Vol. 25: 39–52, 1996 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published May 9

Cytological alterations in the liver of rainbow trout *Oncorhynchus mykiss* after prolonged exposure to low concentrations of waterborne endosulfan

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ABSTRACT: In order to elucidate sublethal cytopathological alterations in hepatocytes, mature male rainbow trout *Oncorhynchus mykiss* were exposed to 1, 10, 50, and 100 ng l^{-1} technical grade endosulfan (Thiodan™, 70% α- and 30% β- isomers) for 28 d. Whereas stereological parameters, i.e. relative volumes and numbers of cell organelles, were unaffected, qualitative ultrastructural alterations were detectable from 10 ng l^{-1} endosulfan. The No-Observed-Effect Concentration (NOEC) for cytological alterations was determined to be 1 ng l^{-1} endosulfan, i.e. 3 orders of magnitude below the LC_{50} value. Cytological effects that were probably of an adaptive nature included proliferation of SER and circular arrays of RER indicating induction of mixed-function oxygenases (MFO) as well as an increase in lysosomal elements at 50 and 100 ng l^{-1} endosulfan, probably due to enhanced cellular turnover. In addition, at ≥50 ng l^{-1} endosulfan, degenerative effects such as dilation of intermembranous spaces in mitochondria, deformation of mitochondria, myelin formation in peroxisomes and cytoplasm, and vesiculation and dilation of RER cisternae were observed. Although there was no indication of specific sublethal modes of toxic action except for MFO induction, the present study indicates that endosulfan has toxic impacts at concentrations of environmental relevance.

KEY WORDS: Liver · Endosulfan · Sublethal toxicity · Rainbow trout · Fish · MFO

INTRODUCTION

The cyclodiene insecticide endosulfan was developed by Hoechst AG in 1954 (Thiodan™) and ranges among the most toxic pesticides for aquatic life, especially fish; it therefore has been registered as a 'priority pollutant' by the US Environmental Protection Agency (Holcombe et al. 1982, Janardan et al. 1984). In a more recent report, the WHO (1986) classified endosulfan in the category of technical products that are moderately hazardous. Acute toxicity for fish (96 h LC_{50}) varies from 0.1 µg l^{-1} in striped bass *Morone saxatilis* (Korn & Earnest 1974) and mosquito fish *Gambusia affinis* (Joshi et al. 1981) to 20 and 42 µg l^{-1} in eel *Anguilla anguilla* at 29 and 22°C, respectively (Ferrando et al. 1987). The LC_{50} in rainbow trout *Oncorhynchus mykiss* is 1.4 µg l^{-1} (Johnson & Finley 1980). For reviews on acute toxicity data for endosulfan, see Schimmel et al. (1977), Johnson & Finley (1980), Goebel et al. (1982), Verschueren (1983), Baier et al. (1985), Mayer & Ellersieck (1986), and Paul & Raut (1987).

Particularly in developing countries, endosulfan is in general use for pest control in jute, cotton, sugar cane and vegetable culture. Due to constant leaching, as well as superficial runoff during rainfall and accidental spillage, endosulfan has repeatedly been reported as a contaminant in surface waters of developing countries (Herzel 1972, van Dyk & Greeff 1977, Wall et al. 1978, Frank et al. 1982, Baier et al. 1985). In the Dutch section of the Rhine River, mean endosulfan concentrations of 0.1 to 0.3 µg l^{-1} have been measured, with maximum values of 0.81 µg l^{-1} occurring in 1969; since then decreasing concentrations have been reported

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(Wegmann & Greve 1978). However, endosulfan has been causally implicated in fish kills in the Rhine River (Braunbeck et al. 1990a, Greve & Wit 1971). Since bioaccumulation factors of endosulfan in fish may reach values ≥1000 after only 96 h and ≥2000 after 28 d (Schimmel et al. 1977, Verschueren 1983), the concentrations measured in the Rhine River after the chemical spill in 1986 (Deutsche Kommission zur Reinhaltung des Rheins 1986) could well be considered a cause of sublethal effects in fish (Braunbeck et al. 1994).

However, in contrast to acute toxicity data, relatively little information exists on the nature and degree of damage to fish after prolonged subacute and sublethal exposure to endosulfan. Pathological changes in brain and liver of fish exposed to endosulfan in the field revealed neurotoxic effects along with hepatocellular necrosis, oedema and toxic accumulations of lipids (Matthiessen & Roberts 1982).

In both mammals (Gupta & Gupta 1979, Tyagi et al. 1985) and fish (Jensen et al. 1991) endosulfan has been shown to function as a phenobarbital-type inducer of cytochrome P-450. In general, fish have repeatedly been shown not to respond to induction by phenobarbital-type compounds (Goksøyr et al. 1987, Kleinow et al. 1987, Gooch et al. 1989, Winston et al. 1989), although they express corresponding cytochrome P-450 IIB genes (Elskus & Stegeman 1989, Stegeman 1989, Miranda et al. 1990; for nomenclature, see Nebert et al. 1991). Since endosulfan is biotransformed in the liver of fish and excreted via the bile (Schoettger 1970), the liver may be expected to represent a suitable monitor organ for revealing sublethal changes of endosulfan intoxication. In fact, ultrastructural alterations in fish liver have been shown to be of high selectivity and sensitivity in detecting chronic toxicity and underlying toxic mechanism(s) of several groups of chemicals (Hacking et al. 1978, Couch & Courtney 1987, Hinton et al. 1987). Thus, in the present study ultrastructural alterations in the liver of mature male rainbow trout *Oncorhynchus mykiss* following subchronic exposure to endosulfan were used (1) to further diagnose the potential hazard by endosulfan in fish, (2) to provide morphological evidence of its potential cytochrome P-450 induction, and (3) to search for specific sublethal modes of action of endosulfan.

MATERIALS AND METHODS

Fish. Since most fish display pronounced sexual dimorphism in ultrastructural reactions to xenobiotics (Braunbeck et al. 1989), only male fish were used in this study. Mature male rainbow trout had been reared at the Institute for Water, Air and Soil Hygiene (Berlin, Germany) and were kept in lots of 20 in aquaria containing 220 l of continuously aerated water at 14.5 ± 0.5°C. Water was replaced in a flow-through system at a rate of 6 l h^{-1} controlled by rotameters. The photoperiod was 12:12 h. Fish were fed with commercially available trout chow (Trouvit™) at a rate of 1% body weight. No mortality could be recorded during the acclimation period. Average body weight and length at the beginning of the experiment were estimated to be 300 g and 30 cm, respectively.

Contaminant and treatment. Technical grade endosulfan (Thiodan™, 70% α- and 30% β-isomers; 6,7,8,9,10,10'-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzo-dioxathiepin-3-oxide, CAS no. 115-29-7) was kindly provided by Hoechst (Frankfurt, Germany). One mg endosulfan was added to 10 l double-distilled water and vigorously stirred for 24 h at 20°C. This stock solution (100 µg l^{-1}) was continuously added to the water input of the aquaria by peristaltic pumps to give final concentrations of 1, 10, 50, and 100 ng l^{-1} endosulfan. Concentrations of stock solutions and actual concentrations in the aquaria were determined by standard gas chromatography (capillary spectrophotometry) after concentration by liquid:liquid extraction; deviations from nominal concentrations were <10%. After an acclimation period of 14 d, fish were exposed to waterborne endosulfan for 28 d. To control for diurnal variation, all sampling was performed at midmorning.

