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Toxicity of 4-Chloroaniline in Early Life Stages of Zebrafish (*Danio rerio*): II. Cytopathology and Regeneration of Liver and Gills After Prolonged Exposure to Waterborne 4-Chloroaniline

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Abstract. Ultrastructural alterations in liver and gills of embryonic and larval zebrafish (*Danio rerio*) following prolonged exposure to waterborne 0.05, 0.5, and 5 mg/L 4-chloroaniline for up to 31 days as well as after a 14-day regeneration period were investigated by means of light and electron microscopy. Acute toxicity was also tested at 25 and 50 mg/L. Survival of zebrafish embryos and larvae was only impaired from 25 mg/L 4-chloroaniline, but—after a transient stimulation following exposure to 0.5 mg/L—4-chloroaniline hatching was retarded after exposure to $\geq$5 mg/L, and fish displayed increasing rates of abnormal development and pigmentation. In contrast, hepatocytes displayed a time- and dose-dependent response from 0.05 mg/L 4-chloroaniline, including changes in nuclei, mitochondria, peroxisomes, endoplasmic reticulum, Golgi fields, lysosomes, and hepatic glycogen and lipid stores, as well as invasion of macrophages. In gills, dose-dependent effects were evident from 0.5 mg/L 4-chloroaniline and included deformation of secondary lamellae due to vacuolization and desquamation of respiratory epithelial cells in conjunction with dilation of intercellular spaces. Respiratory epithelial cells displayed progressive mitochondrial changes, induction of cytoplasmic myelinated structures, augmentation of lysosomes, and modifications of Golgi fields. Erythrocytes were severely deformed. A 14-day regeneration period was sufficient for almost complete recovery of pathological symptoms in both liver and gills. Only minor volumetric changes in hepatocellular organelles and a limited number of myelinated bodies, lysosomes, and cytoplasmic vacuoles were reminiscent of prior 4-chloroaniline exposure. In both qualitative and quantitative terms, most effects in hepatocytes after exposure of embryonic and larval zebrafish to waterborne 4-chloroaniline are comparable to the reaction of hepatocytes in adult zebrafish liver after prolonged sublethal exposure as well as in larval zebrafish after microinjection. Morphological changes in erythrocytes indicate disturbance of respiration as an additional mode of action of 4-chloroaniline.

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In recent years, considerable information has been accumulated on bioconcentration, biotransformation and effects of chlorinated anilines in fish (for literature, see Verschueren 1983; Braunbeck *et al.* 1990a; Rippen 1995; Oulmi and Braunbeck 1996). In the course of a series of more-in-depth studies on sublethal cytological effects in selected monitor organs of fish by xenobiotic compounds, ultrastructural alterations were studied in the liver of adult zebrafish (*Danio rerio*) and fingerling rainbow trout (*Oncorhynchus mykiss*) following long-term exposure to waterborne 4-chloroaniline (Braunbeck *et al.* 1990a) as well as in the liver and kidney of zebrafish after microinjection of newly fertilized eggs with minute doses of 4-chloroaniline (Oulmi and Braunbeck 1996). In addition, electron microscopical modifications by 4-chloroaniline were investigated *in vitro* in primary cultures of hepatocytes isolated from rainbow trout (Braunbeck 1993) as well as the permanent cell line R1 derived from primary rainbow trout liver cell cultures (Zahn *et al.* 1993). In all of these studies, cytological alterations could be demonstrated to react with extraordinary sensitivity to 4-chloroaniline concentrations far below those exerting acutely lethal effects (for toxicological data of 4-chloroaniline, see Braunbeck *et al.* 1990a; Rippen 1995).

At least in hepatocytes, most ultrastructural alterations at sublethal toxicant concentrations have been interpreted as adaptive reactions in the course of compensatory processes (Braunbeck and Völkl 1991, 1993; Braunbeck *et al.* 1992a, 1992b; Braunbeck 1993, 1994). Since, by definition, such compensative morphological modifications should be reversible, the majority of ultrastructural alterations by sublethal concentrations of 4-chloroaniline may be expected to disappear upon termination of toxicant exposure. Since our knowledge on the reversibility of xenobiotic effects in fish is poor not only with respect to histological and cytological alterations but also with reference to most other toxicological endpoints, the major purpose of the present study was to investigate the regeneration potency of liver and gills in early larval stages of zebrafish after sublethal exposure to 4-chloroaniline. Regeneration capacities may be of particular interest in field studies on effects after single or discontinuous release of xenobiotics, and data on the regeneration potency are indispensable prerequisites for the

prediction of the maximum acceptable dosage in individual organisms, species, communities, or ecosystems.

Since, to the best of our knowledge, no information is available on the influence of different routes of application on the quality and quantity of cytological effects, the present study was carried out in parallel to a microinjection study (Oulmi and Braunbeck 1996), thus facilitating a comparison between effects by waterborne 4-chloroaniline with hepatic alterations by microinjection of 4-chloroaniline. Furthermore, comparison of results with liver modifications in adult zebrafish (Braunbeck *et al.* 1990a) after prolonged exposure to 4-chloroaniline should allow for discrimation of age-specific reactions of the liver. Since, as pointed out by Hinton *et al.* (1987), the full integration of morphologic approaches into (eco)toxicology with more quantitatively oriented disciplines is limited by the subjective nature of the micrograph interpretation, morphometric techniques were integrated into this study.

Gill reactions were selected as a further monitor system to account for potential organ-specific peculiarities. Since gills are in continuous, close contact to the surrounding water and potentially hazardous substances and thus represent an important route for uptake, bioconcentration, and excretion of toxicants, a wide variety of rapid and sensitive cytological reactions to such substances may be assumed (Mallat 1985; Evans 1987; Wendelaar Bonga and Lock 1992; Randall *et al.* 1996). However, in contrast to the liver, there is only a limited number of reports on electron microscopical changes in the gills in toxicological studies (Chevalier *et al.* 1985; Hawkins *et al.* 1984; Liu and Wong 1986; Mallatt *et al.* 1985; Roncero *et al.* 1990; Youson and Neville 1987). Even though numerous pollutants are known to affect branchial structure (Abel and Skidmore 1975; Gingerich and Rach 1985; Mallat *et al.* 1985), the majority of investigations has been restricted to the light microscopical level (Skidmore and Tovell 1972; Hughes 1979; Hughes *et al.* 1979; Kumaraguru *et al.* 1982; Leino and McCormick 1984; Jauhar and Kulshrestha 1985; Leino *et al.* 1987a, 1987b; Oronsaye 1989; Richmonds and Dutta 1989). Since in the liver most histopathological syndromes described so far consist of predominantly unspecific phenomena (Braunbeck 1998), the present communication attempts to elucidate whether cytological reactions in the gills can also be classified as a complex mixture of substance-specific and -unspecific cellular reactions.

Materials and Methods

Origin and Maintenance of Parental Fish

Sexually mature zebrafish (*D. rerio;* formerly referred to as *Brachydanio rerio,* Meyer *et al.* 1993) of the ''AB'' and the ''Mixed'' strains were kindly provided by the Max Planck Institute for Developmental Physiology in Tübingen, Germany. Fish were kept in 25-L full glass aquaria and reared in a flow-through system (flow rate of 10 to 20 L/h). Well-aerated water was adjusted to 26.5 ± 1°C (pH 7.2 ± 0.2; total hardness 24°dH) and purified by passage through an activated carbon filter. Light and dark phases were 14 and 10 h each, and fish were fed a commercially available artificial diet (TetraMin™ flakes; Tetra Werke, Melle, Germany) twice daily. Spawning was performed as described by Westerfield (1997).

Toxicant and Exposure Procedures

Prior to 4-chloroaniline exposure, eggs were kept in petri dishes at 27.5°C until the ''germ-ring'' stage of larval development (Westerfield 1997), when unfertilized eggs could easily be sorted out. 4-Chloroaniline concentrations were selected on the basis of concentrations used in previous studies to determine acute and sublethal toxicities in adult zebrafish (for literature, see Braunbeck *et al.* 1990a). For prolonged sublethal exposure, test concentrations of 0.05, 0.5, 5, 25, and 50 mg/L 4-chloroaniline (1-amino-4-chlorobenzene; 99% purity; Merck, FRG) were obtained by direct solution of the toxicant in tap water; test solutions were replaced daily. Triplicate groups of 15 eggs were placed in separate glass dishes for 10 and 31 days at a water temperature of 27.5°C. In each experiment, hatching occurred approximately 96 h after injection. From day 2 after hatching, larvae were fed commercially available powdered food (TetraMikromin, Tetra Melle, FRG) immediately before renewal of the test solutions. Regeneration of liver and gills was studied at 45 days, *i.e.,* 14 days after termination of 4-chloroaniline exposure. For determination of acute 4-chloroaniline toxicity to zebrafish embryos, triplicate groups of 10 eggs were exposed to 0.5, 5, 25, and 50 mg/L 4-chloroaniline for 96 h in separate 8-cm glass dishes at 27.5°C.

Mortality and Teratogenic Effects

Mortality and macroscopically overt signs of teratogenicity were recorded over a 4-day exposure period plus a 12-day recovery period.

Electron Microscopy

Zebrafish larvae at the age of 10 and 31 days (exposed groups) as well as 45 days (regeneration) were sacrificed by an overdose of anesthetic (4-amino benzoic acid ethyl ester, benzocaine, Sigma Chemicals, Munich, FRG). Liver- and gill-containing portions of the fish were dissected and fixed in 2.5% glutardialdehyde in 0.1 M sodium cacodylate buffer (pH 7.6) containing 4% polyvinylpyrrolidone (Serva, Heidelberg, FRG) and 0.05% calcium chloride. After primary fixation, larvae were rinsed in 0.1 M cacodylate buffer and postfixed for 1 h with 1% osmium ferrocyanide (1:1 mixture of 2% aqueous OsO_4 and 2% $K_4[Fe(CN)_6]$ (Karnovsky 1971). After rinsing in 0.1 M cacodylate and 0.05 M maleate buffers (pH 5.2), liver and gill samples 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 (Spurr 1969). Semithin plastic sections of 0.5 µm were stained with methylene blue-Azur II and used for light microscopical localization of liver and gill tissues. 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. Ultrathin sections of 60–80 nm thickness were stained with alkaline lead citrate for 30 s or 1 min (Reynolds 1963) and examined in a Zeiss EM 9S-2 electron microscope.

Stereology

Multistage sampling and morphometric evaluation were performed according to the principles of Weibel *et al.* (1969) and Weibel (1979). At each tier, four fish per group were evaluated with hepatocytes serving as reference space. For details of sampling for stereological analysis, see Table 1 and Segner and Braunbeck (1990).

