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Histopathological changes induced by lindane (γ-HCH) in various organs of fishes*

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SUMMARY: This paper reports the histological analysis of various organs of *Mugil* sp., *Cyprinus carpio* and *Barbus* sp. after an accidental discharge of lindane (γ-Hexachloro-cyclohexane – γ-HCH-) into the Barbate River (Cádiz, SW Spain). Histopathological alterations of gills, liver and kidney were detected. Fusion of the secondary lamella, increased raising of the branchial epithelium and intraepithelial edema in gills, reduction of the diameter of the hepatocytes and, in advanced cases, cellular vacuolisation with hypertrophy of the hepatocytes in liver, as well as tubular necrosis, desquamation and vacuolisation of tubular epithelial cells in kidney, were observed. Quantification of lindane in water and whole fish samples was performed using a gas chromatograph equipped with an electron capture detector. The results of this histological analysis of various fish tissues indicate a direct correlation between pesticide exposure and the histopathological disorders observed. Chemical analysis of the lindane in the water and fish tissues confirmed a causal relationship between exposure to this pesticide and the fish damage found.

Key words: lindane, histological disorders, environmental pollution, fish.

RESUMEN: CAMBIOS HISTOPATOLÓGICOS INDUCIDOS POR LINDANO (γ-HCH) EN VARIOS ÓRGANOS DE PECES – En este trabajo se realiza un análisis a nivel histológico de los efectos producidos en varios órganos de *Mugil* sp., *Cyprinus carpio* y *Barbus* sp. tras una descarga accidental de lindano (γ-Hexaclorociclohexano –γ-HCH-) en el río Barbate (Cádiz, S0 España). Se detectaron alteraciones histopatológicas principalmente en branquias hígado y riñón. En branquias fueron frecuentes las fusiones en las laminillas secundarias, las descamaciones en el epitelio branquial, así como la presencia de edemas. Por otro lado, en hígado se detectó una disminución del diámetro de los hepatocitos y, en casos avanzados, vacuolización celular e hipertrofia. Por último, en riñón se observó necrosis y descamación del epitelio del sistema tubular renal, así como una intensa vacuolización de las células epiteliales tubulares. Conjuntamente al análisis histológico, se cuantificó el contenido de lindano en agua y peces usando un cromatógrafo de gases equipado con un sistema de captura de electrones. Los resultados de los análisis histológicos pusieron en evidencia una correlación directa entre la exposición al pesticida/lindano y las alteraciones histopatológicas observadas. En resumen, la cuantificación del lindano en agua y muestras biológicas, confirmó una relación causa–efecto entre la exposición a este pesticida y los daños observados en los organismos.

Palabras clave: lindano, alteraciones histopatológicas, polución ambiental, peces.

INTRODUCTION

Pesticide pollution of natural water resources has become more frequent with more extensive use of pesticides. Organochlorides represent common pollutants in aquatic systems and could be present in different environments as a result of a combination of run-off from use in agriculture, atmospheric transfer and accidental discharge (Verma *et al*., 1975; Edwards, 1976; Pentreath, 1999). Organo-

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chloride insecticides, in particular, are stable compounds that persist in the environment and, therefore, have been increasingly identified as agents of chronic exposure in aquatic species (Verma *et al*., 1975; Janardan *et al*., 1984; Kungolos *et al*., 1999). Aquatic organisms quickly accumulate and store lindane. Fish are very susceptible to bioaccumulation in their fatty tissues, as they take up lindane residues from the water through the gills and skin (Gopal *et al.,* 1993; Ortiz *et al*., 2002).

In fish, gills are critical organs for their respiratory and osmoregulatory functions. Respiratory distress is one of the early symptoms of pesticide poisoning (McDonald, 1983). According to Skidmore and Tovell (1972), in the gills these toxicants appear to break down the adhesion between epithelial branchial cells and the underlying pillar cells; this is accompanied by a collapse of the structural integrity of the secondary lamellae and subsequent failure of the respiratory functioning of the gills.

The liver is a very important organ performing vital functions such as detoxification, synthesis of several components of blood plasma, glycogen storage and release of glucose to the blood. Morphological, histological and histopathological alterations related to pesticide presence in the liver of fish have been studied, showing that these substances cause severe damage to the liver cells (Ahmad and Srivastava, 1985; Dutta *et al*., 1993; Ortiz *et al*., 2002).

