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Brown trout (*Salmo trutta f. Fario*) liver ultrastructure as a biomarker for assessment of small stream pollution

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The effects of environmental pollutants from two small streams in south-west Germany on the liver of brown trout (*Salmo trutta f. Fario*) were studied as biomarkers by means of quantitative and semi-quantitative electron microscopy, and quantitatively by morphometrical measurements. Cellular damage was assessed semi-quantitatively based on a classification of ultrastructural responses. Both methods revealed more severe cellular effects in the liver of trout which had been exposed to the highly polluted stream than in those exposed to the lightly polluted river. Morphometrical studies showed a significant reduction of glycogen storage and a significant increase in number of mitochondria, peroxisomes and cisternae of the endoplasmic reticulum. The biomarker responses of this study were correlated with the results obtained by limnological and analytical investigations, and reflect the levels of pollution in each stream.

Keywords: Brown trout, liver ultrastructure, biomarker, aquatic toxicology, monitoring, teleost, *Salmo trutta f. Fario.*

Introduction

The aim of this study was to assess the toxicity of water from small streams to indigenous fish by means of biomarker research. Biomarkers represent useful tools to characterize the state of health of important members of the aquatic system (Triebskorn *et al.* 1996). They can be applied directly in the field, but also to organisms exposed by "active monitoring" to the test water (Arndt *et al.* 1987). Generally, fish are suitable organisms with which to monitor environmental pollution. They are located at the top of the aquatic food chain, and are known to accumulate toxicants (Meyer 1990, Rowan and Rasmussen 1992). Secondly, they are in direct contact with polluted water via their gills and their body surface (Arndt *et al.* 1987). The best established monitor organ in fish is the liver (e.g. Braunbeck *et al.* 1992, Braunbeck and Völkl, 1993). As a major metabolic organ, the liver plays an important role in uptake, accumulation (Couch 1975, Gluth *et al.* 1985), biotransformation (Achazi, 1989, Braunbeck and Völkl 1991), and excretion (Pentreath 1976, Köhler 1990) of xenobiotics. Functional changes are known to be reflected in structural changes of hepatocytes (Couch 1975, Meyers and Hendricks 1982, Arias *et al.* 1988), which in turn can be used as biomarkers to trace environmental pollution caused by chemicals (McCarthy and Shugart 1990).

In this study, the ultrastructure of hepatocytes was investigated in four groups of brown trout exposed over a two year period to water from two streams in southern Germany. Limnological and analytical surveys revealed one of these streams to be highly affected by the output from six sewage plants. The pollutants

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detected in surface water, sediments, and sewage plant effluents include nitrogen and phosphorous compounds, pesticides, heavy metals, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). The second stream was shown to be less polluted and unaffected by sewage plants. By means of a valuation system of cytopathological changes, the "state of health" of hepatocytes or hepatic parenchyma of exposed trout were characterized. These results were correlated to the pollution of the corresponding stream. Further, the different cellular alterations are discussed on the basis of the occurrence of contamination in the streams.

Materials and methods

Animals

Three to four month old brown trout (*Salmo trutta f. Fario*) were obtained from a hatchery (Belser, Haigerloch-Gruol, Germany). They were fed on a commercially available pelleted feed (ALMA, Botzenhard GmbH, Kempten, Germany) at a daily rate corresponding to 1.5% of the body weight. The species was chosen as monitor organism, because it is an indigenous fish in the investigated streams, and there are many published data from toxicological experiments (also for rainbow trout, *Oncorhynchus mykiss*). Only sexually immature trout were used, so sex-specific alterations in the ultrastructure of liver were excluded.

Characterization of the streams

The fish-monitoring took place at two small streams (Körsch and Krähenbach) in south-west Germany. The Körsch runs through a densely populated and intensively used agricultural area near Stuttgart. Beside assorted inputs from agriculture, distinct inputs from six sewage plants are present. The Krähenbach runs through sparsely populated countryside and has no sewage-plant influx. There is only a little agriculture along the stream. Possible sources for pollution are a road next to the stream, and air traffic. The mean water run off amounts to 0.6–1.8 $m^3 s^{-1}$ (Körsch) and at least 0.01 $m^3 s^{-1}$ (Krähenbach). Trout are indigenous fish in both streams. The data on the occurrence of contaminants in water and sediment are shown in table 1.