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 profiles of the structure investigated (P_i). Test

Table 1. Tiers of stereological analysis and cytological parameters investigated

Stage	Magnification	Section Thickness (nm)	Test System PT Square	Test System d (µm) Lattice	Parameters Measured Volume Density	Parameters Measured Numerical Density
Tier	×1,100	500–750			Nuclei	Nuclei
	(LM)	(semithin)	391	9.091	Glycogen	
Tier	×7,200	150–200			Mitochondria	Mitochondria
	(EM)	(ultrathin)	391	1.388	Lysosomes	Lysosomes
					Lipid	Lipid
Tier	×18,000	150–200			Peroxisomes	Peroxisomes
	(EM)	(ultrathin)	391	0.555	RER	

PT = test points on grid; d = distance between test points

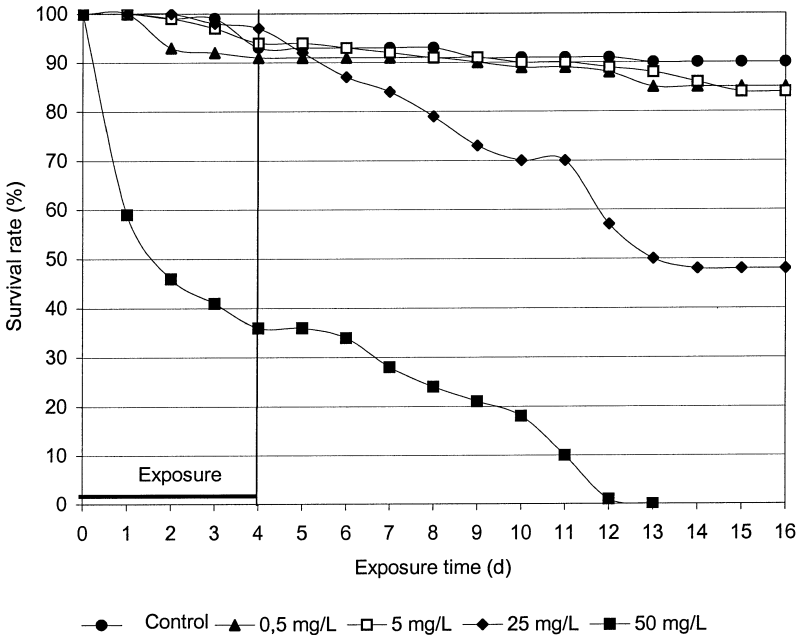


Fig. 1. Cumulative mortality of zebrafish (*Danio rerio*) eggs and larvae following exposure to various concentrations of 4-chloroaniline

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 (1,100×) using a test point lattice with 391 systematically spaced points at a distance of d = 10 mm (=9.091 µm). For measurement of the volume density of hepatic mitochondria, lipid droplets and lysosomes, electron micrographs (7,200×) and a test point lattice with 391 points spaced at d = 10 mm (=88 µm) were employed. The volume densities of peroxisomes and the endoplasmic reticulum fields were measured on micrographs at a magnification of 18,000 × using the same lattice (=0.555 µm).

Numerical densities (N_V) of particulate hepatocellular structures within the test area were calculated according to the formula (Weibel 1979; Weibel *et al.* 1969) $N_V = b^{-1} \cdot N_A^{1.5}/V_V^{0.5}$, with N_A as the number of actually counted particles divided by the test area, with V_V as the volume density of the particles, and with b as a shape-dependent coefficient. This coefficient b was assumed to be 1.38 for lipid droplets, peroxisomes, and lysosomes, and 1.75 for mitochondria (Weibel *et al.* 1969). Numerical densities of mitochondria, lipid droplets, and lysosomes were calculated from electron micrographs at a magnification of 7,200×, that of peroxisomes from micrographs at 18,000 ×.

The numerical density N_V of nuclei was used to calculate the volume of hepatocytes $V_{hep} = 1/N_{Nu}$. Absolute volumes and numbers of mitochondria, lysosomes, lipid droplets, and peroxisomes per hepatocyte were estimated as $V_i \cdot V_{hep}$ and $N_V \cdot V_{hep}$, respectively. The volumes and total numbers of organelles per hepatocyte were estimated as $V_V \cdot V_{hep}$ and $N_V \cdot V_{hep}$, respectively. Hepatocytic and nuclear diameters were calculated as the third root of the relevant volume, assuming spherical shape of the particles. Cell components not measured individually mainly comprise Golgi fields, smooth endoplasmic reticulum, and cytoplasm.

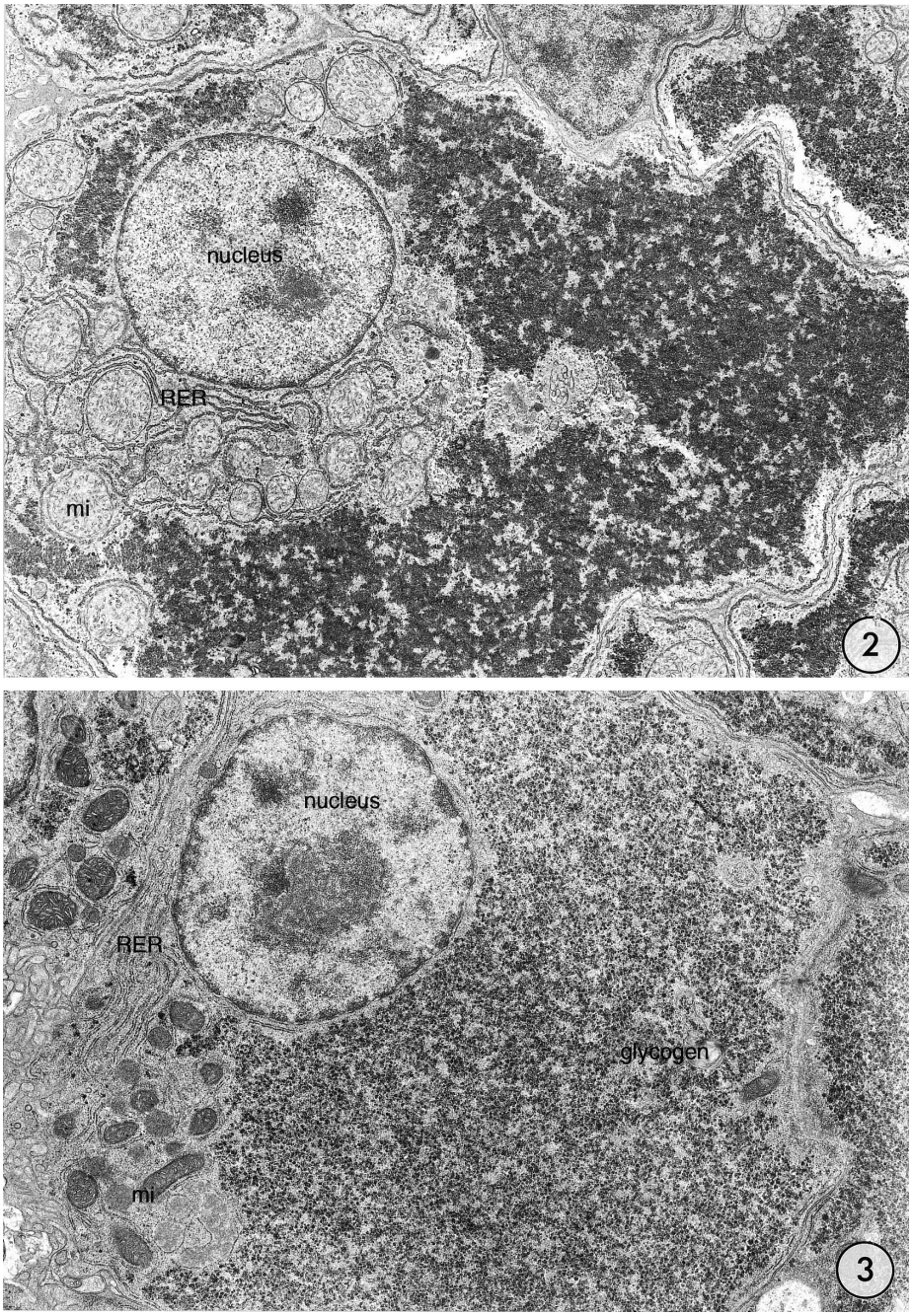
In the gills, 50 erythrocytes from six different sites per individual (n = 4) were measured with respect to section area, maximal and minimal diameters, and diameter of an idealized circle with the corresponding section area. To avoid errors due to erythrocyte distortion in small-sized blood spaces, only erythrocytes from vessels of the larger primary lamellae were selected.

Mean values from all stereological measurements were compared using the nonparametric Wilcoxon-Mann-Whitney U test.

Results

Survival and Macroscopically Overt Signs of Sublethal Toxicity in Larval Zebrafish Exposed to 4-Chloroaniline

Survival of control zebrafish over 4 days during the exposure period was 93% and decreased to 90% in a postexposure



Figs. 2 and 3. The ultrastructure of hepatocytes in control zebrafish (*Danio rerio*) larvae 10 days (Figure 2) and 31 days (Figure 3) after fertilization is characterized by a clear-cut separation into a central, perinuclear organelle-containing portion containing rough endoplasmic reticulum (RER) cisternae, spherical mitochondria (mi), and peroxisomes surrounding the central nucleus and large peripheral glycogen fields. In 31-day-old larvae (Figure 3), the dominance of glycogen deposits is even more prominent than after 10 days. Figure 2: 10-day controls, magnification ×8,800; Figure 3: 31-day controls, magnification ×8,100

observation period (Figure 1). Hatching was complete after 96 h; all individuals were free of pathological symptoms. Following exposure to 0.5 mg/L 4-chloroaniline, there was no change in mortality; all individuals had hatched after 72 h, indicating an acceleration of development by 0.5 mg/L 4-chloroaniline. Except for one individual with spinal deformation out of 30 specimens, no pathology could be observed. Exposure to 5 mg/L 4-chloroaniline failed to increase mortality and hatching rate, but resulted in a slight retardation of hatching and induced 100% spinal deformation of larvae, especially of those not hatching before 120 h. Hatching rates after 72 and 96 h exposure to 25 mg/L 4-chloroaniline were reduced to 59 and 75%, respectively, and larval deformations comprised formation of edema in the trunk region, abnormal eye and trunk pigmentation, lack of eye and mouth formation, as well as spinal malformation eventually resulting in complete disorientation and inability to swim. Twenty-four hours after termination of acute exposure to 25 mg/L, the rate of larvae showing severe teratogenic effects was 41%. After exposure to 50 mg/L 4-chloroaniline, survival was reduced to 59, 46, 41, and 36% after 24, 48, 72, and 96 h, respectively (Figure 1). All hatched larvae displayed severe pathology and died within 12 days. LC_{50} values of 4-chloroaniline for exposure of larval zebrafish over 4, 8, and 28 days could be calculated as 44, 38, and 28 mg/L, respectively.