In fish, as in higher vertebrates, the kidney performs an important function related to electrolyte and water balance and the maintenance of a stable internal environment. The kidney excretes nitrogen-containing waste products from the metabolism such as ammonia, urea and creatinin. Following exposure of fish to toxic agents such as pesticides, histological alterations have been found at the level of the tubular epithelium and glomerulus (Teh *et al*., 1997).

The Barbate River (Cádiz, SW Spain) is surrounded by agricultural areas, mostly ricefields, which are sprayed to a great extent with a number of pesticides, especially insecticides such as lindane (Ortiz *et al*., 2002). Thus, fish populations living in the water are chronically exposed to these substances. Moreover, accidental discharges increase the environmental concentrations of these pesticides, causing massive mortalities. The present study was undertaken to evaluate the histopathological alterations induced in several species of fish, with special emphasis on the changes in the liver, gills and kidney, following an accidental discharge of lindane into the Barbate River, in order to distinguish between chronic and acute alterations and to quantify the concentration of this pesticide in pooled samples from whole fish.

MATERIALS AND METHODS

Immediately after the discharge, abnormal behavioural responses were seen in the fish from the Barbate River (Cadiz, SW Spain) (Fig. 1a). About one hundred fishes appeared concentrated in the surface water showing uncoordinated movements. Massive mortalities were observed. Two hours after the spill, several moribund adult specimens of *Mugil* sp*., Cyprinus carpio* and *Barbus* sp (average weight 30 g and average length 20 cm) were collected. Gills, liver and kidney from 15 adult organisms were immediately extracted, fixed in Bouin's fluid and taken to the laboratory. Control fish were obtained in upper water of the same river from the discharge points. Tissues were washed in running tap water, dehydrated in alcohol and acetone, cleared in benzene, and embedded in paraffin wax. Sections (6-7 μm) were cut and mounting on gelatinised slides using a rotary microtome. Sections were rehydrated in distilled water and Haematoxylin-eosin and Haematoxylin-VOF (light green, orange-G and acid fuchsin). Morphological techniques were performed according to Gutiérrez *et al*.(1986) and Sarasquete *et al*. (1995).

For organochloride quantification, whole fish were lyophilised before being finely crushed and homogenised. Lindane was extracted using an automatic Soxhlet system with a 1:1 mixture of hexane:dichloromethane. The analyses were performed with a gas chromatograph equipped with an electron capture detector.

RESULTS

The lindane contents found in water and fish samples are shown in Table 1. There was a strong relationship between the chromatograph peaks obtained from the well-known organochloride insec-

TABLE 1. – Organochloride lindane levels in fish (μg/kg dry weight, whole body content) and water (μg/L) from Barbate river (Cádiz, SW Spain). The results are expressed as mean values (n=15, ± st. dev.).

Parameter	Water (μg/L)	*Cyprinus carpio* (μg/kg)	*Barbus* sp. (μg/kg)	*Mugil* sp. (μg/kg)
γ-HCH	0.30 (±0.05)	<0.07	<0.2	0.07 (±0.01)