Table 1. Occurrence of contamination (mean value ±standard deviation) in the two streams, in which the fish monitoring took place

Concentrations of contaminants in the two streams		
Water	Krähenbach	Körsch
Pesticides [µg l^{-1}]	0.01 ±0.02	0.17 ±0.15 (***)
PCB [µg l^{-1}]	0 ±0	0 ±0
PAH [µg l^{-1}]	0.06 ±0.02	0.11 ±0.04 (**)
Heavy metals [µg l^{-1}]	86 ±76.7	119 ±73.9
Sediment		
Pesticides [µg kg^{-1}]	6.5 ±8.2	25.6 ±22.8 (**)
PCB [µg kg^{-1}]	29.3 ±40.3	102.5 ±81.5 (**)
PAH [mg kg^{-1}]	3.6 ±2.3	12.1 ±4.4 (***)
Heavy metals [mg kg^{-1}]	50.5 ±19	346.5 ±123.6 (***)

The analyses were performed monthly during the exposure of the fish. Presented as background data (after Honnen *et al.* 1997b). (**) real difference ($p < 0.01$), (***) $p < 0.001$. Pesticides, PCB, PAH and heavy metals are defined and indicated in the text (*Materials* and *Methods: Pollutants chosen for analyses*).

Pollutants chosen for the analyses (Honnen et al. 1997b)

Herbicides (after DIN 38407F12)**:** atrazine, desethylatrazine, isoproturon, linuron, propazine, simazine, terbuthylazine

Organochlorine insecticides (after DIN 38407F2)**:** o,p´-DDT, p,p´-DDT, chlorfenvinphos, β-endosulfane, endrine, alpha-, beta-, gamma-, delta-, epsilon-hexachlorocyclohexane, pentachlorophenol, tri-allate

Acid amides: metazachlor, metolachlor

Phenylurea derivates (after DIN 38407F12), **sulfonylureas:** diuron, isoproturon, rimsulfuron

Phenoxyalkanoic acids (after DIN 38407F14)**:** mecoprop, dichlorprop, fluazifop

Chlorobenzoles: mono-, di-, tri-, tetra-, penta-, hexachlorobenzole

Pyrethroids: cyhalothrin, deltamethrin, cypermethrin

Phosphoric acid esters: dimethoate, parathion-methyl

Nitrogen-containing herbicides (carbamates, thiocarbamates)**:** mercaptodimethur, pirimicarb, pyridate, trifluralin

PCBs (polychlorinated biphenyls)**:** PCB 28, PCB 52, PCB 101, PCB 153, PCB 138, PCB 180

PAHs (polyaromatic hydrocarbons)**:** naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, indeno(1,2,3–*c,d*)pyrene, dibenzo(*a,h*)anthracene, benzo(*g,h,i*)-perylene

Metals: cadmium, copper, zinc, lead

Details of the analyses methods have been published by Honnen *et al.* (1997b).

Exposure conditions

Fish were kept in 250-litre flow-through aquaria (Bypass-Systems) connected to the Körsch and Krähenbach streams. Stream water flowed through the aquaria at a constant rate of 200 l h^{-1}. The exposure aquaria contained river sediment and the flow was imitated by stream pumps. Control fish were kept in the laboratory with uncontaminated water. In the laboratory, water temperature and light/dark phases were adjusted to field values. Four groups of fish (F1, F2, F3, F4) were exposed to these conditions for different lengths of time.

Exposure time and sampling

From each group of fish, exposed as well as controls, samples (n = 6 fish) were taken at the end of each exposure. A total number of 126 trout were examined. Time of exposure and codes for the exposed fish groups are shown in table 2.