Electron microscopy. Fish were anaesthetized in 4-amino benzoic acid ethyl ester (benzocaine; 50 mg l^{-1}). *In situ* cardiac perfusion fixation was accomplished through the ventricular wall using a 3.5 mm I.D. Tygon™ tube (Ismatec) and a blunt 1.2 mm steel needle with a terminal opening of 0.8 to 1.0 mm (Microlance™; Becton & Dickinson, Dublin, Ireland). The vasculature was flushed with fish physiological saline (0.9%; 4°C) containing 2% polyvinylpyrrolidone (PVP; Merck, Darmstadt, Germany) and 0.5% procaine hydrochloride (Serva, Heidelberg, Germany) for 30 s to remove blood cells. This was followed by 1.5% glutardialdehyde and 1.5% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer (pH 7.6) containing 2.5% PVP (4°C). Initial perfusion rate was adjusted to 12-15 ml min^{-1}. Livers were excised immediately after perfusion, immersed in perfusion fixative for at least 20 min, and cut into slices of 60 to 70 µm using an Oxford vibratome. Fixation was continued for 20 min in 2.5% glutardialdehyde in 0.1 M sodium cacodylate buffer (pH 7.6) containing 4% PVP and 0.05% calcium chloride (4°C). To distinguish peroxisomes from lysosomes, catalase peroxidatic activity was demonstrated using alkaline 3,3'-diaminobenzidine (DAB; Le Hir et al. 1979): Tissue slices were incubated in 10 mM Teorell-

Stenhagen buffer (pH 10.0) containing 5 mM DAB and 0.5% H_2O_2 for 60 min at 37°C in a shaking water bath. In control experiments, tissue slices were either incubated in an equivalent medium without H_2O_2, or preincubated for 30 min at 4°C in 10 mM Teorell-Stenhagen buffer containing 0.2 M 3-amino-1,2,4-triazole followed by incubation in the complete DAB medium, which also contained 0.2 M aminotriazole. After repeated rinsing in Teorell-Stenhagen and cacodylate buffers, tissue slices were postfixed for 1 h with 1% osmium ferrocyanide (Karnovsky 1971). After washing in 0.1 M cacodylate and 0.05 M maleate (pH 5.2) buffers, tissues were stained *en bloc* with 1% uranyl acetate in maleate buffer for 1 h. Specimens were dehydrated in a graded series of ethanol and embedded in Spurr's medium. Ultrathin sections of 60 to 80 nm thickness were stained with alkaline lead citrate for 30 s or 1 min and examined in a Zeiss EM 9 or a Zeiss EM 10 electron microscope.

Light microscopy. Semithin plastic sections of 0.5 to 0.75 µm thickness were stained with methylene blue-Azur II and used for orientation. For visualization of glycogen, semithin sections were incubated in an alkaline 1% solution of silver diamine for 1.5 h at 60°C (Singh 1964). After rinsing in distilled water, sections were mounted in Entellan and examined in a Leitz Aristoplan photomicroscope.

Stereological analyses. Multistage sampling and morphometrical evaluation were performed according to the principles of Weibel (1979). At all tiers, hepatocytes were selected to serve as a reference space. At each tier, 4 fish per group were evaluated. For details on sampling for stereological analysis, see Table 1.

Volume densities (V_i) were estimated by placing a lattice of P_T test points on micrographs and by determining the fraction (P_i/P_T) of these points enclosed within the profiles of the structure investigated (P_i). Test points falling on extracellular space (biliary tract, sinusoids, endothelia, space of Disse, etc.) were subtracted from the total number of test points. The volume density of nuclei was determined on light micrographs (1100×) using a test point lattice with 391 points spaced at regular intervals (d) of 10 mm (= 9.091 µm). For measurement of the volume density of hepatic mitochondria, lipid droplets and lysosomes, electron micrographs (7200×) and a test point lattice with 391 points spaced at d = 10 mm (= 1.388 µm) was employed. The volume densities of peroxisomes and the endoplasmic reticulum fields were measured on micrographs at a magnification of 18000× using a test point lattice with 391 points spaced at d = 10 mm (= 0.555 µm).

Numerical densities N_V of particulate hepatocellular structures within the test area were calculated according to the formula $N_V = b^{-1} N_A^{1.5}/V_V^{0.5}$ (Weibel 1979), where N_A is the number of actually counted particles divided by the test area, V_V is the volume density of the particle and b is a shape-dependent coefficient. This coefficient b was assumed to be 1.38 for lipid droplets, peroxisomes and lysosomes (Weibel 1979). Numerical densities of lipid droplets and lysosomes were calculated from electron micrographs at a magnification of 7200×, that of peroxisomes from micrographs at 18000×.

The numerical density of nuclei (N_{Nu}) was used to calculate the volume of hepatocytes: $V_{hep} = 1/N_{Nu}$. Absolute volumes and numbers of lysosomes, lipid droplets and peroxisomes and absolute volume of mitochondria per hepatocyte were estimated as $V_i \times$ cell volume and $N_V \times$ cell volume, respectively. The volumes and total numbers of organelles per hepatocyte were estimated as $V_V V_{hep}$ and $N_V V_{hep}$, respectively. Hepatocytic and nuclear diameters were calculated as the third root of the relevant volumes, assuming a spherical shape of the particles.

Rough endoplasmic reticulum (RER) surface densities (S_V) were calculated by using a line test grid (d = 10 mm) as the ratio of twice the number of intersections with RER cisternae to the total length of lines on the hepatocytes being examined (Weibel 1979). Total hepatocellular surface of RER cisternae was estimated as $S_V V_{hep}$.

Cell components not measured individually mainly included Golgi fields, smooth endoplasmic reticulum (SER), and cytoplasm. Mean values from stereological measurements were compared using Student's double-tailed t-test.

Table 1. Magnification of micrographs and stereological parameters investigated with respect to rainbow trout *Oncorhynchus mykiss* hepatocellular organelles. LM: light microscopy; EM: electron microscopy

Magnification	Section thickness	Volume density of	Numerical density (coefficient b)
1100× (LM)	500–700 nm	Nuclei Glycogen	Nuclei (1.38)
7200× (EM)	150–200 nm	Mitochondria Lysosomes Lipid droplets	Lysosomes (1.38) Lipid droplets (1.38)
18000× (EM)	150–200 nm	Peroxisomes RER	Peroxisomes (1.38)

RESULTS

Macroscopic observations

Following exposure to 1–100 ng l^{-1} endosulfan, no mortality was observed. However, at 100 ng l^{-1} endosulfan, male

rainbow trout showed reduced food intake in the initial phase of the experiment, whereas towards the end of the experiment and at lower endosulfan concentrations no differences from controls were evident.

Liver morphology and stereology

Qualitative and quantitative alterations of liver ultrastructure in controls and fish exposed to endosulfan are listed in Tables 2 & 3.

Controls

The liver of rainbow trout was characterized by uniform hepatocytes with extended, peripheral glycogen fields clearly separated from an organelle-containing perinuclear and peribiliary area (Fig. 1). Hepatocytes were regularly arranged around bile canaliculi (tubular architecture of rainbow trout liver; cf. Hampton et al. 1985, 1988) and closely associated with sinusoids (Fig. 1). Mean diameter of hepatocytes was 15.6 ± 0.8 µm; mean volume was about 1990 µm^3 (Table 3). Lipid inclusions were scant.