Table 2. Semiquantitative evaluation of ultrastructural alterations in livers of larval zebrafish (*Brachydanio rerio*) exposed to 4-chloroaniline for 10 and 31 days, as well as after a regeneration

	10 Days (mg/L)				31 Days (mg/L)				Regeneration (mg/L)			
	Co	0.05	0.5	5	Co	0.05	0.5	5	Co	0.05	0.5	5
Cytoplasmic compartmentation												
Disturbance of compartmentation	−	−	−	±	−	−	++	++	−	−	+	+
Increasing intercellular heterogeneity	−	−	−	+	−	−	+	++	−	−	±	±
Nucleus												
Irregular outline	−	−	−	±	−	−	±	++	−	−	−	±
Mitochondria												
Increase in size	+	+++	++	+++	+	+	+	+	+	+	+	+
Irregular profiles	−	±	++	+++	+	+	++	++	+	+	+	+
Branching profiles	−	+	+	+++	+	+	++	+++	+	+	+	+
Club-shaped deformation	−	−	−	+	−	−	±	++	−	−	−	−
Spherical deformation	−	−	−	−	−	−	−	++	−	−	−	±
Number of mitochondrial granules	+	+++	+++	+++	++	++	+++	+++	++	++	++	++
Peroxisomes												
Decrease in number	−	−	−	−	−	−	−	+	−	−	−	±
Increased morphological heterogeneity	−	−	−	±	−	−	−	+	−	−	−	±
Rough endoplasmic reticulum												
Overall reduction	−	+	++	+++	−	+	+	+	−	+	+	+
Stacking of cisternae	+++	++	+	+	++	+	+	+	+++	+++	+++	++
Fenestration of cisternae	−	+	++	+++	−	−	+	++	−	−	−	±
Fragmentation of cisternae	−	+	++	++	−	+	+	+	−	−	−	+
Vesiculation of cisternae	−	+	+	+	−	+	+	++	−	−	−	±
Dilation of cisternae	−	−	−	±	−	±	+	++	−	−	−	−
Induction of steatosis	−	−	−	−	−	+	+	++	−	−	−	−
Association with other organelles	++++	++++	+++	+++	++++	++++	++	++	++++	++++	++++	+++
Smooth endoplasmic reticulum												
Proliferation	−	−	−	−	−	−	+	+	−	−	−	±
Golgi fields												
Number of Golgi fields	+	++	+++	+++	+	++	++	+++	+	+	+	++
Length of Golgi cisternae	+++	++	+	+	++	+	±	±	++	+	+	+
Vesiculation of cisternae	−	+	++	++	−	+	+	++	−	+	+	++
Fenestration of cisternae	−	+	++	+++	−	+	++	+++	−	−	−	±
Secretory activity	+	++	++	++	+	+	++	++	++	++	++	++
Lysosomes												
Overall amount	+	++	++	++	+	++	++	+++	+	+	+	++
Number of myelinated bodies	+	++	+++	++++	+	++	+++	++++	+	+	+	+++
Size of myelinated bodies	+	++	+++	++++	+	+	++	+++	+	+	+	+
Induction of autophagosomes	−	−	−	−	−	−	−	+	−	−	−	+
Lipid inclusions												
Number of lipid droplets	+	+	+	+	+	+++	+++	++	+	+	+	+
Size of lipid droplets	±	±	±	±	±	+++	++	++	±	±	+	±
Cholesterol crystals												
Amount of cholesterol	−	−	−	−	−	+	++	++	−	+	+	+
Glycogen												
Overall amount	+	+	+	+	++++	++++	+++	+	++++	++++	+++	++++
Macrophages												
Macrophage invasion	−	−	−	−	−	−	++	+++	−	−	−	+

Data are given as means from 4 specimens. Co = controls. − = absent; ± = very little developed; + = little developed; ++ = moderately developed; +++ = strongly developed; ++++ = very strongly developed

Liver Ultrastructure of Control Zebrafish Larvae

Basically, liver ultrastructure in zebrafish larvae (Figures 2 and 3; Tables 2–4) closely resembles that of adult male zebrafish, *i.e.*, nonvitellogenic fish (van der Gaag *et al.* 1977; Peute *et al.* 1978, 1985; van Bohemen *et al.* 1981, 1982; Braunbeck *et al.* 1989, 1990b). In brief, hepatocytes of control larvae were characterized by a strict cytoplasmic compartmentation into (1) a central, perinuclear organelle-containing portion containing small stacks of up to three layers of short rough endoplasmic reticulum (RER) cisternae, few spherical mitochondria (up to 1.75 µm in diameter; mean volume: 1.07 µm^3; Table 4) of subspherical to elongated shape and a small number of small (0.1–0.7 µm), spherical peroxisomes surrounding the central nucleus, which contained little heterochromatin, but a conspicuous nucleolus; and (2) peripheral storage areas mainly comprising glycogen fields (about 21% of total hepatocyte volume; Table 3) and very few lipid droplets (Figure 4). Otherwise, storage areas were free of organelles except for lysosomes as well as Golgi fields and vesicles in the peribiliary area. Smooth endoplasmic reticulum as well as multivesicular bodies and autophagic vacuoles could not be observed.

Alterations between livers of 10-, 31-, and 45-day-old zebrafish larvae were restricted to variations in the quantities of most cell components (Tables 2–4). Most striking differences were a considerable increase in cell size due to a more than twofold increase in the extension of glycogen fields (Figure 3). Mitochondria were slightly more irregular in shape, and the amount and size of RER cisternae significantly increased with age (Table 3). Single RER cisternae consistently displayed an intimate association with other mitochondria and peroxisomes. A slight, yet not significant proliferation could be seen in lysosomal elements; no consistent changes were evident in volumes and numbers of mitochondria and peroxisomes (Figure 4; Tables 3, 4).

Liver Ultrastructure of Zebrafish Larvae After Exposure to 4-Chloroaniline

0.05 mg/L 4-Chloroaniline: Reactions in 10- and 31-day-old larvae were very similar; however, effects in mitochondria and the RER system were more pronounced in 10-day-old than in 31-day-old larvae (Tables 2–4), thus indicating a higher susceptibility of early larval changes and partial recovery during exposure. The age-dependent increase in mitochondrial heterogeneity appeared stronger than in controls and displayed an increase in both absolute and relative volumes (cf. Figure 5), but a slight decrease in the number per hepatocyte (Figure 4). Whereas no qualitative and quantitative alterations could be detected in peroxisomes, the RER system displayed an overall reduction in 31-day-old larvae. From a qualitative point of view, RER cisternae were less regularly arranged (tendency to fenestration, fragmentation, and vesiculation), and cisternal stacking was less conspicuous. In contrast to mitochondria and RER, 0.05 mg/L 4-chloroaniline induced a 30% increase in the nuclear volumes in 31-day-old larvae only, which resulted in a corresponding increase in the nuclear-cytoplasmic ratio (Table 4).

Golgi fields were increased in number, but single cisternae were reduced in length, tended to fenestrate and vesiculate;

Table 3. Alterations in percentile composition of hepatocytes from juvenile zebrafish (*Brachydanio rerio*) following exposure to 4-chloroaniline for 10 and 31 days, as well as after a 14-day regeneration period subsequent to a 31-day exposure to 4-chloroaniline

	Nuclei	Mitochondria	Peroxisomes	Endoplasmic Reticulum	Lysosomes	Glycogen	Lipid	Rest
10-day exposure								
Controls	4.61 ± 0.82	10.12 ± 1.12	2.41 ± 0.20	9.20 ± 1.17	0.93 ± 0.31	21.53 ± 4.56	2.11 ± 0.31	49.21 ± 6.34
0.05 mg/L	4.84 ± 0.49	12.43 ± 1.98	2.63 ± 0.26	8.83 ± 1.12	3.63 ± 0.40***	20.21 ± 3.56	2.54 ± 0.41	45.12 ± 5.87
0.5 mg/L	5.83 ± 0.93	16.12 ± 2.08*	2.54 ± 0.56	8.58 ± 2.08	5.80 ± 1.21***	18.72 ± 2.67	3.34 ± 0.88*	36.69 ± 6.89
5 mg/L	6.72 ± 1.23	18.23 ± 2.65**	4.54 ± 0.87**	6.43 ± 0.66**	5.91 ± 1.38***	17.53 ± 1.99	2.13 ± 0.51	33.67 ± 3.87**
31-day exposure								
Controls	3.81 ± 0.93	9.18 ± 2.15	2.33 ± 0.29	8.27 ± 1.01	1.43 ± 0.33	32.77 ± 8.76	1.11 ± 0.44	39.10 ± 4.66
0.05 mg/L	4.84 ± 0.49	11.28 ± 0.99	2.65 ± 0.29	7.99 ± 0.89	2.78 ± 0.23	28.21 ± 3.78	6.45 ± 1.12***	36.80 ± 4.45
0.5 mg/L	4.77 ± 0.62	15.33 ± 2.98***	2.14 ± 0.26	6.34 ± 1.54*	6.80 ± 0.93***	23.45 ± 5.57*	7.39 ± 1.72***	32.72 ± 5.01
5 mg/L	6.72 ± 1.23*	16.23 ± 2.33***	4.77 ± 1.34**	5.99 ± 0.79**	4.91 ± 0.23***	9.53 ± 1.35***	6.43 ± 1.38***	47.42 ± 8.98
31-days exposure + 14-day regeneration								
Controls	4.31 ± 0.53	12.77 ± 1.17	2.54 ± 0.46	13.56 ± 1.88	1.23 ± 0.22	36.65 ± 6.78	2.02 ± 0.22	25.42 ± 2.38
0.05 mg/L	4.34 ± 0.50	14.66 ± 3.89	2.63 ± 0.26	10.10 ± 1.54	3.03 ± 0.28***	34.55 ± 5.00	2.58 ± 0.31	28.11 ± 3.23
0.5 mg/L	4.39 ± 1.00	15.12 ± 0.99*	2.35 ± 0.33	10.09 ± 1.15*	6.98 ± 1.42***	35.65 ± 7.99	2.78 ± 0.27	23.24 ± 4.29
5 mg/L	4.26 ± 0.67	14.11 ± 1.75	2.59 ± 0.22	10.43 ± 1.99	6.78 ± 0.56***	32.87 ± 9.77	2.63 ± 0.61	26.03 ± 5.46