Cytological techniques

Prior to fixation for electron microscopy, fish were anaesthetized with ethyl-4-aminobenzoate (benzocaine). They were perfused *in situ* via the ventricle with ice-cold 1.5% glutaraldehyde and 1.5% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer (pH 7.6) containing 2.5% polyvinylpyrrolidone (PVP, Merck, Darmstadt). The anterior portion of the liver was excised immediately after perfusion and cut into pieces of 1 mm length. After fixation in the perfusion fixative for 60 min (at 4°C), samples were rinsed in three portions of cacodylate buffer. Then they were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.6), containing 4% PVP and 0.05% calcium chloride for at least 24 h. After rinsing in cacodylate buffer, the tissue pieces were postfixed in 1% osmium ferrocyanide (Karnovsky 1971) for 1 h at 4°C. After washing in 0.1 M cacodylate and 0.05 M maleate buffer (pH 5.2), samples were stained *en bloc* with 1% uranyl acetate in maleate buffer for at least 1 h at 4°C. The specimens were dehydrated in a graded series of ethanol solutions and embedded in Epon medium. Ultrathin sections (50–100 nm) were stained with alkaline lead citrate (Reynolds 1963)

Table 2. Codes for the different fish groups, exposed to stream water.

Code for sampling	Group	Time of exposure in weeks
05	F1	13 (July 13 – Oct. 12 1995)
07	F1	21 (July 13 – Dec. 7 1995)
05	F2	8 (Aug. 17 – Oct. 12 1995)
07	F2	16 (Aug. 17 – Dec. 7 1995)
14	F3	12 (May 3 – July 29 1996)
16	F3	20 (May 3 – Sept. 18 1996)
14	F4	6 (June 13 – July 24 1996)

Table 3. Semiquantitative assessment of cytopathological alterations in hepatocytes of trout

	Cytopathological categories		
	1	2	3
Parenchyma			
Bile canaliculi	• Microvilli short and regularly arranged • Few external cells	• Slight increase in number of external cells • Augmentation of apical vesicles	• Microvilli prolonged and diffusely arranged • Reduction in number of microvilli • Many apical vesicles • Many external cells
Space of Disse	• Microvilli short and regularly arranged • Few external cells	• Slight increase in number of external cells	• Many external cells • Reduced microvilli • Presence of ito-cells
Hepatocyte			
Cytoplasmic compartmentation	• Strongly developed	• Disturbance	• No compartmentation
Nucleus	• Round to oval shaped • Only mononucleated cells • Little heterochromatin • Nucleolus not organized in pars fibrosa and pars granulosa.	• Slight deformation of the nuclear envelope • Slight swelling of perinuclear cisternae	• Deformation of the nuclear envelope • Fractionation of nuclear membrane • Perinuclear cisternae enlarged • Inclusions in the nucleoplasm (lipid, myelin-like structures)
Endoplasmic reticulum	• Long, parallell stacked cisternae surrounding the nucleus • Absence of fenestration	• Presence of short and fenestrated cisternae portions • Slight ER-alterations	• Short cisternae • Degranulation of cisternae • Dilation of cisternae • Fenestration of cisternae • Vesiculation of cisternae • Strong proliferation of cisternae • Steatosis • Formation of myelin-like bodies or membrane whorls • Breakdown of ER-membrane
Golgi apparatus	• 3–4 cisternae per dictyosome • Few vesicles located at *cis*- and *trans*-side	• Increased morphological heterogeneity	• Hypertrophy • Many vesicles of different size at *cis*- and *trans*-side • Dilation of cisternae • Fenestration of cisternae • Breakdown of membrane
Mitochondria	• Exclusively located close to the nucleus • Polymorphism	• Slight increase in volume • Decrease in the number of cristae	• Strong proliferation in number • Dilation of intermembrane space

Table 3—*cont.*

	Cytopathological categories		
	1	2	3
		• Slight proliferation in number	• Disintegration • Absence of mitochondrial cristae • Formation in matrix of myelin-like structures • Increase in volume
Peroxisomes	• Limited number	• Slight proliferation in number	• Massive proliferation in number • Formation of clusters
Lysosomes	• Limited number	• Slight proliferation in number	• Massive proliferation in number
Storage products	• Large glycogen areas in the cell periphery • Very few lipid-droplets	• Reduction of glycogen storage • Few lipid-droplets	• Much glycogen reduction • Increase of lipid-droplets
Macrophages, granulocytes	• Small number in the parenchyma	• Increased number, only in the parenchyma	• Greatly increased number
Myelin-like bodies	• Few	• Slight increase	• Prominent increase

1: control conditions, 2: slight changes and/or reactions are visible, 3: strong reactions and/or destruction are visible.

for 30 s to 1 min and examined with a Zeiss CEM 9 electron microscope. For quantification of glycogen, semithin sections were stained according to the method of Singh (1964).