Table 2. *Oncorhynchus mykiss*. Semiquantitative analysis of ultrastructural alterations in the liver of rainbow trout following exposure to endosulfan. (Control = 0 ng l^{-1} of endosulfan). Data are given for 4 fish. Abbreviations: – not present, (+) very little developed, + little developed, ++ moderately developed, +++ strongly developed

	Control	1 ng l^{-1}	10 ng l^{-1}	50 ng l^{-1}	100 ng l^{-1}
Parenchymal organization					
Cellular heterogeneity	–	–	–	–	++
Nucleus					
Irregular outline	+	+	++	++	+++
Dilation of nuclear envelope	–	–	–	++	++
Two nucleoli	–	–	(+)	++	++
Mitochondria					
Structural integrity	+++	+++	+++	++	+
Myelin formation in intermembranous space	–	–	–	++	+++
Dilation of intermembranous space	–	–	–	++	++
Deformation	–	–	–	–	++
Peroxisomes					
Structural integrity	+++	+++	+++	+++	+++
Cluster formation	–	–	–	++	++
Heterogeneity of shape	+	+	++	++	++
Heterogeneity of matrix	–	–	–	–	++
Myelin formation in matrix	–	–	–	–	++
Lysosomes					
Structural integrity	+++	+++	+++	+++	+++
Peribiliar localization	++	++	++	(+)	(+)
Myelin formation	–	–	–	++	++
Autophagosomes	–	–	+	++	+++
Multivesicular bodies	–	–	–	++	++
Cytoplasmic myelin bodies	–	–	–	++	++
RER					
Vesiculation of cisternae	–	–	–	+	+
Dilation of cisternae	–	–	+	+	+
Parallel stacks of cisternae	+++	+++	+++	+++	+++
Concentric arrays	–	–	–	+	+
SER					
Peribiliar localization	++	++	++	(+)	(+)
Localization within glycogen field	–	–	(+)	++	+++
Quantity	+	+	+	++	+++
Golgi fields					
Structural heterogeneity	–	–	+	++	++
Activity	+++	+++	+++	+++	+++
VLDL particles	+++	+++	+++	++	++
Storage materials					
Lipid	–	–	–	–	–
Glycogen	+++	+++	+++	+++	+++
Macrophages					
Macrophage centers	–	–	–	–	++
Quantity	–	–	–	++	+++

Table 3. *Oncorhynchus mykiss*. Stereological analyses of ultrastructural alterations in hepatocytes of male rainbow trout following subchronic exposure (28 d) to 10, 50 and 100 ng l^{-1} endosulfan. (Control = 0 ng l^{-1}.) Data given as means ± SE for 4 fish. Differences of experimental groups from controls were evaluated by Student's double-tailed *t*-test (n = 4)

	Control	10 ng l^{-1}	50 ng l^{-1}	100 ng l^{-1}
Hepatocyte				
Total volume (μm^3)	1990.4 ± 284.7	2037.9 ± 223.8	2042.5 ± 361.2	2054.0 ± 442.5
Diameter (μm)	15.6 ± 0.8	15.7 ± 0.6	15.7 ± 0.9	15.7 ± 1.2
Nucleus				
Total volume (μm^3)	135.8 ± 23.6	128.7 ± 8.0	144.3 ± 26.8	111.7 ± 10.7
Diameter (μm)	6.4 ± 0.4	6.3 ± 0.1	6.5 ± 0.4	6.0 ± 0.2
Cytoplasm				
Total volume (μm^3)	1854.6 ± 265.1	1909.2 ± 217.6	1898.2 ± 335.6	1942.3 ± 436.8
Nuclear-cytoplasmic ratio	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.01
Mitochondria				
Total volume (μm^3)	105.3 ± 9.1	105.6 ± 11.6	120.9 ± 28.4	113.3 ± 13.2
Peroxisomes				
Total volume (μm^3)	28.3 ± 1.6	27.0 ± 7.8	34.5 ± 4.9	27.1 ± 7.5
Diameter (μm)	0.8 ± 0.03	0.8 ± 0.05	0.8 ± 0.05	0.8 ± 0.1
Lysosomes				
Total volume (μm^3)	10.8 ± 5.1	16.9 ± 3.7	6.9 ± 2.3	12.1 ± 6.0
Diameter (μm)	0.8 ± 0.1	0.9 ± 0.03	0.7 ± 0.06	0.7 ± 0.11
RER				
Total volume (μm^3)	516.9 ± 62.5	505.2 ± 74.7	535.3 ± 129.6	460.3 ± 188.3
Surface area (μm^2)	5223.1 ± 920.8	4352.2 ± 486.1	4854.7 ± 1047.8	4869.7 ± 1440.7
Lipid				
Total volume (μm^3)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Glycogen fields				
Total volume (μm^3)	737.1 ± 219.2	740.6 ± 85.0	711.4 ± 189.1	672.0 ± 203.1
Other cell components				
Total volume (μm^3)	456.1 ± 41.7	514.1 ± 103.0	489.1 ± 61.8	657.6 ± 164.0

The nuclei of hepatocytes were spherical with a mean diameter of 6.4 μm and showed slightly corrugated outlines and small amounts of heterochromatin randomly scattered over the nucleoplasm. The slightly eccentric nucleolus was distinctly separated into a *pars fibrosa* and a *pars granulosa*. The perinuclear area was characterized by a sheath consisting of stacks of up to 15 non-fenestrated parallel cisternae of the RER. This RER envelope was usually interrupted in the peribiliary area and free of glycogen, but interspersed and bordered by numerous mitochondria and peroxisomes. RER surface area and volume were ca 5200 μm^2 and 500 μm^3, respectively (Table 3). Mitochondria appeared as spherical or elongated profiles with numerous cristae and some intramitochondrial granules. As estimated by stereology, the mean volume of mitochondria was ca 105 μm^3 (Table 3). Each hepatocyte contained about 600 spherical peroxisomes, which accounted for about 28 μm^3 of the hepatocellular volume. Peroxisomes were usually closely associated with mitochondria at the outer border of the RER envelope and had a homogeneous, finely granular matrix.

Lysosomes, Golgi fields and SER were concentrated in the peribiliary area of the hepatocytes. SER, typically developed as an irregular network of tubular or vesicular profiles, was restricted to minute areas in the cytoplasm. Golgi fields were small and few in number and possessed 3 to 5 cisternae budding off numerous vesicles containing VLDL (Very Low Density Lipoprotein) granules. Lysosomes were large (0.8 ± 0.1 μm) but few in number (Figs. 1 & 21), and were restricted to the vicinity of bile canaliculi. Their heterogenous matrix was of high electron density.