Data are given as means from 4 fish per group ± SD. Differences between controls and exposed fish were evaluated by the Wilcoxon–Mann–Whitney U-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 4. Alterations in absolute composition of hepatocytes from larval zebrafish (*Brachydanio rerio*) following exposure to 4-chloroaniline for 10 and 31 days, as well as after a 14-day regeneration period subsequent to a 31-day exposure to 4-chloroaniline

	10-Day Exposure				31-Day Exposure				14-Day Regeneration			
	Controls	0.05 mg/L	0.5 mg/L	5 mg/L	Controls	0.05 mg/L	0.5 mg/L	5 mg/L	Controls	0.05 mg/L	0.5 mg/L	5 mg/L
Hepatocyte												
Mean volume of a mononuclear hepatocyte (μm^3)	2233	2328	2199	1877	3231	3412	3287	2188	3416	3489	3210	3512
Mean diameter of a mononuclear hepatocyte (µm)	16.22	16.44	16.13	15.30	18.34	18.68	18.45	16.11	18.69	18.82	18.30	18.86
Nucleus												
Mean nuclear volume (μm^3)	103	113	128	126	123	165	157	147	147	151	141	150
Mean nuclear diameter of a mononuclear hepatocyte (µm)	5.82	6.00	6.25	6.22	6.17	6.81	6.69	6.55	6.55	6.61	6.46	6.59
Nuclear–cytoplasmic ratio	0.046	0.049	0.058	0.067	0.038	0.048	0.048	0.067	0.043	0.043	0.044	0.043
Mitochondria												
Total mitochondrial volume per hepatocyte (μm^3)	226	289	354	342	317	351	504	455	436	511	485	496
Mitochondria per hepatocyte	212	187	175	168	286	244	292	274	264	244	265	283
Mean volume of a single mitochondrion (μm^3)	1.07	1.55	2.02	2.04	1.11	1.44	1.73	1.66	1.65	2.09	1.83	1.75
Mean diameter of a single mitochondrion (µm)	1.27	1.43	1.57	1.57	1.28	1.40	1.49	1.47	1.47	1.59	1.52	1.50
Peroxisomes												
Total peroxisomal volume per hepatocyte (μm^3)	34	41	36	65	45	70	60	74	47	92	75	81
Peroxisomes per hepatocyte	343	357	399	360	342	366	278	243	328	354	322	275
Mean volume of a single peroxisome (μm^3)	0.10	0.11	0.09	0.18	0.13	0.19	0.22	0.30	0.14	0.26	0.23	0.29
Mean diameter of a single peroxisome (µm)	0.57	0.60	0.56	0.70	0.63	0.71	0.74	0.83	0.65	0.79	0.76	0.83
Endoplasmic reticulum fields												
Total volume of ER fields per hepatocyte (μm^3)	205	206	187	121	267	219	208	131	497	352	350	366
Lysosomes												
Total lysosomal volume per hepatocyte (μm^3)	12	75	108	111	21	75	136	117	18	76	144	158
Lysosomes per hepatocyte	422	506	497	530	466	530	605	618	437	599	621	699
Mean volume of a single lysosome (μm^3)	0.03	0.15	0.22	0.21	0.05	0.14	0.22	0.19	0.04	0.13	0.23	0.23
Mean diameter of a single lysosome (µm)	0.38	0.66	0.75	0.74	0.44	0.65	0.75	0.71	0.43	0.62	0.76	0.76
Lipid inclusions												
Total lipid volume per hepatocyte (μm^3)	37	59	55	50	46	220	243	141	69	90	89	92
Lipid inclusions per hepatocyte	21	23	18	27	32	74	85	78	24	25	20	27
Mean volume of a single lipid inclusion (μm^3)	1.76	2.57	3.06	1.85	1.44	2.97	2.86	1.81	2.88	3.60	4.45	3.41
Mean diameter of a single lipid droplet (µm)	1.50	1.70	1.80	1.52	1.40	1.78	1.76	1.51	1.76	1.90	2.04	1.87
Glycogen fields												
Total volume of glycogen fields per hepatocyte (μm^3)	481	470	412	329	1059	963	771	209	1251	1205	1144	1154
Volume of components not measured individually (µm)	1135	1075	919	733	1353	1349	1208	914	951	1012	782	1015

Data are given as means from 6 fish per group. For significances of differences between experimental groups and controls, see Table 4. "Components not measured individually" comprise Golgi complexes, cytoplasmic vesicles, and bile canaliculi

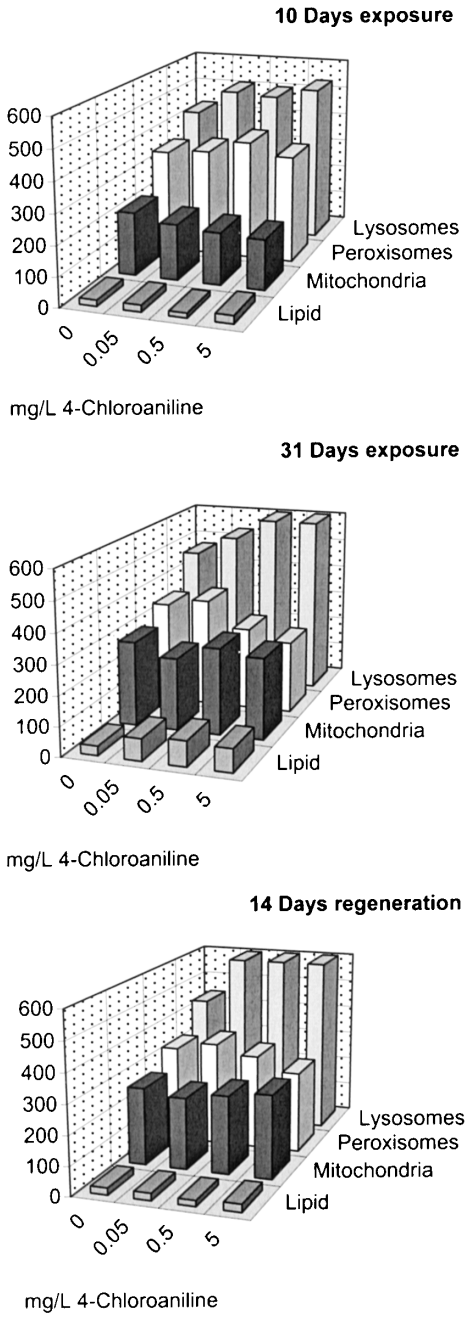


Fig. 4. Alterations in the number of selected particulate organelles in hepatocytes of larval zebrafish (*Danio rerio*) after 10 and 31 days' of exposure to 4-chloroaniline as well as 14 days after cessation of exposure (regeneration), *i.e.*, at 45 days

overall secretory activity of Golgi fields as evidenced by the number of Golgi vesicles appeared to be higher than in controls (Figures 6 and 7). Both lysosomes and myelinated bodies displayed significant increases over controls, both with respect to relative and absolute volumes (Figure 8; Tables 3 and 4) as well as number of single profiles (Figure 4). Only in 31-day-old larvae, both number and volume of lipid droplets were significantly increased by factors of 2 and 6, respectively (Tables 2–4), whereas glycogen seemed unaffected by 4-chloroaniline exposure. In parallel to cytoplasmic lipid accumulation, 31-day-old zebrafish displayed an aggregation of lipid droplets within cisternae (Figure 9) (steatosis, microvesicular lipid accumulation; Baglio and Farber 1965) as well as a slight accumulation of cholesterol crystals within glycogen fields (Figure 10).

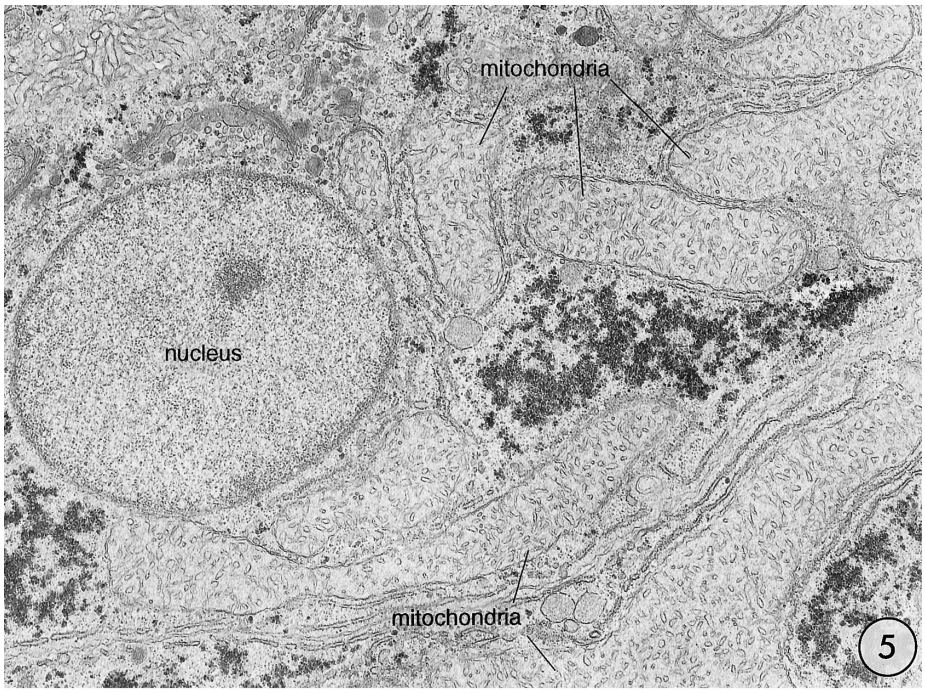
0.5 and 5 mg/L 4-Chloroaniline: Cytological alterations listed for zebrafish exposed to 0.05 mg/L 4-chloroaniline were clearly enhanced following exposure to 0.5 and 5 mg/L, thus indicating a positive dose-response relationship (Tables 2–4). In contrast, the inverse time-response relationship described for 0.05 mg/L 4-chloroaniline was no more evident in larvae exposed to 0.5 and 5 mg/L. As a consequence of the less regular arrangement of most cell components, the cytoplasmic compartmentation into central organelle-containing areas and peripheral storage fields was slightly modified in 10-day-old larvae exposed to 5 mg/L, but severely disturbed at $\geq$0.5 mg/L in 31-day-old larvae.