Semiquantitative assessment of cytopathological changes

To characterize the 'state of health' of hepatocytes or hepatic parenchyma, the ultrastructural symptoms in liver tissue were classified as belonging to one of the following three categories: **1**, the control state, **2**, slight changes and/or reactions visible, **3**, strong reactions and/or destruction visible.

Criteria upon which this classification is based are listed in table 3. These criteria were derived from toxicological publications on fish liver ultrastructure in response to single toxicants (Couch 1975, Hacking *et al.* 1978, Gingerich 1982, Hampton *et al.* 1989, Köhler 1990, Braunbeck *et al.* 1990a, Braunbeck *et al.* 1990b, Hinton and Lauren 1990, Braunbeck and Völkl 1991, Heining and Hoffmann 1993).

For each test specimen, liver sections were investigated and the conditions of the cell organelles were allocated to one of the cytological categories listed in table 3. From these values (1, 2, 3) mean values for each fish were calculated. Mean values for each group (see table 2) were calculated from the mean values of the individual fish.

Morphometric procedures

Sampling and morphometric evaluation were performed according to the methods of Weibel *et al.* (1969) and Weibel (1979). Three levels of magnification were employed. Light micrographs were recorded from liver sections of 0.5 μm at a final magnification of ×1100. Electron micrographs of ultrathin sections were taken at magnification levels of ×7200 and ×18000. The volume density was estimated by placing a test point lattice with a defined number of test points on a micrograph and determining the proportion of these points enclosed within boundaries of the structure investigated. Test points on extra-hepatocellular structures (bile canaliculi, sinusoids, etc.) were subtracted from the total number of test points. For measurement of volume density of hepatocellular glycogen fields, light micrographs (magnification ×1100) with a test point lattice of 391 points spaced at a distance of d=10 mm (equivalent to 9 μm in the tissue) was employed. The volume densities of hepatic mitochondria, lipid and lysosomes were determined on electron micrographs (magnification

lattice with 391 systematically spaced points at a distance of d=10 mm on the test point lattice (equivalent to 1.4 µm in the tissue). Volume densities of endoplasmic reticulum and peroxisomes were determined on electron micrographs (magnification ×18000) with a test point lattice of 391 points spaced at a distance of d=10 mm (equivalent to 0.6 µm in the tissue).

Statistics

Differences between groups were evaluated according to the method of the nonparametric Wilcoxon-Mann-Whitney U-test (Sachs 1984). The control datasets of the electronmicroscopic investigations were used for the comparison with the corresponding fish group datasets of Krähenbach and Körsch. Following significance levels were chosen: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Results

Histology and ultrastructure in hepatocytes of control trout

Normal hepatocytes from fish exposed to control water were characterized by a system of parallel stacked cisternae of the rough endoplasmic reticulum (RER) in the vicinity of the centrally located round nucleus; few mitochondria and few other cell-organelles, e.g. lysosomes and peroxisomes (figure 1a). A pronounced cellular compartmentation into a central, organelle-rich area and a peripheral cell area with storage materials, mainly glycogen, was observed (figure 1a). The nuclei contained only a little heterochromatin. They were usually surrounded by stacks of five to fifteen non-fenestrated lamellae of the RER. The dictyosomes usually consisted of three to four cisternae. A few Golgi-vesicles of different size were found on the *cis*- as well as on the *trans*-side of the dictyosomes. Mitochondria were almost exclusively located close to the nuclei and between the cisternae of the RER. Peroxisomes were very rare and were randomly distributed over the cytoplasm. Few lipid inclusions were present. Macrophages and granulocytes were found only in small numbers along the bile-canaliculi or in the sinusoids. In the space of Disse, the liver cell membrane (adjacent to the endothelial cells of the sinusoids) exhibited numerous microvilli. Collagen fibres and a distinct type of fat-storing cell (*ito-cell*) were also found in the space of Disse.

Qualitative changes in exposed trout

Lightly polluted stream (Krähenbach). The liver ultrastructure of fish exposed to the lightly polluted stream water did not differ significantly from controls. One obvious reaction was the increase of apical vesicles close to the bile canaliculi. A few fish showed some changes to the cytoplasmic compartmentation and slight reduction in glycogen content of the hepatocytes (figure 1b). Rarely, vesiculation of the RER was observed. In these fish, the observed effects were much less pronounced than in fish exposed to the highly polluted stream water.