Livers of fish exposed to endosulfan

Response in rainbow trout liver to technical grade endosulfan displayed clear dose-dependent cytological effects starting at 10 ng l^{-1} endosulfan (Table 2). No histological alterations in liver ultrastructure were evident after exposure to 1 ng l^{-1} endosulfan, thus indicating a No-Observed-Effect Concentration (NOEC) of 1 ng l^{-1}. The uniform appearance of hepatocytes was well preserved up to 50 ng l^{-1} endosulfan, whereas exposure to 100 ng l^{-1} endosulfan resulted in conspicuous structural heterogeneity of hepatocytes. However, by stereological methods, only minor quantitative

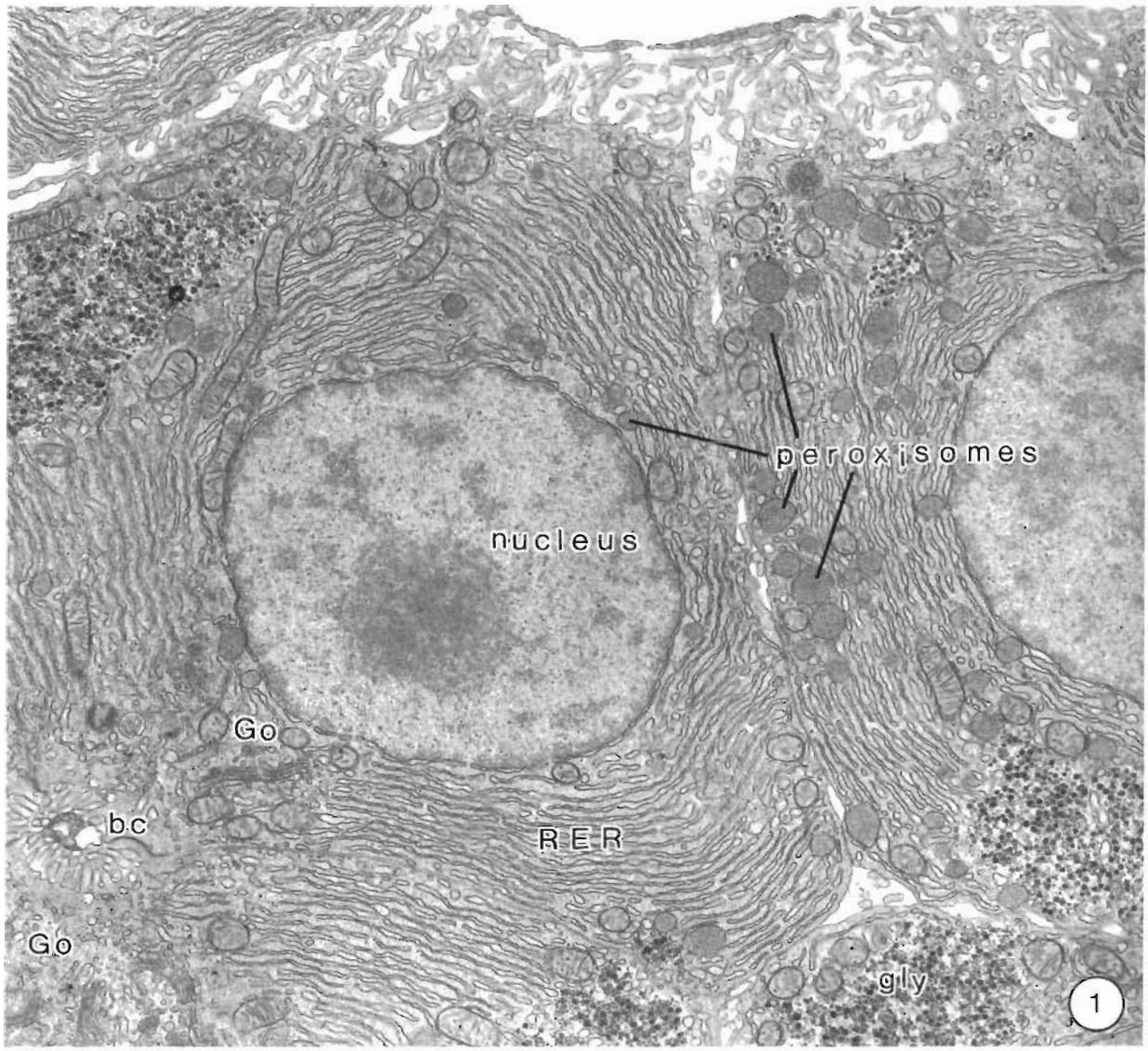


Fig 1 *Oncorhynchus mykiss* The ultrastructure of rainbow trout liver was characterized by a central nucleus surrounded by extensive stacks of parallel, non-fenestrated, rough endoplasmic reticulum (RER) bordered by numerous mitochondria and peroxisomes. The RER envelope was interrupted in the peribiliary area, where Golgi fields (Go), smooth endoplasmic reticulum and a small number of lysosomes were located close to the bile canaliculus (bc). gly: glycogen. 8000×

changes following exposure to endosulfan could be recorded (Table 3, Figs. 21 & 22).

Subchronic exposure to 10 ng l^{-1} endosulfan resulted in irregularities of the nuclear outline, amplification of nucleoli and an increase in structural heterogeneity of peroxisomes and Golgi fields. Furthermore, a few autophagosomes and a slight dilation of RER cisternae were discernible (Table 2) Following exposure to ≥50 ng l^{-1} the endosulfan effects already described for 10 ng l^{-1} endosulfan occurred, but were generally more pronounced (Table 2). In addition, from 50 ng l^{-1} onwards, the nuclear envelope appeared dilated and less regular (Fig 2). The RER basically preserved its regular arrangement in parallel stacks (Figs. 2 & 3). In contrast, dilation of cisternae, as was already evident from 10 ng l^{-1} endosulfan, was associated with slight vesiculation (Figs. 2 & 3) and formation of concentric arrays of RER cisternae. In addition, the relative volume of RER stacks slightly decreased from 26% in controls to about 22% following exposure to 100 ng l^{-1} endosulfan (Fig 22). The SER was clearly increased in volume from 50 ng l^{-1} endosulfan, with the strongest induction at 100 ng l^{-1} endosulfan (Table 2, semiquantitative data). In conjunction with the induction, SER profiles spread from the peribiliary area to a wider distribution within glycogen fields (Fig. 4).

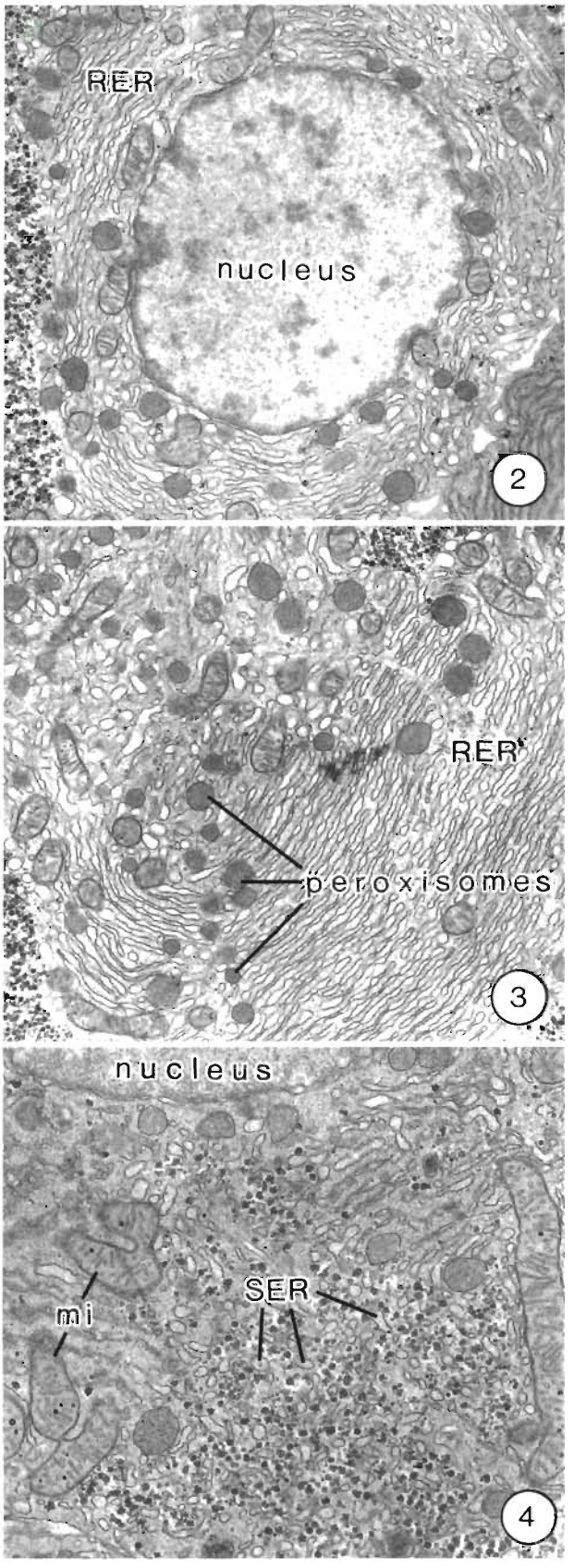
Effects in peroxisomes comprised cluster formation (Figs. 5 & 6) and, at 100 ng l^{-1} only, an increased heterogeneity of the matrix partly accompanied by conspicuous intraperoxisomal myelin formations (Fig. 7). Due to considerable variability in the stereological data, no changes could be documented in number and relative volume of peroxisomes (Figs. 21 & 22).