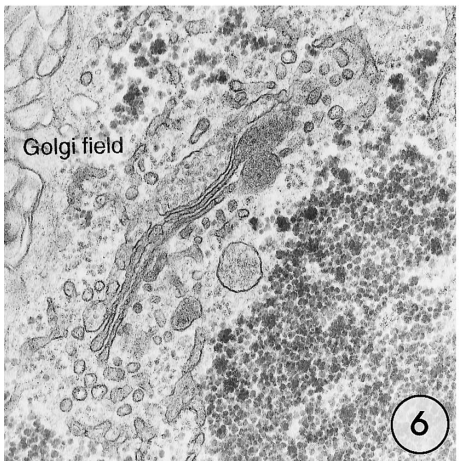
Additional alterations at higher 4-chloroaniline concentrations comprised club-shaped and spherical deformation of mitochondria (Figure 11) as well as an increase in the number of mitochondrial granules. In some hepatocytes of 31-day-old larvae, the outline of the nuclei appeared less regular. The volumetric and numeric proliferation of mitochondria became statistically significant at $\geq$0.5 mg/L. Especially after exposure to 5 mg/L 4-chloroaniline, 31-day-old zebrafish larvae displayed a decrease in the number of peroxisomes, but an increase in relative and absolute volumes due to a conspicuous increase in the size of single peroxisomes (Tables 2–4). In contrast to the augmentation of steatotic RER profiles, the increase in the volume of cytoplasmic lipid droplets was less conspicuous at 5 mg/L than at 0.5 mg/L 4-chloroaniline (Tables 2–4). Moreover, in 31-day-old zebrafish larvae only, there was a progressive decline in the size of hepatocellular glycogen fields.

A feature unique of 31-day-old larvae exposed to 0.5 mg/L 4-chloroaniline is a dose-dependent invasion of macrophages into the hepatic parenchyma via the space of Disse as well as the connective tissue and intercellular spaces along the biliary tracts.

Liver Ultrastructure of Zebrafish Larvae After a 14-Day Recovery Subsequent to 31-Day 4-Chloroaniline Exposure

Most effects induced by 4-chloroaniline were no longer significantly different from controls following a 14-day recovery period after termination of exposure. Statistically significant differences between larvae formerly exposed to 0.5 mg/L 4-chloroaniline and corresponding controls were restricted to mitochondrial (increase) and RER (decrease) volumes. Additional remnants of preceding exposure were slight (statistically insignificant) disturbances in hepatocellular compartmentation; some nuclei with a less regular outline; an insignificant decrease in the number of peroxisomes; an elevated number of cholesterol deposits, lysosomes, and myelinated bodies; as well as a limited number of macrophages invading the liver parenchyma. Without knowledge of the alterations present during acute exposure, however, the latter differences might not have been discovered.





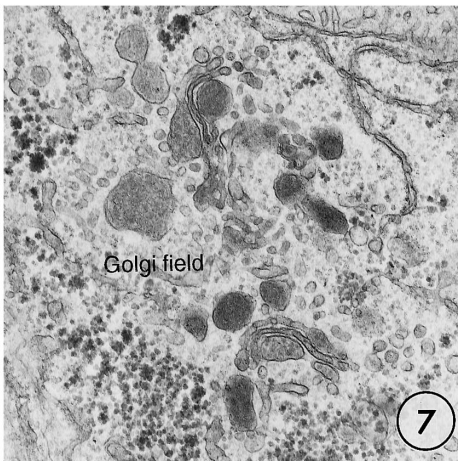


Fig. 5. Following exposure to waterborne ≥0.5 mg/L 4-chloroaniline, hepatocytes of larval zebrafish (*Danio rerio*) display giant mitochondria measuring up to 12 µm in length and containing a multitude of small, irregular mitochondrial cristae. 10 days, 5 mg/L 4-chloroaniline, magnification ×9,700

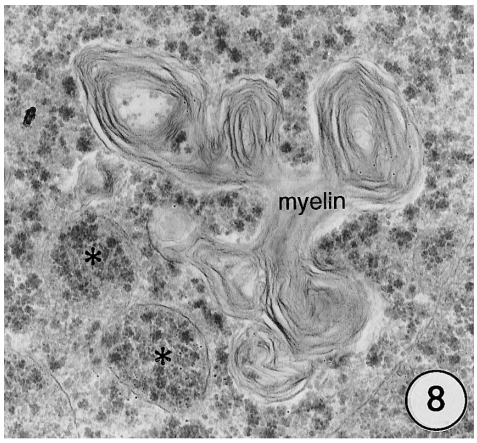
Figs. 6 and 7. If compared to controls (Figure 6), Golgi fields in hepatocytes of zebrafish (*Danio rerio*) exposed to 4-chloroaniline were increased in number, but single cisternae were reduced in length and appeared fenestrated and vesiculated (Fig. 7). The elevated number of large, irregularly shaped Golgi vesicles indicated increased secretory activity of Golgi fields. Figure 6: 10-day control, magnification ×26,000; Figure 7: 10 days, 5 mg/L 4-chloroaniline, magnification ×18,300

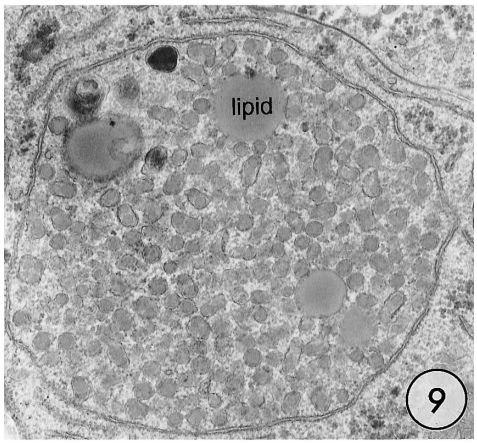
Gill Ultrastructure of Control Zebrafish Larvae

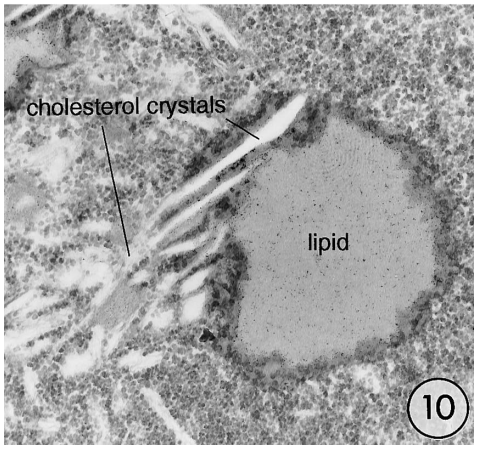
The ultrastructure of gills in control zebrafish was similar to that described by Karlsson (1983). In brief, primary gill lamellae were lined by an epithelium consisting of two cell types, flattened respiratory epithelial cells and chloride cells. Respiratory epithelial cells were prominent on secondary lamellae and form a duplicate layer limited by a basal lamina (Figure 12). On gills of larval zebrafish, apical microridges of respiratory cells were scant (Figure 13). Only close to adjacent cells, microridges were as prominent as described by Karlsson (1983). Respiratory cells regularly displayed flattened nuclei, Golgi fields, few lysosomes, cisternae of the rough endoplasmic reticulum, ribosomes, and mitochondria (Figure 13). In pillar cells, the large nucleus was centrally located, and peripheral flanges containing few mitochondrial and vesicles, but many microfilaments lined the central blood spaces of the secondary lamellae. In contrast to the description by Karlsson (1983), part of the controls in the present experiment showed minor desquamation of the epithelium, dilation of marginal canals, and electron-lucent vesicles in the cytoplasm of respiratory epithelial cells. Chloride cells concentrate at the base of secondary lamellae and were characterized by numerous mitochondria and an elaborated tubulovesicular system (Figure 14) in open connection to the cell surface. In the blood spaces, biconvex erythrocytes (Figure 15) and leukocytes could be observed.

Gill Ultrastructure of Zebrafish Larvae After 31-Days' Exposure to 4-Chloroaniline

Reactions of gill ultrastructure could only be observed from ≥0.5 mg/L 4-chloroaniline and displayed considerable inter- and intraindividual variability, even in adjacent cells. Prominent alterations were only observed in epithelial cells and erythrocytes, whereas the ultrastructure of pillar cells appeared unaffected (Table 5).







Figs. 8–10. After exposure to ≥0.5 mg/L 4-chloroaniline, increased numbers of myelin bodies (Figure 8), accumulations of steatotic vesicles (Figure 9) as well as deposition of crystalline materials resembling cholesterol crystals (Figure 10) can be observed in the cytoplasm of hepatocytes from zebrafish (*Danio rerio*) larvae exposed to 4-chloroaniline. At asterisks, note small glycogenosomes in Figure 8. Figure 8: 31 days, 5 mg/L 4-chloroaniline, magnification ×16,700; Figure 9: 31 days, 5 mg/L 4-chloroaniline, magnification ×17,600, Figure 10: 31 days, 0.5 mg/L 4-chloroaniline, magnification ×23,500

0.5 mg/L 4-Chloroaniline: After exposure to 0.5 mg/L 4-chloroaniline, part of the secondary lamellae showed irregular outlines due to bowl-shaped deformation of the respiratory epithelial cells, with the nuclei located in the curvature. In the plasmalemma below this bowl-shaped depression, electron-lucent vesicles and vacuoles appeared. Microridges were few and transformed into hook-like protrusions. These alterations, most likely due to changes in the permeability of the plasma membrane, lead to focal rupture of the basal cell membrane, occasional dilation of ER cisternae, and focal distension of Golgi cisternae and vesicles. Single respiratory epithelial cells of the outer epithelial layer were lifted off. However, adjacent cells were consistently connected by intact desmosomes. As a consequence of this desquamation, intercellular spaces appeared dilated. Even in areas without epithelial lifting, distension and invagination of intercellular spaces into the respiratory epithelial cells were prominent. Intercellular spaces were filled with cell debris, membranous material, myelinated bodies, or even intact organelles. Invasion of macrophages was common in distended extracellular spaces.