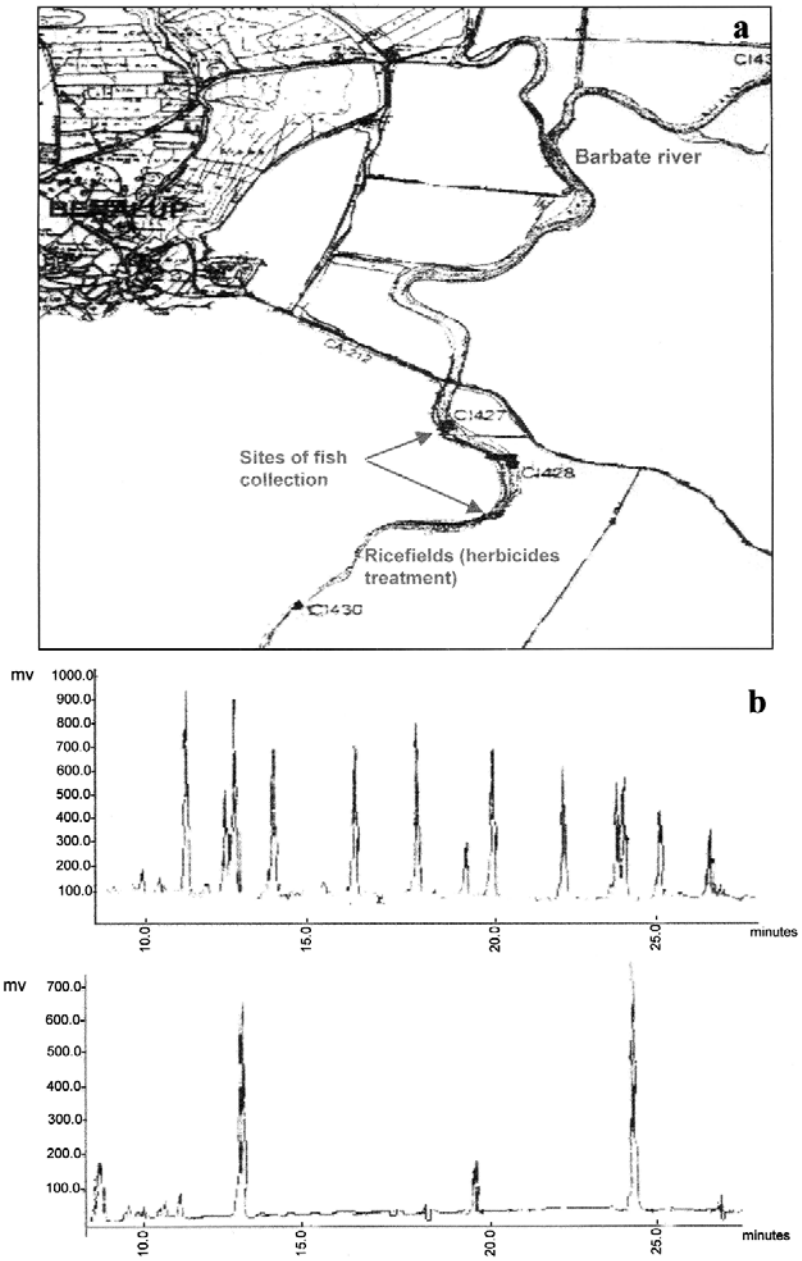

FIG. 1. – **a**, sampling sites in the Barbate river (Cádiz, SW Spain); **b**, lindane concentrations in water and whole fish.

ticide, Lindane (Fig. 1b, bottom) and those corresponding to the lyophilised fish (Fig. 1b, top).

The main histopathological alterations recorded were similar in all three examined species. A brief description of the histological characteristics of reference fish is given before the histopathological disorders found are described.

The liver, gill and kidney morphology of the reference fish is similar to that of other teleost fish species (Myers *et al*., 1987; Richmonds and Dutta, 1989; Hinton, 1994). The gill is made up of filaments or primary lamellae arranged in double rows. Secondary lamellae arise from these filaments. The secondary lamellae are lined by a squamous epithelium. Below that epithelium are lamellar blood sinuses separated by pillar cells. Between the secondary lamellae, the primary lamella is lined by a thick stratified epithelium. This region contains numerous mucous and chloride cells (Fig. 2a).

Contamination with lindane produces some degenerative changes in gills. Dilatation of blood capillaries, hiperplasia of the epithelial lining of the secondary lamellae, necrosis and shortening of the secondary lamella (Fig. 2b), abnormal raising or