Mitochondria displayed severe loss of structural integrity as a result of myelin formation and dilation of the intermembranous space (Figs. 8 to 10). After exposure to 100 ng l^{-1} endosulfan only, mitochondria became severely deformed (Fig. 10). Again, however, no significant changes were seen in the stereological parameters for hepatocellular mitochondria following endosulfan exposure (Table 3, Figs. 21 & 22). Mean and relative volumes were similar to controls or only slightly changed, to a maximum of 120 μm^3 and 5.9% at 50 ng l^{-1} endosulfan, respectively.

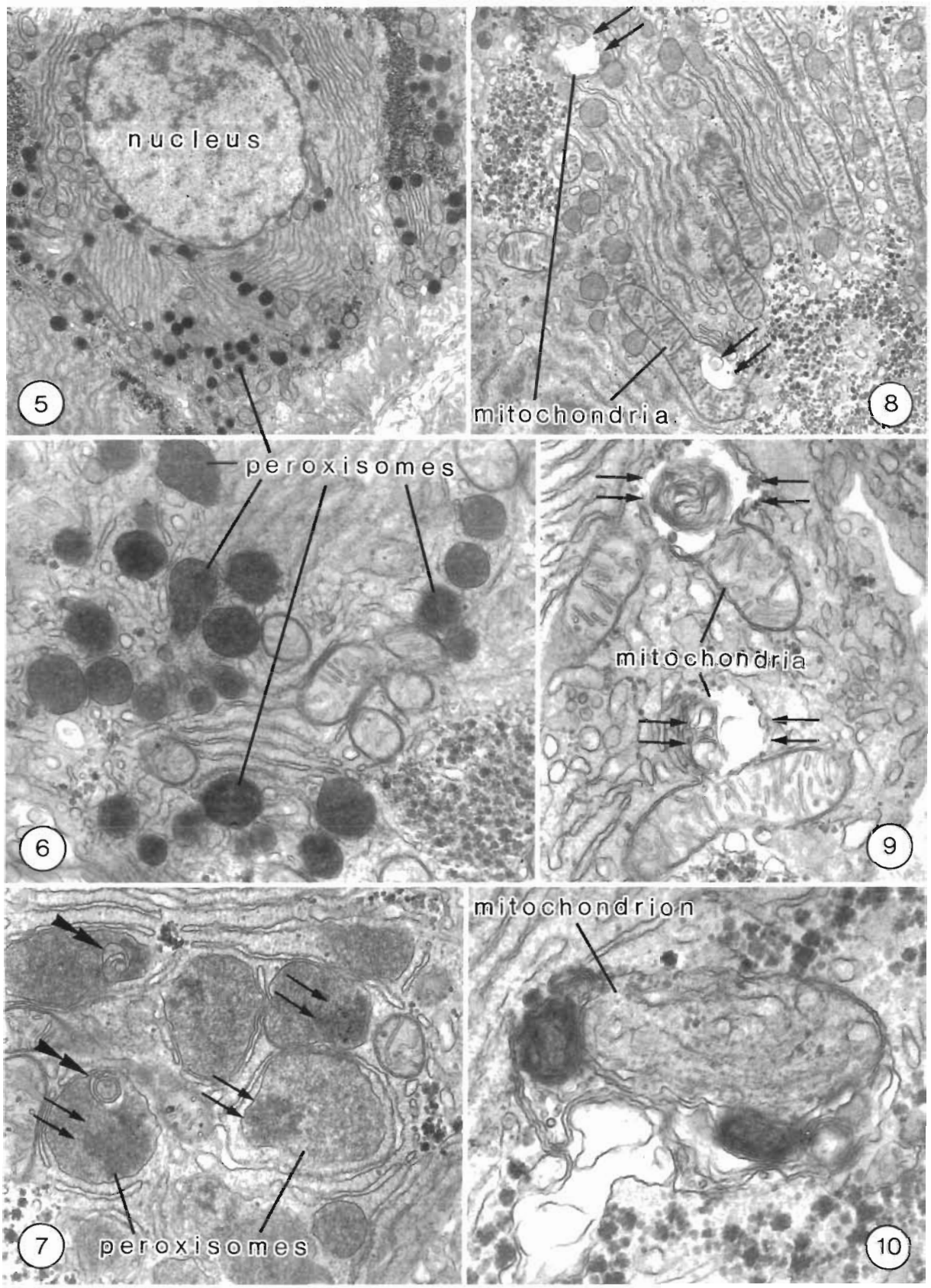
Lysosomes showed major morphological changes due to higher endosulfan exposure levels when compared to controls and the effects described for 10 ng l^{-1} endosulfan. As was true for SER, most lysosomes displayed a more random distribution within hepatocytes (Fig. 11). Furthermore, lysosomes with myelin formations (Fig. 12), multivesicular bodies (Fig 15) and cytoplasmic myelinated bodies (Fig. 16) were strongly developed at the 2 highest concentrations tested. The number of autophagosomes showed a clear dose-dependent increase (Figs. 13 & 14) In addition, the relative volume of macrovesicular lysosomes increased to an average of 20 profiles per hepatocyte following 100 ng l^{-1} endosulfan (Fig. 21).

Compared to 10 ng l^{-1} endosulfan, under higher exposures the structural heterogeneity of Golgi fields was further increased and VLDL secretion was reduced, whereas the activity of Golgi fields as estimated by vesicle formation was not affected by endosulfan exposure. Hepatic glycogen contents were not affected by endosulfan and remained at about 700 μm^3 per cell, accounting for 34% of the hepatocellular volume (Table 3, Fig. 22). As in controls, lipid inclusions were scant.