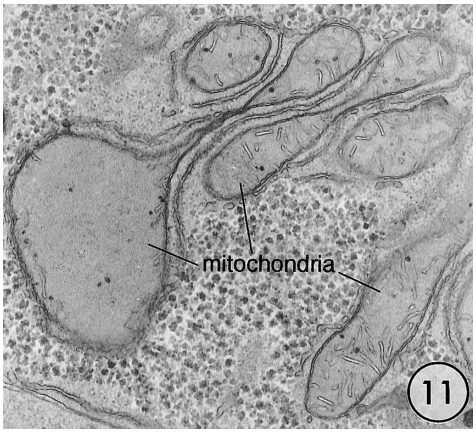
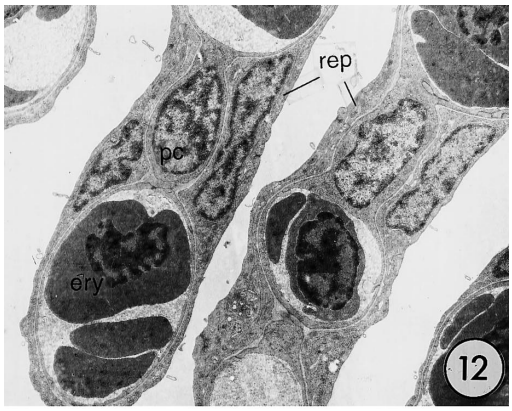


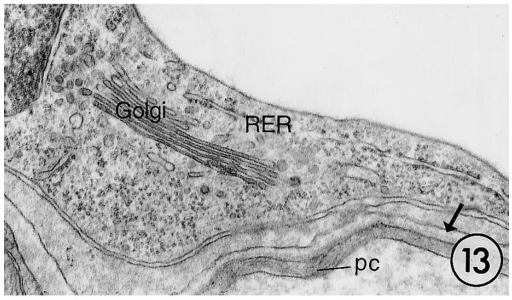
Fig. 11. In hepatocytes of zebrafish (*Danio rerio*) exposed to 4-chloroaniline for 31 days, formation of dumbbell-shaped mitochondria can frequently be documented from 0.5 mg/L 4-chloroaniline. Magnification ×17,500

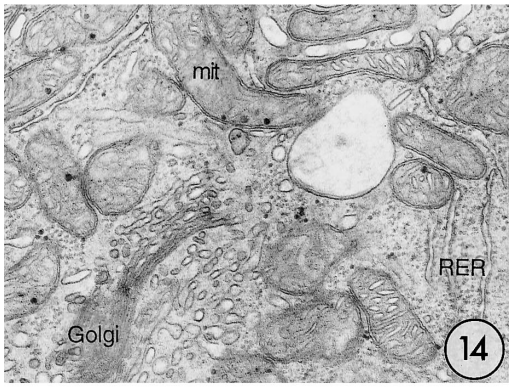
In both respiratory epithelial and chloride cells, myelinated bodies were prominent in the intermembranous space of mitochondria as well as between ER cisternae and Golgi fields. In addition, respiratory cells displayed proliferation and enlargement of lysosomes, which were heterogenous with respect to their myelin-like, crystalline, and/or membranous contents and electron density. Mitochondria frequently showed cup- or dumbbell-shaped deformation. Dilation and destruction of cristae, formation of translucent matrices, and swelling was indicative of mitochondrial degeneration, finally resulting in electron-optically translucent mitochondria and incorporation into lysosomes.

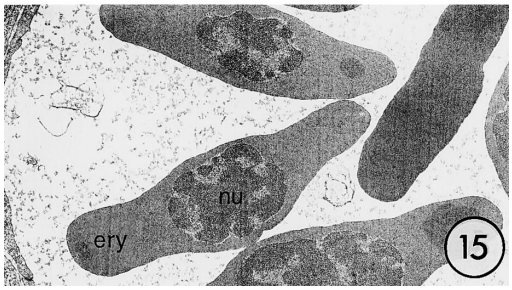
In the blood spaces, deformed erythrocytes with knob-shaped peripheral edges were occasionally found. A higher number of lymphocytes was present, and in rare occasions, the marginal canal appeared dilated.

5 mg/L 4-Chloroaniline: Following exposure to 5 mg/L 4-chloroaniline, changes described for lower concentrations were more frequent and more pronounced. In particular, the irregular outline of the secondary lamellae with protruded and lifted epithelial cells, the proliferation of electron-lucent vacuoles and myelinated bodies, Golgi alterations, and the disruption of the basal cell membrane in respiratory epithelial cells, as









Figs. 12–15. In the gills of 31-day-old control zebrafish (*Danio rerio*), blood spaces of secondary lamellae with erythrocytes (ery) are limited by pillar cells (pc), and respiratory epithelia are formed by a duplicate layer of respiratory epithelial cells (rep) with flattened nuclei (Figure 12). Respiratory epithelial cells are characterized by regularly arranged Golgi fields (Golgi) and slightly distended cisternae of rough endoplasmic reticulum (RER). Microridges are lacking. The basal membrane (→) is in contact with a pillar cell (pc), in which microfilaments are visible (Figure 13). Control chloride cells show extended Golgi fields (Golgi), few cisternae of rough endoplasmic reticulum (RER) and mitochondria (mit; Figure 14). Erythrocytes (ery) in control fish appear as regularly shaped biconvex nucleated (nu; Figure 15) cells. Figure 12, magnification ×3,300; Figures 13 and 14, magnification ×21,800, Figure 15, magnification ×6,300

well as the dilation of blood spaces (especially the marginal canals) were very prominent (Figures 16–19; Table 5). Part of the lysosomes took huge dimensions, were of very high electron density and displayed a multitude of different inclusions (Figure 19, inset). However, undamaged or mildly affected epithelial cells could still be observed in rare occasions.

In chloride cells, number and size of myelinated bodies were increased (Figure 20). Additional alterations at 5 mg/L 4-chloroaniline comprised thickening of the basal lamina especially below dilated intercellular spaces and a conspicuous deformation of erythrocytes (Figure 21) correlating with a significant reduction of the mean section area of erythrocytes (Figure 22). Since erythrocyte deformation was similar in vessels of secondary and primary lamellae, mechanical deformations as a consequence of the smaller diameter of blood spaces in secondary lamellae was not likely. In the blood spaces, cellular debris and membrane aggregates were seen in considerable amounts. Both lymphocytes and macrophages were found in blood spaces as well as invading the gill epithelium.

Gill Ultrastructure of Zebrafish Larvae After a 14-Day Recovery Subsequent to a 31-Day 4-Chloroaniline Exposure

Following a 2-week regeneration period, gills displayed far-ranging regeneration. However, part of the respiratory epithelial cells still showed an elevated number of lysosomes, myelinated bodies, and enlarged Golgi vesicles. Likewise, in chloride cells, the number of myelinated bodies was still increased. Moreover, dilation of the marginal canals, mild desquamation of the epithelium, and a slight increase in cytoplasmic vesicles and vacuoles reminded of former 4-chloroaniline exposure.

Discussion

Ecotoxicology has been defined as the science on adverse effects of chemical compounds on populations, communities, and ecosystems. Thus, in ecotoxicological research, emphasis should be put at the population and ecosystem levels. However, given the usually relatively low concentrations of contaminants in aquatic systems, the comparatively low sensitivity of parameters measured at higher levels of biological organization may frequently result in a failure to detect the presence and effects of trace contaminations. Since, in recent years, continuous exposure to low toxicant concentrations of complex chemical mixtures has become more important than events of acute toxicity (Segner and Braunbeck 1998), the development of more sensitive and more rapid methods is required (Braunbeck 1998). One such approach is the concept of biomarkers, which is based on the measurement of molecular, cellular, or organismic parameters as indicators of sublethal effects at higher levels of biological organization (Hinton and Laurén 1990; McCarthy and Shugart 1990; Huggett *et al.* 1992).

However, the assessment of the ecological importance of biomarkers, *i.e.*, the interpretation of sublethal effects of contaminants with respect to their relevance for populations and the environment, is extremely difficult, since most reactions induced by very low doses of toxicants represent adaptive rather than degenerative phenomena (Braunbeck 1994, 1998).

Table 5. Semiquantitative evaluation of ultrastructural alterations in gills of larval zebrafish (*Brachydanio rerio*) exposed to 4-chloroaniline for 31 days and after a regeneration period of 14 days

	31 Days (mg/L)				Regeneration (mg/L)			
	Co	0.05	0.5	5	Co	0.05	0.5	5
General organization of secondary lamellae								
Irregular outline	±	−	+	+++	±	+	±	±
Epithelial cells								
General organization								
Cytoplasmic protrusions	±	−	+	+++	±	±	±	±
Desquamation of epithelial cells	+	±	+	++	±	+	±	+
Formation of vacuoles and vesicles	+	+	++	++	+	+	±	+
Mitochondria								
Dilation of cristae	−	−	+	+	−	−	−	−
Formation of myelinated bodies	−	−	+	++	−	−	−	−
Degeneration of mitochondria	−	−	+	++	−	−	−	−
Golgi fields								
Focal dilation of Golgi cisternae	−	−	+	++	−	−	−	−
Enlargement of Golgi vesicles	−	−	+	++	−	−	+	+
Lysosomes								
Proliferation of lysosomes	−	−	++	++	−	−	+	+
Increase in size of lysosomes	−	−	++	+++	−	−	+	+
Number of myelinated bodies	−	−	++	++	−	−	−	−
Formation of crystalline structures	−	−	+	+	−	−	−	−
Plasmalemma								
Disruption of basal cell membranes	−	−	++	+++	−	−	−	−
Basal membrane								
Thickening of basal membranes	−	−	−	+++	−	−	−	−
Chloride cells								
Number of myelinated bodies	−	−	++	±	−	−	+	+
Blood spaces								
Dilation of marginal canals	−	−	±	++	−	+	+	+
Irregular outline of erythrocytes	−	−	+	+++	−	−	−	−

Data are given as means from 4 specimens. Co = controls; − = absent; ± = very little developed; + = little developed; ++ = moderately developed; +++ = strongly

Most likely, such sublethal reactions evolved to compensate for disturbances in metabolic homeostasis and, thus, to avoid detrimental consequences at higher levels of biological organization. As a result, the correlation of sublethal changes with effects at the individual, population, or even ecosystem level may well be poor, although there is strong indication of interference of contaminants with biological systems (Braunbeck 1998).

One important criterion for the ecotoxicological assessment of sublethal effects is the question as to their reversibility, *i.e.* their persistence after cessation of exposure. In aquatic toxicology, histological and cytological modifications of selected monitor organs such as the liver of fish have repeatedly been shown to represent extremely sensitive tools to detect traces of organic micropollutants (for reviews, see Hinton and Laurén 1990 and Braunbeck 1998). Since none of the studies performed so far provides information on the reversibility of morphological changes, the primary purpose of the present study was not only to record effects by water-borne 4-chloroaniline in liver and gills of early life stages in zebrafish and to compare them to reactions in adult zebrafish (Braunbeck *et al.* 1990a), but also to investigate the recovery of zebrafish liver. Moreover, the study was designed to compare alterations by waterborne 4-chloroaniline to those induced by microinjection (Oulmi and Braunbeck 1996) as well as effects produced *in vitro* in primary cultures of hepatocytes isolated from rainbow trout (Braunbeck 1993) as well as the permanent fish cell line R1 (Zahn *et al.* 1993).