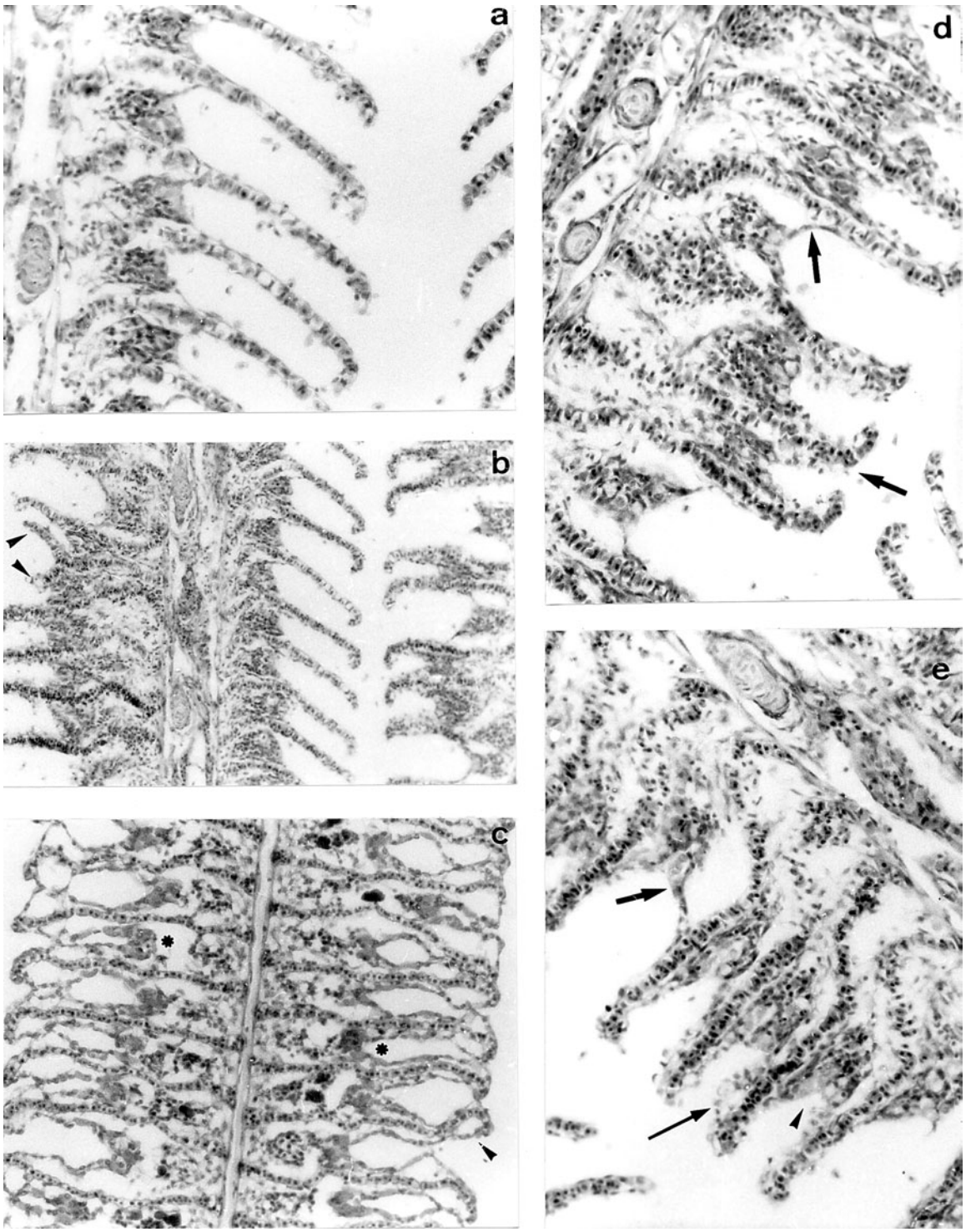

FIG. 2. – **a**, gills from reference (control) fish showing primary lamellae and secondary lamellae arising from these, parallel with them and perpendicular to the filament axis (H&E x400); **b**, shortening of the secondary lamellae (➢) (H&E x200); **c**, Edematous separation of the epithelial layer (➢) with necrotic cells occupying the interlamellar space (✳) (H&E x400); **d**, swelling of the epithelium and fusion of secondary lamella (➞)(H&E x400); **e**, hyperplasia (➢) and epithelial lining in the base (➞) and tips (→) of secondary lamella (H&E x400).

swelling of the epithelium (Fig. 2c), as well as fusion of the secondary lamellae (Fig. 2d and e) and excessive mucus secretion, were observed in contaminated fish.