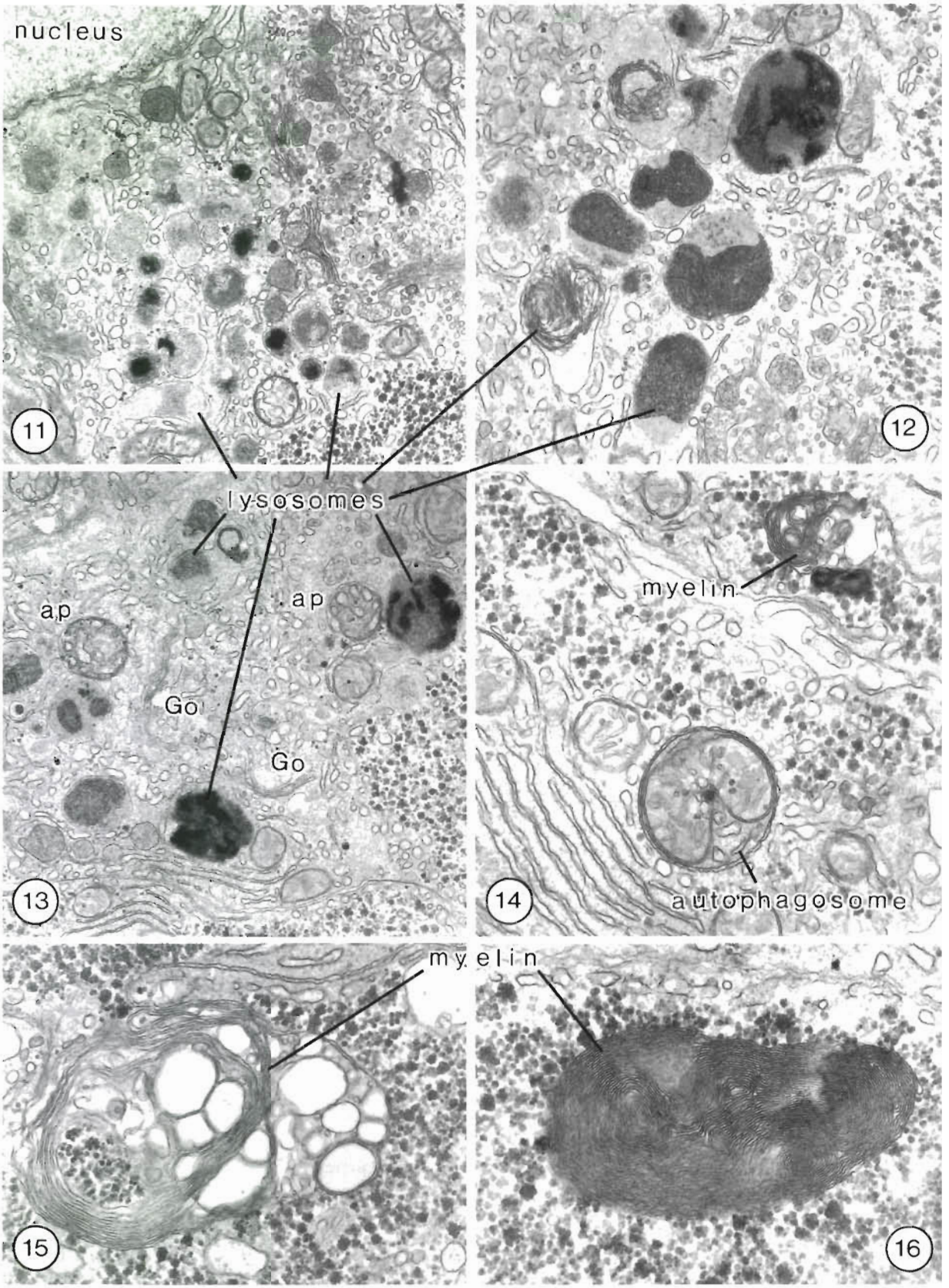
In addition to hepatocellular changes, numerous macrophages containing large amounts of materials ingested by phagocytosis, mainly in lysosomes and



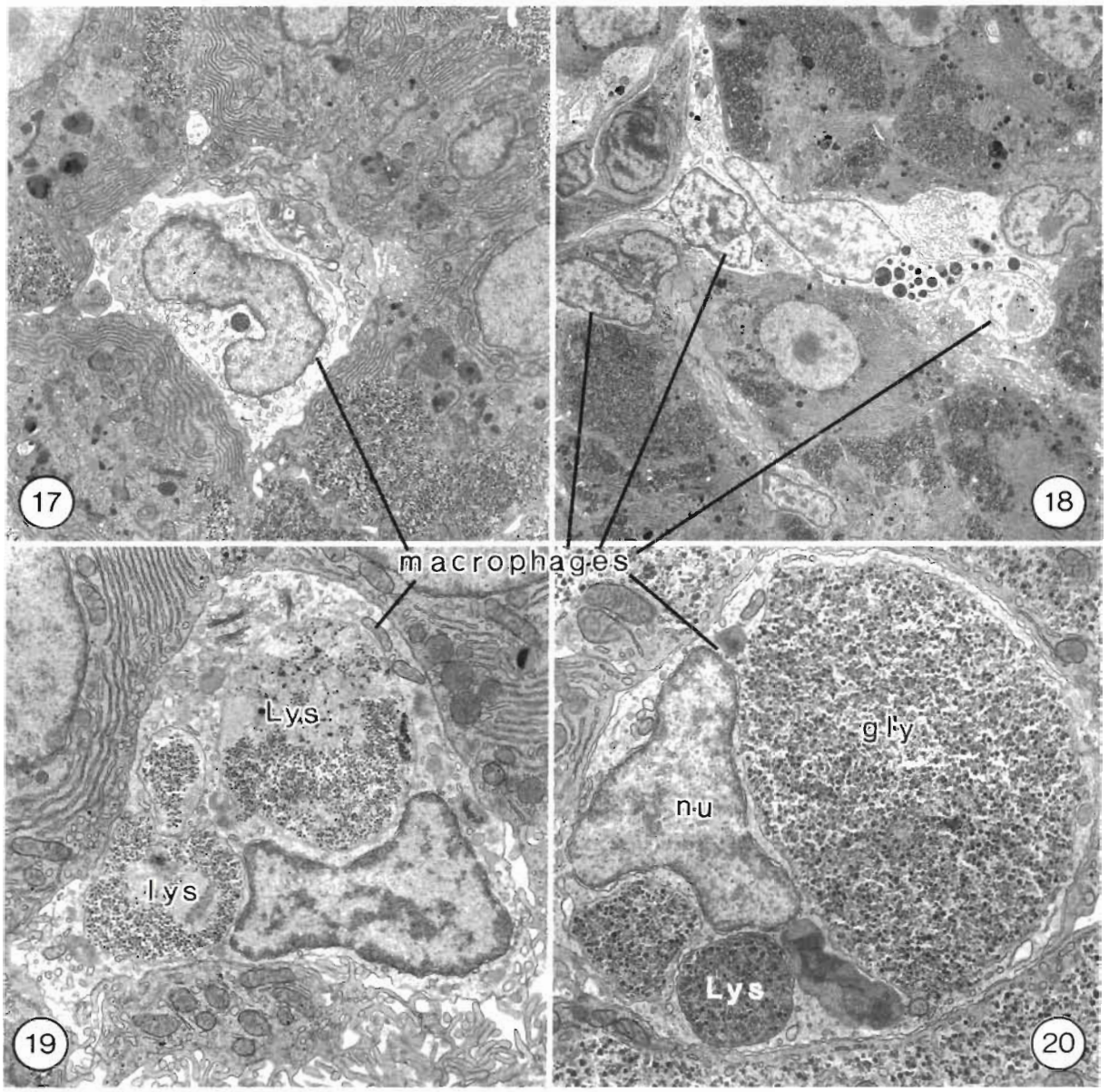
Figs. 2 to 4. *Oncorhynchus mykiss*. Typical alterations in rainbow trout hepatocytes due to endosulfan exposure were dilation (Figs. 2 & 3) and vesiculation (Fig. 3) of rough endoplasmic reticulum (RER) cisternae as well as a slight dilation of the nuclear envelope (Fig. 2). Furthermore, a strong augmentation of smooth endoplasmic reticulum (SER) along with a more random cellular distribution could be observed following endosulfan exposure (Fig. 4). mi: mitochondria Exposure level = 50 ng l^{-1}. Fig. 2: 7400×; Fig. 3. 9100×; Fig. 4: 14 500×