Highly polluted stream (Körsch). In comparison to controls, hepatocytes of trout exposed to highly polluted stream water appeared more homogeneous due to a loss of the cellular compartmentation (figure 1c). Organelles were distributed all over the cytoplasm and only small spots of glycogen could be found. The RER was increased compared to the controls and the ER was seldom arranged in long and parallel cisternae. The short cisternae were distributed all over the cytoplasm. Moreover the RER showed numerous structural alterations; cisternae were found to be dilated, vesiculated or degranulated. The same was

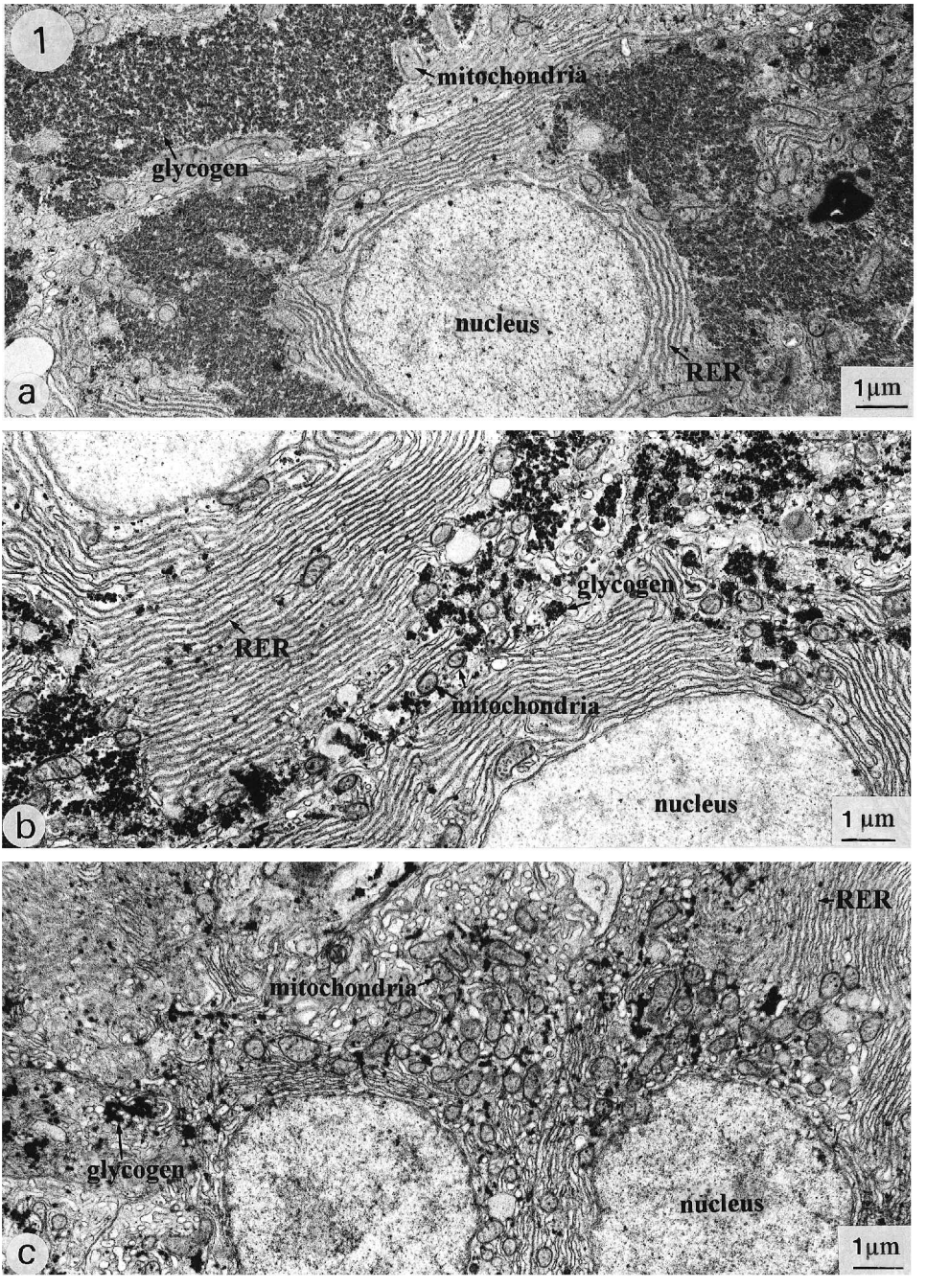

Figure 1. Brown trout 05F1 (exposed for 13 weeks). a) Hepatocyte of a control with pronounced cellular compartmentation. Around the centrally located nucleus, few non-fenestrated lamellae of the RER and only few mitochondria can be observed. Large amounts of glycogen are found in the peripheral cell areas. b) The cellular compartmentation of fish exposed to the lightly polluted water (Krähenbach) shows slight changes in comparison to controls. Further cellular storage products (glycogen) are slightly decreased, cell organelles, e.g. RER, show an increase in number. c) The cellular effects on fish exposed to the highly polluted water (Körsch) are much more pronounced than in the fish of the Krähenbach. The cellular compartmentation is completely lost, and a strong augmentation of mitochondria and RER can be noticed. G