The liver from reference fish is a large bi-lobed organ, having a homogeneous mass of polygonal hepatic cells or hepatocytes with centrally-located nuclei and a granular cytoplasm. The hepatocytes

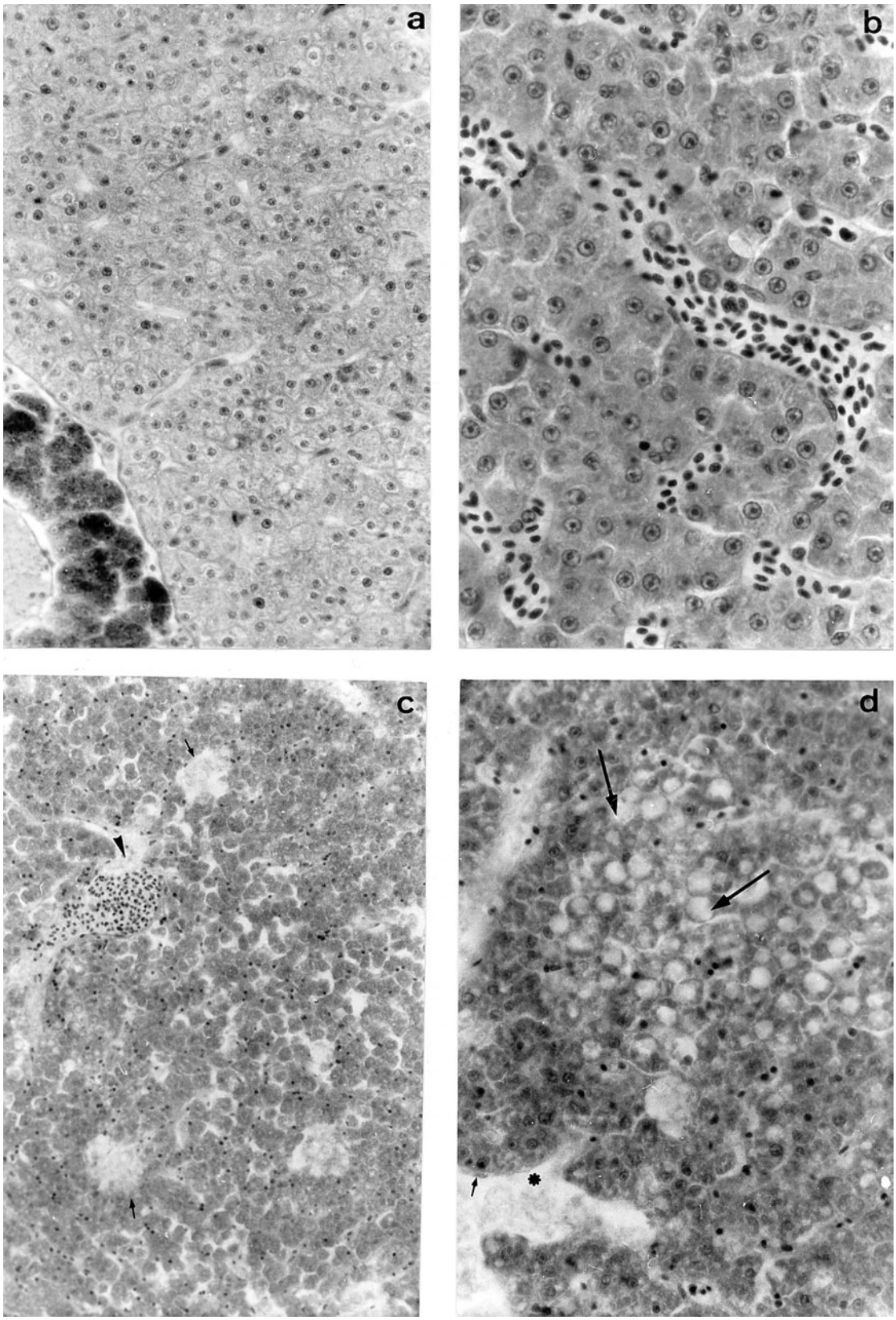

FIG. 3. – **a**, liver from control fish showing the exocrine pancreas around the blood vessels (H&E x200); **b**, parenchymatous distribution of the hepatocytes in cords around the sinusoids in control fish (H&E x400); **c**, dilation of blood sinusoids (➛) and parenchymal vacuolisation (→) in fish exposed to lindane (H&E x200); **d**, Cytoplasmatic vacuolisation (→), basophilia in some hepatocytes (✳) which have some pyknotic nuclei (→)(H&E x200).