Results clearly document multiple cytological alterations in both liver and gills after sublethal exposure to 4-chloroaniline and, thus, confirm the extraordinary susceptibility especially of liver cytology to 4-chloroaniline shown in earlier experiments with adult and larval zebrafish (Braunbeck *et al.* 1990a; Oulmi and Braunbeck 1996). A comparison of electron microscopical changes by 4-chloroaniline in larval and adult zebrafish reveals striking similarities for numerous cytopathological changes (Table 6). However, since in a comparative study on cytopathology of rainbow trout liver after exposure to seven different organic xenobiotics (Braunbeck 1994) most of the electron microscopically detectable changes listed in Table 6 could be demonstrated to be not substance-specific, the similarity of effects in different test systems cannot be interpreted as evidence of substance-specificity.

Although the 4-chloroaniline dose set during microinjection in a fertilized egg or an embryo cannot be directly correlated to a specific water concentration (Metcalfe and Sonstegard 1984, 1985; Black *et al.* 1985, 1988; Grizzle *et al.* 1988; Metcalfe *et al.* 1988; Mizell and Romig 1997), the LOECs (lowest observed effect concentrations) for specific cytological changes were quite similar in all test systems (Table 6). This might indicate that these concentrations and doses really represent the lower detection limit for the changes listed for 4-chloroaniline.

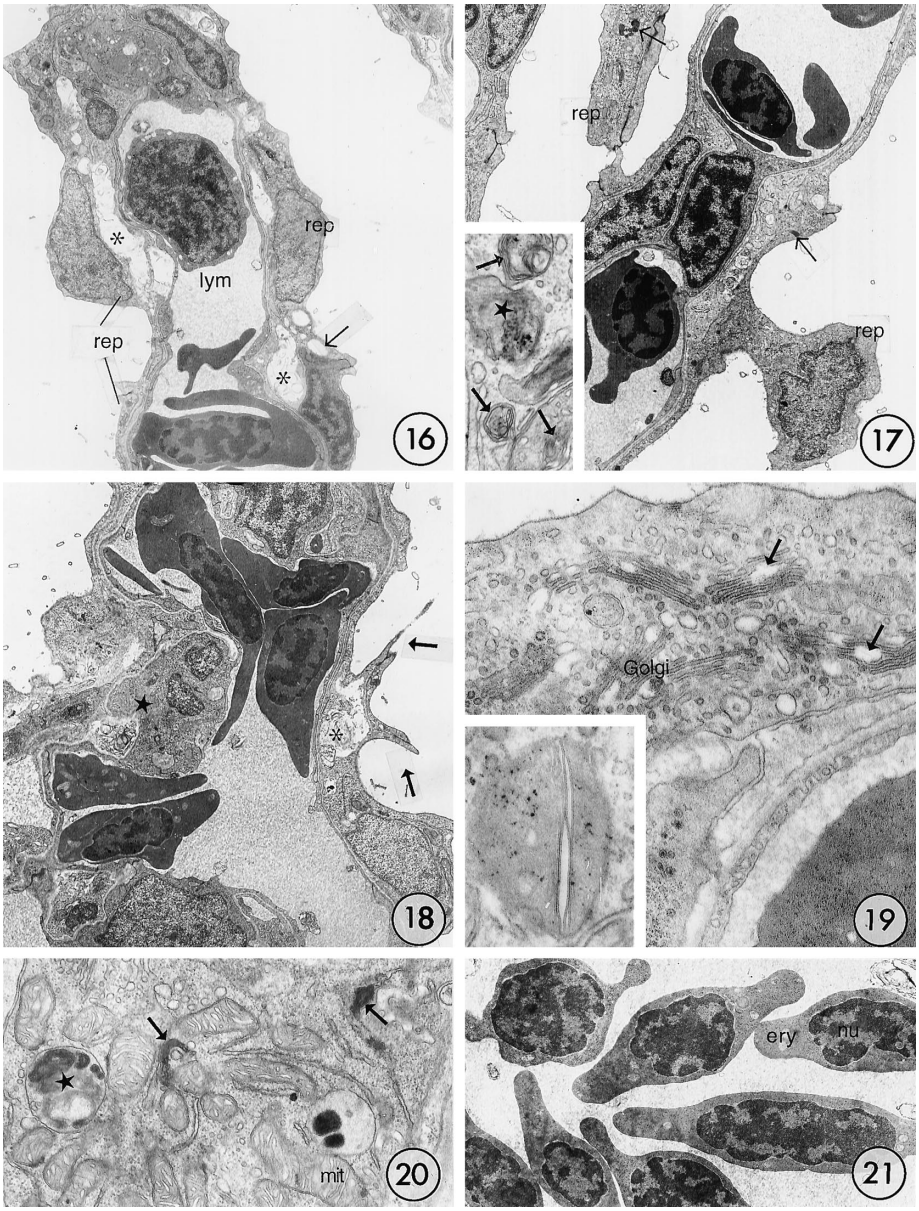


Fig. 16. Desquamation of the epithelium with cellular debris in the dilated intercellular spaces (*) and lymphocyte in the blood space (lym). In the respiratory epithelial cells (rep), vacuoles are visible (→). Magnification ×3,300

Fig. 17. Irregular outline of the secondary lamella with protruded respiratory epithelial cells (rep). Note proliferation of myelinated bodies in respiratory epithelial cells (→). Inset: Myelinated bodies in the cytoplasm and mitochondria (→) as well as proliferation of lysosomes (*). Figure 17, magnification ×3,500; inset, magnification ×25,900

Fig. 18. Disorganization of the secondary lamella with dilated intercellular spaces filled with debris (*); note hook-like protrusions of the microridges (→) and necrotic pillar cell (*). Magnification ×3,200

Fig. 19. Focal dilation of Golgi cisternae (→) and enlargement of Golgi vesicles. Magnification ×21,800. Inset: Lysosome with crystalline structures. Magnification ×21,800; inset, magnification ×54,400

Fig. 20. In chloride cells, myelinated bodies are prominent in the cytoplasm (→) as well as within lysosomes (*) and mitochondria (mit). Magnification ×13,100

Fig. 21. After 31 days of exposure to 5 mg/L 4-chloroaniline, erythrocytes (ery) take an irregular shape. Magnification ×4,800

Likewise, whereas it is very difficult to compare the 4-chloroaniline exposure in the *in vitro* systems to that in the *in vivo* experiments in terms of effective concentrations inside the cells, there is a surprisingly high correlation with regard to the qualitative nature of the *in vitro* effects in hepatocytes (Braunbeck 1993).

With respect to the lower detection limit of sublethal effects by 4-chloroaniline, a comparison with ultrastructural alterations in the liver of larval and adult zebrafish reveals that the LOEC data (0.05 mg/L and 0.04 mg/L, respectively) are identical. Since biodegradation of 4-chloroaniline in soil is slow, partly due to transformation into more stable compounds such as chlorinated biphenyls, azo compounds, and triazines (Corke *et al.* 1979; Latorre *et al.* 1984), maximum 4-chloroaniline concentrations measured in surface waters were as high as 1 µg/L in the Rhine river (Wegmann and DeKorte 1981; Malle 1984), and 2.2 µg/L in Dutch waters (Cost 1984) were measured. Thus, electron microscopical changes in hepatocyte structure cannot only be observed well below conventional NOEC (no observed effect concentration) values given for zebrafish (1.8 mg/L; Rudolph and Boje 1987), and LOEC data (3.2 mg/L; Munk 1984), but are also within environmentally relevant ranges. With regard to acute toxicity, the LC_{50} for adult zebrafish (46 mg/L) is higher than for other fish species (rainbow trout: 14 mg/L; fathead minnow, *Pimephales promelas:* 12 mg/L; blue-green sunfish, *Lepomis macrochirus:* 2 mg/L; Verschueren 1983). However, LC_{50} values of 44, 38, and 28 mg/L after 4, 8, and 28 days of exposure document that early life stages of zebrafish are more susceptible to 4-chloroaniline exposure than adults.

As the liver, fish gills represent a well-known target organ for toxic agents and are rapidly affected by unfavorable environmental conditions (for literature, see Mallatt 1985; Lock *et al.* 1994; Poleksic and Mitrovic-Tutundzic 1994). If compared with data listed for hepatocytes in Table 6, the ultrastructurally observed alterations of the gills appear to be slightly less sensitive, but

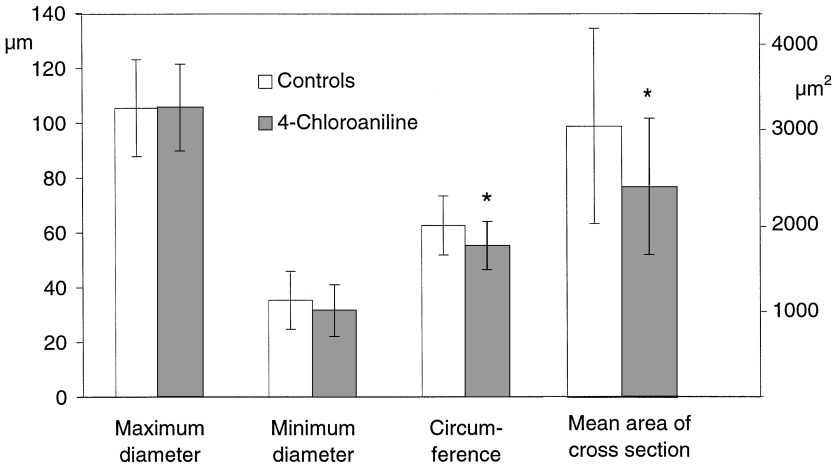


Fig. 22. Morphometric analyses of parameters of erythrocytes in the gills of juvenile zebrafish. Comparison between erythrocytes of control and 5 mg/L 4-chloroaniline–exposed zebrafish show significant differences (*; $p < 0.05$) in the section area and in the diameter of an idealized circle with the corresponding section area

can also be classified into specific and unspecific alterations as well as effects independent of the organ. Alterations such as the proliferation of lysosomes; the induction of myelinated bodies within the cytoplasm, mitochondria, endoplasmic reticulum, nuclear envelope, Golgi cisternae, and lysosomes; and the deformation of mitochondria were not only observed in gill epithelial and chloride cells, as well as in hepatocytes and renal tubular cells of zebrafish embryos after microinjection of 4-chloroaniline (Oulmi and Braunbeck 1996), but also in fish exposed to a multitude of other chemical stressors (Karlsson-Norrgren *et al.* 1985; Mallat *et al.* 1985; Braunbeck *et al.* 1989; Segner and Braunbeck 1998) and have, therefore, to be classified as independent of both substance and organ.

