MC) in the meninges and cerebral cortex (Fig.10), mainly in the telencephalon, associated with degenerate neuronal bodies (some undergoing necrosis and apoptosis) and with glyosis (Fig.11). EGC/MC had size increased with eosinophilic cytoplasmic components of different sizes (both fine granulations and hyaline droplets). In T 0, this finding was localized as opposed to the greater dispersion and number of neurons involved in such processes in T's 1-5, between which there were no differences in severity.

DISCUSSION

This study showed no variations in the water parameters of oxygen and temperature, or in calcium and magnesium concentrations. The differences in pH in T's 3-5, in comparison with T 0, are due to the natural acidity of GP (WHO 1994).

The LC_{50} values of Roundup® in teleost fish vary widely, from 2.3 to 54.8mg L^{-1} (Folmar et al. 1979, Hildebrand et al. 1982, Jiraungkoorskul et al. 2002). In comparison, the cachama blanca (LC_{50}: 97.47mg L^{-1}) is relatively more resistant to the acute toxicity of Roundup® herbicide. Acute toxicity of Roundup® in fish is a consequence of the POEA surfactant (Folmar et al. 1979).

In skin, necrosis of pavimentous cells could be due to a greater permeability of the cellular membrane induced by the surfactant that facilitate loss of intracellular electrolytes (Abel 1976, Stagg & Shuttleworth 1986) and that allow a greater access of GP to the cell where it exerts its cytotoxic effect (Monroy et al. 2005). The proliferative changes of mucous cells with slight degenerative changes in the epidermis observed in the present study indicate a reactive phase (Fig.8) which constitutes a general response to stress conditions (Wendelaar Bonga et al. 1984). Melanin accumulation together with leukocyte infiltration that constitute a defense barrier against microorganisms in front of which the skin can be more susceptible (Bols et al. 2001).

Increase in mucous production on the skin and gills of the Roundup®-exposed animals is a well-known response of fish in cases of pollution of the aquatic environment (Bols et al. 2001). Mucous material of grayish color observed in the gills of the animals of T's 4 and 5 consists of mucus, cellular detritus and blood. Mucous hypersecretion in gills facilitates the clearance, dilution and/or neutralization of toxics and pathogens (Ferguson et al. 1992) but it makes gas exchange more difficult by increasing the distance of branchial diffusion (Bols et al. 2001).

The severity of the branchial injuries increased with the concentration of Roundup®. Necrosis and rupture of branchial epithelium have been described as resulting directly from irritating compound. Lifting of the epithelium, lamellar fusion (Fig.3) and folding of lamellae could constitute a physiological adaptation whereby the fish reduce their vulnerable superficial area so as to maintain their osmoregulatory functions (Mallatt 1985, Cengiz & Ünlü 2002). These modifications reduce the diffusion of the toxic through the gills (Smart 1976). Necrosis of branchial epithelium could occur by a direct cytotoxic effect of the GP (Monroy et al. 2005) and/or in conjunction with alterations of the cellular permeability induced by the surfactant (Abel 1976, Stagg & Shuttleworth 1986).

Hyperplasia of mucous cells, in addition to reduce diffusion of xenobiotics through the branchial epithelium, constitutes a response to possible electrolytic imbalance caused by the greater branchial permeability (Lin & Randall 1995). Increase of size of chloride-compatible cells can constitute an compensating response to osmotic stress (Lin & Randall 1995) and could be implicated in the acclimatization to possible acid stress (Haaparanta et al. 1997) generated in T's 4 and 5 by the acute decrease of the pH by approximately one unit.

The microvascular changes shown in this study have been observed in states of hypoxia (Scott & Rogers 1980, Ishibashi et al. 2002). Additionally, the lamellar hemorrhages and aneurysms may be directly associated with the irritating action of the Roundup®. According to Roberts & Rodger (2001) the lamellar aneurysms occur by rupture of pillar cells, which allows expansion of vascular space and predisposes to the development of hemorrhages when the noxious agents cannot be removed from the surface of the gills (Jiraungkoorskul et al. 2002).

The branchial injuries described here affect breathing, osmoregulation, acid-base balance, and excretion of nitrogenous residues. In cases of transitory or prolonged exposure to sublethal concentrations of toxics, an acute toxic event cannot appear, but such conditions could favor the development of infectious diseases (Bols et al. 2001).