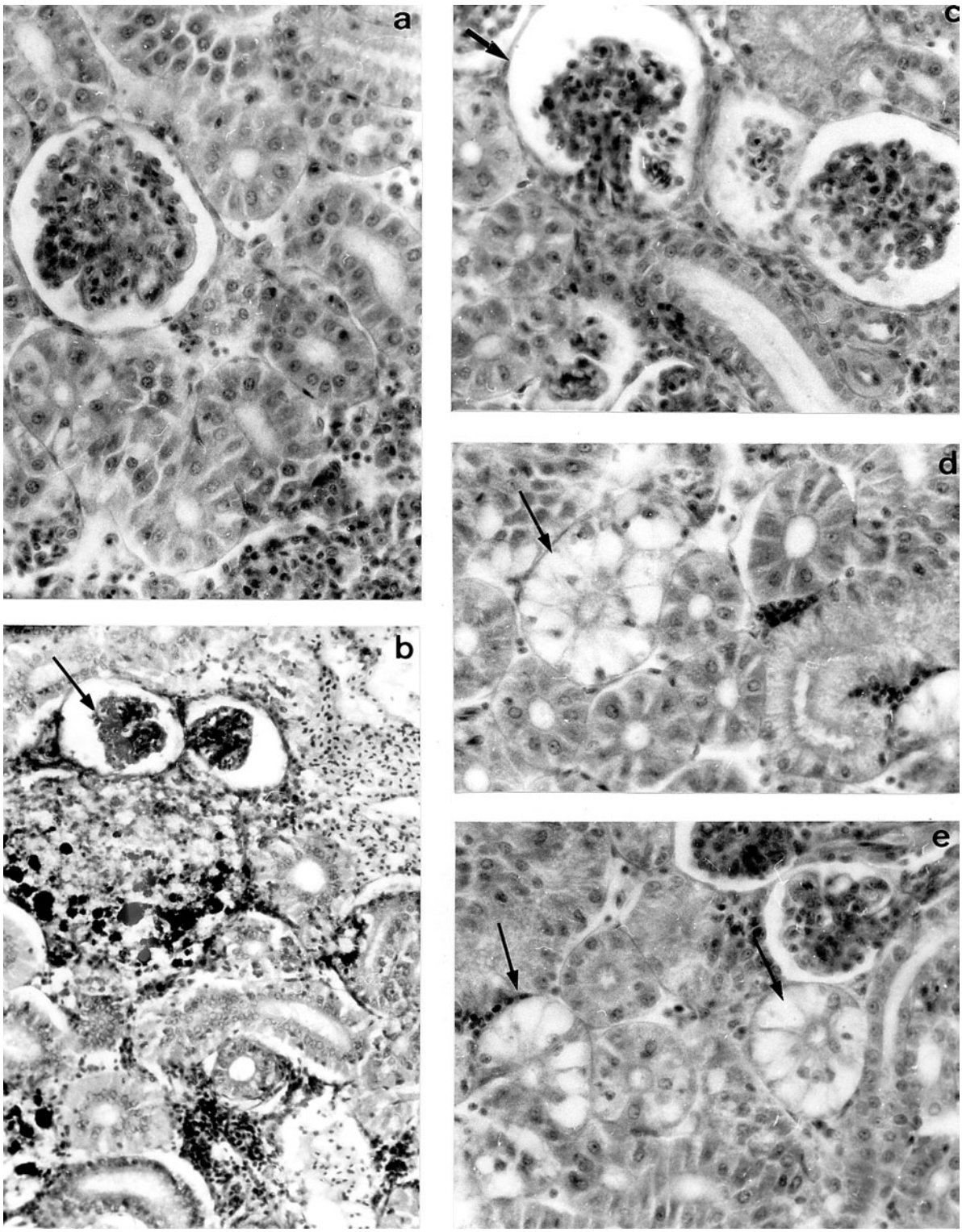

FIG. 4. – **a**, glomerulus and renal tubules from control fish (H&E x400); **b** and **c**, Shrinkage of glomerulus and expansion of space inside the Bowman's capsule (→)(H&E x400, H&Ex200); **d** and **e**, Intracytoplasmatic vacuoles in epithelial cells of renal tubules, with hypertrophied cells and lumen tubules diminished (→)(H&E x400).

enclose the bile canaliculi which open into the hepatic ducts. These cells are supported by a fine reticular network of connective tissue (Fig. 3a and b).