In contrast, effects such as proliferation of irregularly formed gill lamellae accompanied or followed by lifting of the epithelium, thickening of epithelial cells in conjunction with a more pronounced development of cell surface protrusions, dilation of intercellular spaces filled with debris, myelin-like structures, and leukocytes appear more specific of the gills (Karlsson-Norrgren *et al.* 1985; Mallat *et al.* 1985) and can be related to functional consequences. Many of these changes result in increased distances for oxygen diffusion and, consequently, in inadequate gas exchange (Karlsson-Norrgren *et al.* 1985). The reduced diffusion capacity may be at least partly compensated by the dilation of blood spaces, especially the marginal canal, as observed in the present study.

However, morphometric analysis of blood cells has also documented 4-chloroaniline to specifically affect erythrocytes. Since, at least in mammals, hemoglobin plays a role in N- and C-oxidation of 4-chloroaniline (Corbett *et al.* 1980; Golly *et al.* 1984; Lenk and Sterzl 1984; cf. Golly and Hlavica 1985) and hemoglobin adducts are formed (Golly and Hlavica 1983), red blood cells are likely to be another site of bioactivation of aromatic amines apart from the liver. Some of the oxidative metabolites of 4-chloroaniline have been demonstrated to be potent mutagens and carcinogens (Golly and Hlavica 1983; Lenk and Sterzl 1984). The methemoglobin formation induced by aromatic amines results in a decrease of arterial O_2 pressure and O_2 depletion of peripheral organs and may account for the changes in erythrocyte morphology (Jensen *et al.* 1987; Khan *et al.* 1993; Jensen 1996). The reduced O_2 affinity resulting from erythrocyte shrinking (Jensen *et al.* 1987; Jensen 1996) may be a further reason for the damage in mitochondria observed in various organs and cells (Table 6; Braunbeck *et al.* 1990a; Braunbeck 1993; Oulmi and Braunbeck 1996) not only after both *in vivo* and *in vitro* exposure to 4-chloroaniline, but also from nitrite toxicology (Arillo *et al.* 1984).

Although numerous studies have demonstrated that xenobiotic exposure may result in inhibition of transport enzyme systems of chloride cells and subsequent compensatory proliferation of chloride cells (Evans 1987; Bury *et al.* 1996; Lee *et al.* 1996), only few reports have given attention to cytological changes of chloride cells. After exposure to low concentration of cadmium, *e.g.*, chloride cells were the first cells of the gill epithelium to display lesions in form of a reduction in size and a vacuolation of the cytoplasm (Karlsson-Norrgren *et al.* 1985). Since, apart from a proliferation of myelinated bodies, no cytological modifications could be observed in chloride cells of zebrafish larvae exposed to 4-chloroaniline, chloride cell function seems to be restricted to ionic and osmotic regulation, which is apparently not affected by chloroaniline compounds.

A recreation period of 2 weeks in uncontaminated water seems to be sufficient for an almost complete regeneration of cytopathological alterations in both liver and gills. Whereas in gill epithelia elevated rates of mitosis have repeatedly been shown to represent a way of rapid regeneration (Temmink *et al.* 1983; Leino and McCormick 1984; Chretien and Pisam 1986; Zenker *et al.* 1987), the rate of hepatocytes in mitosis is far below 1% and cannot account for the rapid restoration of hepatic ultrastructure. However, in the present experiment, there was no conspicuous increase in the mitotic rate of gill epithelial cells as well, and the persistence of increased numbers of lysosomes and myelinated bodies in at least larger portions of the gill epithelium document that mitosis did not play a predominant role during regeneration, and that by far not all cells were replaced, but had truly recovered. Rather, most of the ultrastructural effects described in both hepatocytes and gill epithelial cells have therefore to be classified as fully reversible symptoms of cellular adaptation to stress. Similar observations were made in the livers of various species of ornamental fish exposed to therapeutical agents such as malachite green and chinin for extended periods of time (Braunbeck *et al.* in preparation). As in cells regenerating from exposure to 4-chloroaniline, hepatocytes of fish previously treated with malachite green can be identified by an elevated number of lysosomal elements.

Table 6. Comparison of cytopathological effects by 4-chloroaniline in the liver of larval and adult zebrafish (*Brachydanio rerio*) after long-time exposure to water-borne 4-chloroaniline, in zebrafish larvae following single microinjection, as well as in isolated hepatocytes from rainbow

	Chronic Water-Borne Exposure		Microinjection	*In Vitro* Exposure
	Zebrafish Larvae (10 and 31 days old)	Adult Zebrafish[a] (4 months old)	Zebrafish Larvae[b] (4 and 6 days old)	Isolated Hepatocytes[c] (rainbow trout)
Liver parenchyma				
Disturbance of compartmentation	0.5 mg/L	0.04 mg/L	5 ng/egg	1 mg/L
Increasing intercellular heterogeneity	0.5 mg/L	0.04 mg/L	5 ng/egg	1 mg/L
Invasion of macrophages	—	1 mg/L	12.5 ng/egg	***
Nucleus				
Irregular outline	0.5 mg/L	—	5 ng/egg	1 mg/L
Reduction of heterochromatin	—	—	—	1 mg/L
Reduced electron density of nucleus	—	—	—	3 mg/L
Nucleolus displaced to periphery of nucleus	—	—	—	1 mg/L
Loss of nucleolus	—	—	—	10 mg/L
Mitochondria				
Proliferation of atypical profiles	0.05 mg/L	—	12.5 ng/egg	3 mg/L*
Increase in size	0.05 mg/L	—	5 ng/egg	1 mg/L
Myelin-like inclusions	—	1 mg/L	25 ng/egg	1 mg/L
Increase of mitochondrial granules	0.05 mg/L	—	—	—
Dilation of intermembranous spaces	—	—	—	3 mg/L
Reduced matrix electron density	—	—	—	10 mg/L
Loss of mitochondrial cristae	—	—	—	3 mg/L
Association with cytoplasmic myelin	—	—	—	3 mg/L
Peroxisomes				
Decrease in number	0.5 mg/L	0.2 mg/L	—	—
Proliferation	—	—	—	3 mg/L
Increased morphological heterogeneity	0.5 mg/L	0.2 mg/L	12.5 ng/egg	3 mg/L
Formation of clusters	—	—	—	10 mg/L
Rough endoplasmatic reticulum				
Overall reduction	0.05 mg/L	0.04 mg/L	5 ng/egg	1 mg/L
Less regular arrangement	0.05 mg/L	0.04 mg/L	5 ng/egg	1 mg/L
Fenestration of cisternae	0.05 mg/L	0.2 mg/L	5 ng/egg	3 mg/L
Fractionation/vesiculation of cisternae	0.05 mg/L	0.2 mg/L	5 ng/egg	1 mg/L
Dilation of RER cisternae	0.05 mg/L	—	5 ng/egg	1 mg/L
Induction of steatosis	0.05 mg/L	—	12.5 ng/egg	1 mg/L
Loss of spatial relationship with mitochondria and peroxisomes	0.5 mg/L	0.2 mg/L	5 ng/egg	1 mg/L
Transformation into membrane whorls	—	—	—	1 mg/L
Smooth endoplasmatic reticulum				
Proliferation	0.5 mg/L	—	—	1 mg/L
Golgi fields				
Proliferation of Golgi fields	0.05 mg/L	0.2 mg/L	—	1 mg/L*
Size reduction of Golgi cisternae	0.05 mg/L	—	—	—
Fenestration/vesiculation of cisternae	0.05 mg/L	0.2 mg/L	12.5 ng/egg	1 mg/L
Proliferation of secretory vesicles	0.05 mg/L	0.2 mg/L	—	—
Dilation of Golgi cisternae	0.05 mg/L	0.2 mg/L	12.5 ng/egg	1 mg/L
Altered secretory activity	0.05 mg/L	—	12.5 ng/egg	1 mg/L
Lysosomes				
Overall proliferation	0.05 mg/L	0.04 mg/L	5 ng/egg	1 mg/L**
Increase in size	0.05 mg/L	0.2 mg/L	5 ng/egg	1 mg/L**
Proliferation of atypical profiles	0.05 mg/L	0.2 mg/L	5 ng/egg	1 mg/L**
Induction of crystalline inclusion	—	0.2 mg/L	5 ng/egg	1 mg/L
Proliferation of myelin whorls in cytoplasm	—	0.2 mg/L	5 ng/egg	1 mg/L
Glycogen stores				
Heterogeneous distribution between cells	0.5 mg/L	0.2 mg/L	5 ng/egg	1 mg/L
Overall decrease	0.5 mg/L	0.2 mg/L	5 ng/egg	***
Formation of membrane-glycogen complexes	—	—	—	1 mg/L
Lipid deposits				
Proliferation of lipid deposits	0.05 mg/L	—	5 ng/egg	1 mg/L
Proliferation of cholesterol crystals	0.05 mg/L	1 mg/L	12.5 ng/egg	—

Data presented as lowest concentration of 4-chloroaniline, at which effect could be observed

[a] Data for chronic exposure of adult zebrafish from Braunbeck et al. (1990a)

[b] Data for microinjection of zebrafish eggs from Oulmi and Braunbeck (1996)

[c] Data for isolated rainbow trout hepatocytes from Braunbeck (1993)

* Temporarily after 2-day *in vitro* exposure of rainbow trout hepatocytes to 4-chloroaniline only; ** long-term suppression of the proliferation typical in isolated control hepatocytes of rainbow trout; *** not comparable, since in isolated rainbow trout hepatocytes glycogen contents are only temporarily reduced

Conclusions

The data presented document that both liver and gill cells in early larval stages of zebrafish show a very rapid response to sublethal exposure to 4-chloroaniline, which, in qualitative terms as well as with respect to sensitivity, is comparable to the reaction of adult zebrafish following prolonged sublethal exposure as well as larval zebrafish after microinjection of newly fertilized eggs. In the case of 4-chloroaniline, the liver appears to react more sensitively than the gills. Comparison of the lower detection limits for ultrastructural changes especially in the liver with results from other studies with 4-chloroaniline reveals that electron microscopical changes represent an extremely sensitive source of biomarkers of sublethal chemical exposure. After a regeneration period, most cytopathological modifications proved to be fully reversible. Morphological changes in erythrocytes indicate disturbance of respiration as an additional mode of action of 4-chloroaniline.

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