Figs. 5 to 10 *Oncorhynchus mykiss* Following exposure to 50 ng l^{-1} endosulfan, peroxisomes showed a distinct tendency towards cluster formation (Figs 5 & 6) and increased morphological heterogeneity (Fig 6). At 100 ng l^{-1} endosulfan, the peroxisomal matrix displayed strong heterogeneity in electron density (cf core-like dense bodies in matrix: Fig. 7, arrows), and small myelin figures spread inwards from the peroxisomal membrane (Fig 7, doubled arrowheads). Further effects by endosulfan included dilation of the intermembranous space of mitochondria (Fig 8, arrows) and the development of myelin figures in the resulting spaces (Fig 9, arrows) At 100 ng l^{-1} endosulfan, some of the mitochondria showed strong deformation and highly condensed myelin figures (Fig 10) Fig 5. 50 ng l^{-1}, 5700×, Fig 6. 50 ng l^{-1}, 22 900×, Fig 7 100 ng l^{-1}, 22 600×; Fig. 8: 50 ng l^{-1}, 11 700×; Fig 9 50 ng l^{-1}. 30 000×, Fig 10 100 ng l^{-1}, 46 400×



Figs 11 to 16. *Oncorhynchus mykiss*. Major pathological alterations due to endosulfan exposure were observed in the lysosomal compartment. In addition to shifting from the usual peribiliary location to a more random distribution (Fig. 11), lysosomes developed highly condensed intralysosomal myelin figures (Figs. 12 & 13). Furthermore, an augmentation of autophagosomes (ap, Fig. 13), which usually were observed degrading hepatocellular mitochondria, could be observed at 50 ng l^{-1} endosulfan (Fig 14). Multivesicular bodies (Fig 15) and concentrated myelin bodies (Fig 16) within glycogen fields were conspicuous following endosulfan exposure Go: Golgi fields Fig. 11: 50 ng l^{-1}, 14 000×, Fig. 12. 50 ng l^{-1}, 18 700×; Fig 13 50 ng l^{-1}, 14300×, Fig 14 50 ng l^{-1}, 29 800×; Fig 15: 50 ng l^{-1}, 22 700×, Fig 16. 100 ng l^{-1}, 36 000×



Figs 17 to 20. *Oncorhynchus mykiss* The immigration of single macrophages at 50 ng l^{-1} endosulfan (Fig. 17) was followed by the development of macrophage centres at higher endosulfan concentrations (Fig 18) Macrophages were characterized by large amounts of ingested materials, especially glycogen, in the electron-lucent cytoplasm (Figs. 19 & 20). gly: glycogen; lys: lysosomes; nu nucleus of macrophage Fig 17 50 ng l^{-1}, 3700×, Fig 18 100 ng l^{-1}, 1800×, Fig 19 50 ng l^{-1}, 3700×; Fig. 20: 100 ng l^{-1}, 9400×

glycogenosomes, were found at 50 and 100 ng l^{-1} endosulfan (Figs. 17, 19 & 20). At 100 ng l^{-1} endosulfan, groups of up to 5 macrophages per section had formed large macrophage centres (Fig 18).

DISCUSSION

The toxicity of most organochlorine pesticides often manifests itself by neurological effects such as hyperactivity, muscular twitching or convulsions, resulting in death of animals (WHO 1984). However, no mortality and no such effects were observed in rainbow trout exposed to 10–100 ng l^{-1} waterborne endosulfan. In contrast, numerous cytological alterations due to endosulfan exposure were recorded in rainbow trout liver. Effects were observed starting at 10 ng l^{-1} endosulfan (LOEC, Lowest Observed Effect Concentration), i.e. concentrations 2 orders of magnitude below the 96 h LC_{50} of 1.4 µg l^{-1} endosulfan for rainbow trout (Johnson & Finley 1980) Since 10 ng l^{-1} endosulfan are within the range of field data, these observations are likely to

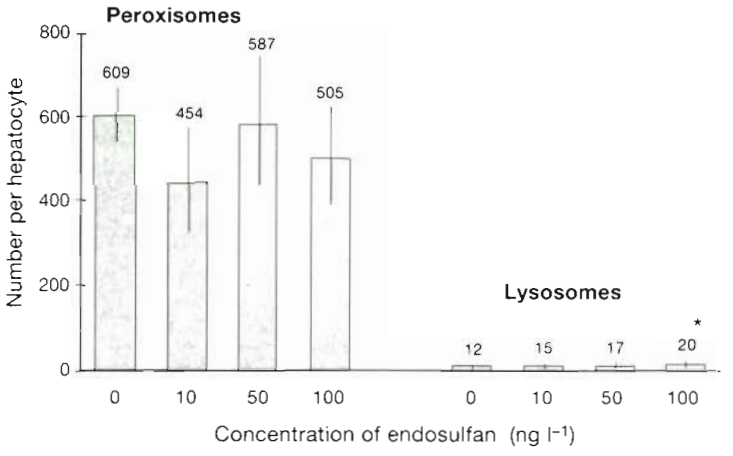


Fig. 21. *Oncorhynchus mykiss*. Changes in numbers of peroxisomes and lysosomes in hepatocytes of male rainbow trout following exposure to 0, 10, 50, and 100 ng l^{-1} endosulfan for 28 d. *$p < 0.05$

be encountered in fish from the field as well. Our results thus confirm earlier conclusions that changes in liver ultrastructure can serve as sensitive biomarkers of exposure to toxicants, integrating a wide spectrum of physiological and biochemical functions of hepatocytes (Hinton & Laurén 1990, Braunbeck 1994).

In rainbow trout liver, alterations due to endosulfan were clearly dose-dependent, showing adaptive as well as degenerative phenomena. The fact that no severe, strictly pathological effects were found emphasizes the adaptive nature of most cellular responses to endosulfan. This view is also supported by the almost complete lack of change in total volumes as well as relative volumes of peroxisomes, mitochondria and lysosomes, which is generally regarded as a typical reaction in necrotic cells (Bowen 1984, Wyllie et al. 1984). Not even degradation of liver glycogen reserves, which is usually consistent with unspecific stress phenomena due to pesticide exposure (Hanke et al. 1983, Gluth & Hanke 1985), was observed. This observation contrasts somewhat with studies which have shown glycogen loss in *Barbus conchonius* (Gill et al. 1991), *Channa punctatus* (Murty & Devi 1982, Verma et al. 1983) and *Tilapia mossambicus* (Dange 1986) following exposure to considerably higher concentrations of endosulfan.