Hepatic lesions demonstrate morphological differences between T 0 and Roundup®-exposed T's. Lipidic vacuolization (Fig.5 and 6), a common finding for all T's, could be induced or favored by a period of starvation. However, it´s was more severe at higher concentrations. Moreover, in T 2 eosinophilic reticular material began to appear in the cytoplasm of the hepatocytes and increased with increasing Roundup® concentrations, generating cytoplasmatic hyaline droplets in T 5 (Fig.6). These hyaline droplets have been described by Szarek et al. (2000) and by Jiraungkoorskul et al. (2003) in others fishes exposed to Roundup®. The hyaline droplets may represent alterations in the protein metabolism and synthesis (Papadimitriou et al. 2000, Jiraungkoorskul et al. 2003) due to failures in the transcription process caused by Roundup® (Marc et al. 2005). Failures in transcription and protein synthesis could be related to lipidic changes of greater severity found in Roundup®-exposed fish due to alterations in lipoprotein synthesis necessary for the release and transport of lipids from the hepatocytes (Jones et al. 1997). Nevertheless, lipidic changes could be a strategy of the fish leading to concentrating lipophylic xenobiotics such as the POEA surfactant with the purpose of reducing its bio-availability (Cooley et al. 2000, Sarkar et al. 2005).

In kidney, significant morphological differences between the Roundup®-exposed fish and T 0 were not found, contrasting with the report of Jiraungkoorskul et al. (2002). The microvascular changes found in the kidneys of animals that died during the experiment would be part of the shock process (Scott & Rogers 1980).

Brain alterations indicate degenerative neuronal processes (Fig.11) that have not been described following exposures to herbicides. These components can gain access to the central nervous system through the olfactory epithelium and then to the olfactory bulb in the telencephalon, this could agree with the focalization of degenerative neuronal processes to the olfactory bulb, and/or through the blood/brain barrier where the surfactant components because of their amphiphylic character and incorporation into biological membranes could facilitate access of GP and its own passage (Stock & Holloway 1993). Predisposition of the olfactory bulb to degenerative foci could be generated by its communication with the olfactory epithelium and/or by the presence of proliferation centers and neural regeneration (Lema et al. 2005) where Roundup® components might induce alterations in the control points of the cellular cycle or in transcription processes that occur during neurogenesis (Marc et al. 2002, 2003, 2004a,b, 2005). The EGC/MC present in meninges and in the telencephalon cortex follow a pattern of satellitosis around neurons with degenerative foci, possibly triggering or accelerating apoptosis (Fig.10-11). However, apoptosis in fish brain occurs physiologically (Zupanc 2006). The pattern observed suggests a very local and specific process in which a generalized reaction in the cerebral parenchyma has not occurred. EGC/MC have been described in teleost fish and are related to mast cells of higher vertebrates (Reite 1998). Recently, it has been shown that GP induces oxidative stress *in vitro* in human cells. Therefore, degenerative neuronal processes could be produced, partly, for oxidative stress (Gehin et al. 2006, Hultberg 2007).

Neuron-associated gliosis found in the telencephalon associated to degenerative neuronal changes in the absence of associated EGC/MC, could indicate that the EGC/MC do not induce degenerative changes of the neurons, but accelerate cell death or are involved in neural remodeling following damage of neuronal bodies in the olfactory bulb based on the synthesis and release capacity of neural growth factor (NGF) by mast cells (Williams et al. 1995).

Although one T 0 animal was also infiltrated by EGC/CM in the telencephalon, it should be emphasized that it showed very slight changes compared to the ones found in Roundup®-exposed animals. Additionally, neuronal degeneration was only observed in somas with associated EGC/MC.

The greater frequency of neuronal degeneration processes and apoptosis in the olfactory bulb could imply a direct effect of Roundup® on the central nervous system that could affect olfaction as well as individual and group behavior, the reproductive performance of the fish and, hence, to have repercussions at the population level.

CONCLUSIONS

In conclusion, exposure of "cachama blanca" juveniles to the Roundup® herbicide produces anatomo-pathological alterations in gills, liver, skin and brain at concentrations evaluated in the present study, suggesting that these organs are targets of the toxic effects of the herbicide. Furthermore, brain effects must be assessed.

Acknowledgements.- The research was supported by Instituto de Investigaciones de la Orinoquia Colombiana (Convocatoria 2004), Universidad de los Llanos, Villavicencio, Colombia. The authors are grateful to Anne-Lise Haenni for help in reviewing the manuscript and the IALL personal’s.

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