In lindane contaminated fish, the hepatic cells appeared compactly arranged with a strong cytoplasmatic vacuolisation (steatosis) and an increased

basophilia within the cytoplasm of some hepatocytes (chronic toxicity responses), which had an eccentric and pyknotic nucleus. Hepatocellular necrosis with parenchymal vacuolisation (acute responses), hypertrophy of hepatocytes, hemorrhages and widening of blood sinusoids were also observed (Fig. 3c and d).

The functional unit of the kidney is the nephron. Morphologically, the nephron from fish of reference sites consists of the glomerulus, tubules and collecting ducts. The glomerulus is a cluster of capillaries surrounded by the Bowman's capsule. The microscopic picture of the glomerulus shows capillary spaces covered by endothelial cells on the inner side, which in their lumen contain nucleated blood cells. The space between capillaries is filled with mesangial cells. On the inner side of the Bowman´s capsule are found epithelial cells. The Bowman´s capsule extends to form the convoluted tubules. The proximal and distal convoluted tubules are covered by tall columnar epithelial cells with oval or round, centrally or basally located nuclei depending on the segment. The cells which cover the tubules are tall and columnar with a weak eosinophilic cytoplasm and showing apical microvilli or "brush border" towards the lumen (Fig. 4a).

In lindane polluted fish, the kidney showed a disintegration of the convoluted tubules, and large intracytoplasmic vacuoles in the epithelial cells of these tubules (acute responses) (Fig. 4d and e). A shrinkage of the glomerulus and increased space within the Bowman´s capsule were also shown (Fig. 4b and c).

DISCUSSION

The histopathological and chemical analyses performed in this study suggest a positive relationship between the accidental discharge of this pesticide and the occurrence of histological alterations in various organs of three different fish species *(Mugil, Cyprinus* and *Barbus)* from the Barbate River (Cádiz, SW Spain).

Several previous studies on the physiological and histological effects of pesticides confirm the toxicity of lindane and its harmful effects in different fish species (*Cyprinus carpio* LC50 (96 h): 90 µg/L; *Fundulus heteroclitus* LC50 (96 h): 60 µg/L; *Mugil cephalus* LC50 (96 h): 66 µg/L; and *Perca fluviatilis* LC50 (96 h): 68 µg/L (MacDonald, 1994).

For the protection of aquatic life, the U.S. Water Environment Federation (1992) established numerical criteria for priority toxic pollutants and stipulated 0.099 µg/L as the toxic level for lindane dissolved in freshwater and 0.16 µg/L in seawater.

Our quantification results for lindane concentration are expressed per unit of whole body dry weight, so that they can be compared with the results from previous studies of water pollution. In a previous study (Geyer *et al*., 1994), the level of lindane in water was 5.5 ng/L and the corresponding levels in fish were 18 µg/kg dry weight in roach and 60 µg/kg dry weight in perch. These results suggest that persistent low levels of these pollutants in the environment lead to high levels of bioaccumulation, and to adverse but sublethal effects in these organisms. In our case, a single event of accidental discharge of a large quantity of this organochloride contaminant resulted, in a short period of time, in high mortality and severe damage, but with short persistence in the environment and in the remaining living species (water content 0.30 µg/L, 0.07 µg/kg dry weight, max. whole body content found).

Histopathological changes in the gills of fishes due to pesticides and other contaminants have been reported by several authors (Mallatt, 1985; Richmonds and Dutta, 1989). Since the gills are the primary route for the entry of pesticide, and the liver is the main organ for detoxification (Dutta *et al*., 1993), these organs are preferentially discussed here.