alterations in mitochondria; as in the controls, their size and structure is very flexible. However, there was a pronounced increase in numbers of mitochondria, which were distributed throughout the cytoplasm (figure 1c). The cisternae of the dictyosomes were partially dilated and surrounded by an increased number of vesicles. Glycogen storage in the periphery of the cell was reduced and the number of cell organelles increased (Figure 1c). Occasionally, large amounts of lipid were found. Close to the bile canaliculi, and to the space of Disse, an increase of apical vesicles was observed in the hepatocytes. The number of microvilli in the space of Disse seemed to be reduced. Macrophages and granulocytes in the bile canaliculi and in the sinusoids increased in number compared to controls. Myelin bodies seldom occurred. The nuclei, however, did not apparently differ between controls and exposed animals.

Semiquantitative evaluation of symptoms in exposed trout

Figure 2 summarizes the semi-quantitative results obtained by investigations of the liver ultrastructure in exposed trout. Comparing the different groups of fish and sampling times reveals a similar pattern. Fish exposed to the lightly polluted stream (Krähenbach) do not differ significantly from controls. Fish exposed to the highly polluted stream (Körsch), however, always show strong alterations in hepatic ultrastructure which significantly differ from those observed in controls. The values of fish exposed to highly polluted water always range between category 2 and 3.