Particularly at concentrations of 10 ng l^{-1} endosulfan, adaptive changes such as induction of autophagosomes—a sign of increased turnover of cell components—were found, but, from 50 ng l^{-1} endosulfan, an increasing number of degenerative effects was seen in addition to adaptive reactions. Further adaptive effects included excessive augmentation of autophagosomes accompanied by increasing numbers of multivesicular bodies and intralysosomal myelin, invasion of macrophages and formation of macrophage centres, indicating the induction of cell-mediated unspecific immune response, which has already been reported in fish exposed to endosulfan (Ellis 1977).

The SER augmentation observed at levels ≥50 ng l^{-1} endosulfan is generally regarded as a sign of the induction of biotransformation processes (Hinton et al. 1978, Braunbeck et al. 1989, 1990a, b, c, Braunbeck & Völkl 1991, 1993). In addition, transition of RER into concentric arrays has also been suggested to be indicative of mixed-function oxygenase (MFO) induction (Klaunig et al. 1979, Hawkes 1980) However, information about endosulfan metabolism by MFO enzyme systems is inconsistent. In rats, endosulfan has been reported to exert no effects (Dorough et al. 1978) or to be a weak inducer following single exposure (Gupta & Gupta 1977, 1979). In contrast, endosulfan significantly stimulates MFO activities following repeated or chronic administration as in our experiment (Tyagi et al. 1984, 1985, Singh & Pandey 1989). There is even more contradictory evidence for induction of cytochrome P-450 (for nomenclature, see Nebert et al. 1991) by phenobarbital-type inducers in fish: whereas

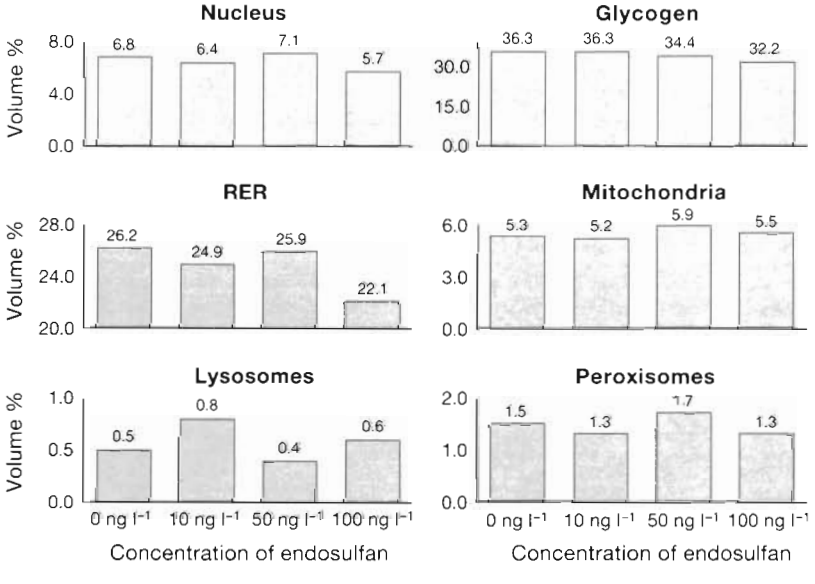


Fig. 22. *Oncorhynchus mykiss*. Changes in relative volumes of selected cell components in hepatocytes of male rainbow trout following exposure to 0, 10, 50, and 100 ng l^{-1} endosulfan for 28 d

Goksøyr et al. (1987), Kleinow et al. (1987), Gooch et al. (1989), and Winston et al. (1989) failed to reveal such induction, other reports have suggested an induction by phenobarbital-type compounds (Gutman & Kidron 1971, Burns 1976, Förlin & Lidman 1978, Eisele et al. 1984, Bach & Snegaroff 1989, van der Oost et al. 1990). Since Jensen et al. (1991) found stimulation not only of cytochrome P-450 IA1-related ethoxycoumarin-O-deethylase and aryl hydrocarbon hydroxylase activities, but also of aldrin epoxidase activities, the data presented in the present communication provide additional morphological evidence that endosulfan most likely does function as a mixed-type inducer of cytochrome P-450. However, since there appears to be a difference between the inducing potentials of technical and analytical grade endosulfan (Jensen et al. 1991), some of the SER proliferation observed in the present study might also have been due to impurities in the technical grade endosulfan used. In the case of endosulfan, biotransformation by rat as well as fish MFO enzyme systems would lead to a detoxification of the parent compound and the sulfate metabolite to less toxic derivatives such as the diol, lactone and ether form, which are readily excreted via bile, faeces, or, to a lesser extent, urine (Schoettger 1970, Dorough et al. 1978).

Increasing cellular heterogeneity, dilation of the nuclear envelope, progressive loss of mitochondrial integrity, myelin formation in peroxisomes and cytoplasm, and vesiculation and dilation of RER cisternae, which were observed at levels ≥50 ng l^{-1}, are clearly degenerative effects. Dilation of intermembranous spaces in mitochondria, for example, was also observed in eel *Anguilla anguilla* exposed to the chemical spill at Basel in 1986 (Braunbeck et al. 1990a) and in eel and golden ide *Leuciscus idus melanotus* exposed to dinitro-*o*-cresol (DNOC; Braunbeck & Völkl 1991, 1993), as well as in isolated hepatocytes of rainbow trout exposed to 4-chloroaniline (Braunbeck 1993). Both DNOC and endosulfan are known to be uncouplers of oxidative phosphorylation (Dubey et al. 1984, Verschoyle et al. 1987). The mitochondrial effects observed may therefore represent the morphological counterpart of disturbances in mitochondrial respiration. Likewise, matrix heterogeneity and myelin formation in peroxisomes, as observed after exposure to 100 ng l^{-1} endosulfan, could also be a sign of physiological degeneration of peroxisomal functions.

The reduction in VLDL secretion by Golgi fields suggests changes in lipid metabolism and indicates a general reduction in the synthetic capacities of hepatocytes. Most of the hepatocellular effects observed in mitochondria, lysosomes, SER and RER are well known responses to other toxicants in the liver of rainbow trout (Arnold & Braunbeck 1995) as well as of other fish species (Braunbeck et al. 1990a, b, c, Braunbeck & Völkl 1993, Braunbeck 1994). These effects must therefore to be classified as unspecific. Although this restriction limits their use for the diagnostic discrimination of particular chemical compounds in the field, they may be used to identify the biological detection limit of toxicant effects. In addition, they represent highly sensitive biomarkers of general environmental contamination well below lethal concentrations.

CONCLUSIONS

The results document toxic effects of endosulfan in rainbow trout liver at concentrations of 10 ng l^{-1}, i.e. well below the LC_{50} of 1.4 µg l^{-1} endosulfan (Johnson & Finley 1980). The almost complete lack of stereological alterations, contrasted with the numerous qualitative changes, indicates that qualitative histo- and cytopathology may not simply be reduced to quantitative data. Whereas effects at 10 ng l^{-1} endosulfan suggested only low toxicity, progressive alterations at higher exopsure levels indicate enhanced toxicity of endosulfan above 50 ng l^{-1}. Pathological changes in mitochondria and peroxisomes most likely represent the morphological counterpart of disturbances in mitochondrial respiration as one possible mechanism of endosulfan intoxication. The results further are consistent with endosulfan as a mixed-type inducer of MFO systems in fish hepatocytes.

Acknowledgements. This investigation was supported by a grant from the Sandoz Rheinfonds under contract from May 1991. The authors express their cordial thanks to Mrs G. Adam for excellent assistance in photographic reproduction.

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Responsible Subject Editor: C. B. Schreck, Corvallis, Oregon, USA

Manuscript first received: March 24, 1995
Revised version accepted: October 27, 1995