According to Leino *et al*., (1987), the gills of pearl dace and fathead minnows from environmentally-polluted Canadian lakes exhibited various cellular, histological and histopathological changes, which may contribute to problems related to respiration and acid-basic balances. The severe damage in terms of necrosis and rupture of the gill epithelium resulted in hypoxia and respiratory failure. In addition, the fish showed problems in relation to ionic and acid-base balance.

The epithelial necrosis and rupture of the gill epithelium observed are direct responses to the action of lindane. The defense responses noticed are excessive mucus secretion, lifting up of the epithelium and lamellar fusion. The lifting of the epithelium increases the distance through which the toxicant has to travel to reach the blood stream. Lamellar fusion could be protective in that it diminishes the amount of vulnerable gill surface area (Mallatt, 1985). The result of these alterations in gills could be understood as a defense mechanism against exposure to pollutants rather than as an irreversible toxic effect.

Alterations in the liver may be useful as markers that indicate prior exposure to environmental stressors. Approximately 85% of teleosts liver volume is occupied by hepatocytes, the most numerous cell type. Stressor-associated alterations of hepatocytes may be found in the nucleus or the cytoplasm or both. Irregular nuclear profiles with scalloped edges have been reported after fish were exposed to pesticides (Hacking *et al*., 1978). With acute toxicity and cell death, necrotic changes in affected nuclei are easily recognised. Coagulative necrosis follows cessation of blood flow to an organ and is seen after exposure to toxicants. In this condition, cell membranes are maintained and the morphology of the organ is recognisable but the nuclei show clear areas centrally and chromatin clumps peripherally (karyolisis), fragmentation of nuclei (karyorrhexis), or condensation of chromatin into a single dense dot (pyknosis).

On the other hand, changes in staining properties of cytoplasm is a signal exposure to lethal levels of toxicants. One example is the loss of cytoplasmic basophilia associated with ribosomal shearing from endoplasmic reticulum and swollen cisternae of the latter. Moreover, loss of cytoplasmic hepatic glycogen is an early toxic response and may cause an apparent increase in cytoplasmic basophilia (Wethaak and Wester, 1996).

Randomly distributed lipid vacuoles are commonly observed in the hepatocyte cytoplasm of affected animals. According to Meyers and Hendricks (1985), after exposure to various toxicans, the cytoplasm of hepatocytes displays vacuoles that appeared as clear vesicles occupying the whole cytoplasm.

Braunbeck *et al.* (1990) observed hepatic steatosis (lipid accumulation) in zebra fish (*Brachydanio rerio*) induced by long-term exposure to γ-Hexachlorocyclohexane. These authors speculated that these clear vesicles of lipid origin might be the morphological expression of a blockage in the metabolism of hepatic triglycerides due to a defective synthesis of very low density lipoproteins, which are involved in the transport and mobilisation of hepatic triglycerides to extrahepatic tissues.

Hypertrophy of hepatocytes is a closely-related condition that occurs under chronic toxicity (Myers *et al*., 1987; Kent *et al*., 1988). Cellular hypertrophy is associated with exposure to pesticides such as Lindane and Aroclor 1,2,5,4 (Hinton *et al*., 1988; Klaunig *et al*., 1979). The histopathological alterations resulting from an exposure to lindane may lead to a reduction in the functional efficiency of the liver, leading to malfunctioning of several organ systems of the fish.

The kidney of fishes receives much the largest proportion of postbranchial blood, and therefore renal lesions might be expected to be good indicators of environmental pollution. Kendall (1975) found tubular degeneration and eosinophilic, proteinacious, intratubular casts and hyaline droplets, and an increase in the amount of hemosiderin-or melanin-like intertubular deposits in catfish (*Ictalurus punctatus*) exposed to methyl mercury.

In medaka specimens exposed to a lindane isomer, Wester and Canton (1986) found prominent glomerular hyalinosis as an indicator of renal toxicity. In *Mugil auratus* exposed to organic and inorganic mercury, Establier *et al*. (1978) observed renal epithelial necrosis, sloughing of the epithelium, and accumulation of necrotic debris within lumen of the renal tubules

As a conclusion, the findings of the present histological investigations demonstrate a direct correlation between pesticide exposure and histopathological disorders observed in several tissues. Chemical analysis of river water and fish tissue confirms a causal relationship between lindane exposure and the fish damage found.

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