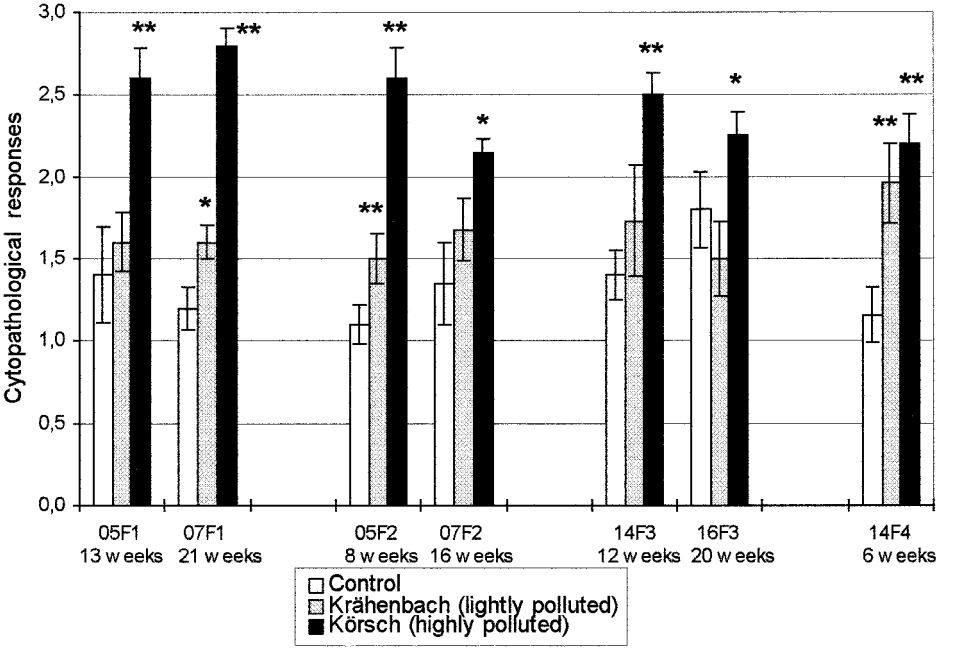

Figure 2. Means and standard deviations of semiquantitative values obtained for cytopathological responses in hepatocytes of the groups of fish. Significance is expressed in comparison to the respective control groups.
X-axis: Exposure group and corresponding exposure time (see Table 2 for the codes).
Y-axis: Cytopathological response scale. 1: control conditions. 2: slight changes and/or reactions are visible. 3: strong reactions and/or destruction are visible.

Due to relatively high values for controls in the exposure group 16F3, the effects on fish exposed to the more heavily polluted stream are not as highly significant as in the other groups, while the control value does not significantly differ from that of fish exposed to the lightly polluted stream. Fish exposed to mildly polluted water in group 14F4 showed an unusually strong reaction, which was significantly higher than in controls.

Morphometric investigations of ultrastructural changes in hepatocytes

In hepatocytes of trout exposed to both highly and lightly polluted stream water, ultrastructural alterations of glycogen, mitochondria, RER, lysosomes, peroxisomes and lipid were quantified. The results are summarized in figures 3 and 4. In fish exposed to highly polluted water the most striking effects were a significant reduction of glycogen storage (figures 3 and 5), and a significant increase of the volume density of mitochondria, RER and peroxisomes (figure 4). There was also an increase of lipid in fish exposed to the highly polluted water. This effect, however, was not significant.

Figure 3 illustrates a significant reduction of glycogen (up to 50%) in exposed fish compared to controls. In controls, up to 48% of the cytoplasm cell area was filled with glycogen, while in fish exposed to the Körsch, only up to 24% of the cell area comprised glycogen. Hepatocytes of fish exposed to the lightly polluted water showed a glycogen content of 33%. A comparable tendency could be observed for groups 05F1, 07F1 and 07F2 (figure 3).

Discussion

Honnen *et al.* (1996, 1997a) characterize the stream Körsch, the water of which contains sewage outspill, as being polluted with several environmental chemicals. The stream Krähenbach is described as a less polluted stream (table 1). Analysis results of the water of Körsch and Krähenbach revealed individual pesticides at concentrations far below the threshold value for drinking water; and the same is true for PAH and heavy metal contamination (Honnen *et al.* 1997b). The total

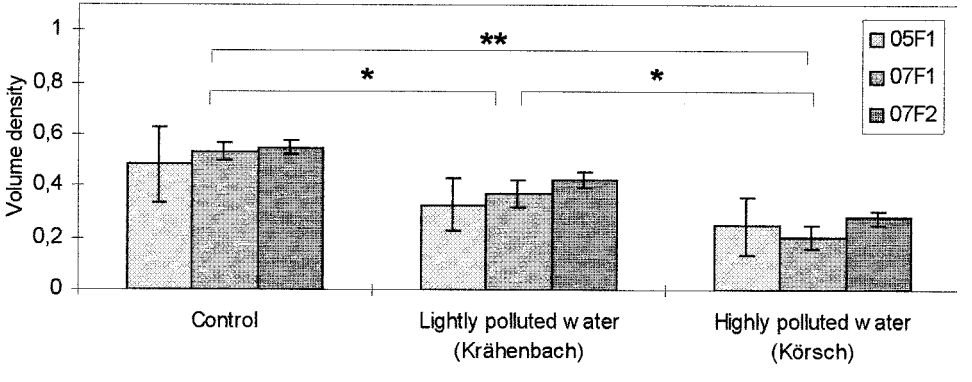

Figure 3. Comparison of glycogen volume densities in hepatocytes of trout after exposure to the lightly and highly polluted streams, with respect to different exposure time (05F1 = 13 weeks, 07F1 = 21 weeks, 07F2 = 16 weeks, for the codes see Table 2). Y-axis: Relative volume densitiy (total cell volume = 1).

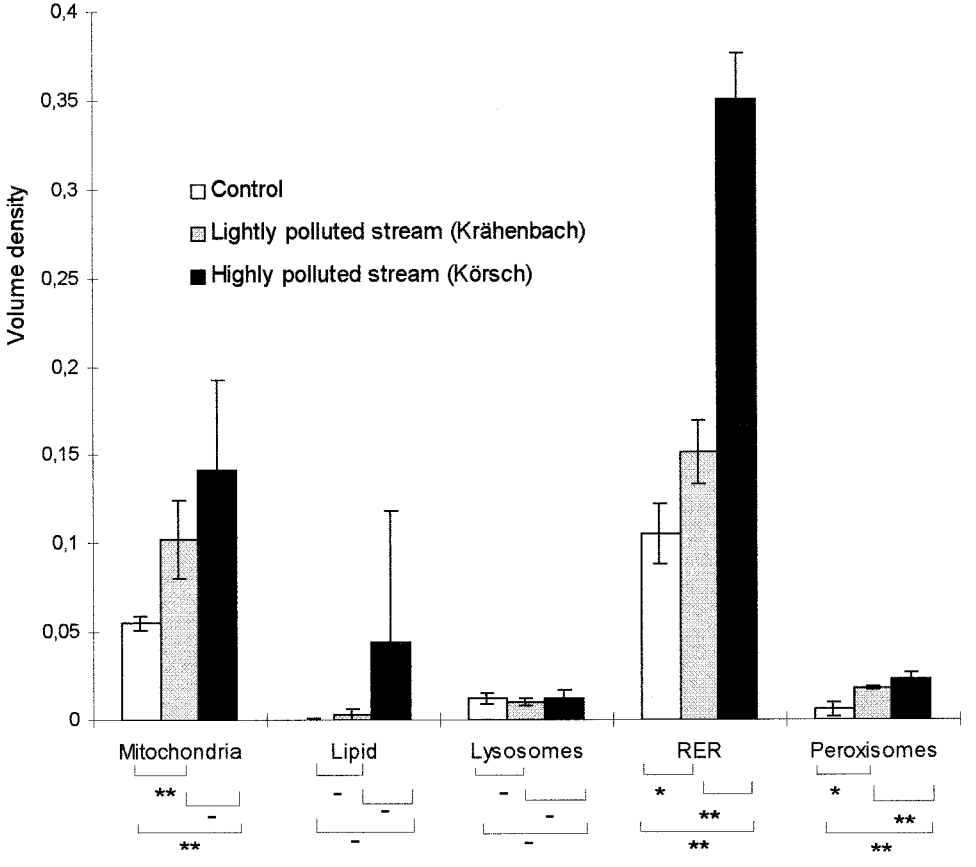

Figure 4. Volume density of cell organelles and storage products in hepatocytes of trout (exposure group 05F1, 13 weeks). Y-axis: Relative volume densitiy (total cell volume = 1).

pesticide concentration however is about 0.1 µg l^{-1} in the Körsch. Analysis of sediment showed higher levels of pollution in the Körsch than in that from the Krähenbach (table 1). In comparison with data from the literature, these values reveal strong PAH contamination of the Körsch sediment (Römpp 1993). Analyses of fish tissue (complete animal, dryweight) showed a bioaccumulation of substances with $\log K_{ow} > 3.4$ *($\log K_{ow}$ = logarithm of the n-octanol-water partition coefficient)* which matches the contamination of the sediments (fish exposed to the Körsch: pesticides 185 µg kg^{-1} dry weight, PCB 126 µg kg^{-1} dry weight, PAH 130 µg kg^{-1} dry weight, heavy metals 114 mg kg^{-1} dry weight; fish exposed to the Krähenbach: pesticides 100 µg kg^{-1} dry weight, PCB 76 µg kg^{-1} dry weight, PAH 94 µg kg^{-1} dry weight, heavy metals 103 mg kg^{-1} dry weight) (Honnen *et al.* 1997a, 1997b). Substances with $\log K_{ow} < 3.4$ could only be detected in water samples but not in the sediments or in the exposed fish. Analyses of autochthone trout caught by electrofishing in the Körsch and Krähenbach confirm the tendency mentioned above. However, in these animals the bioaccumulation of pollutants is up to five times higher than in controls while in animals exposed in the bypass-system, the bioaccumulation was greater by a factor of only 2–3 (Honnen *et al.* 1997a, 1997b).

Since ultrastructural changes in hepatocytes can be

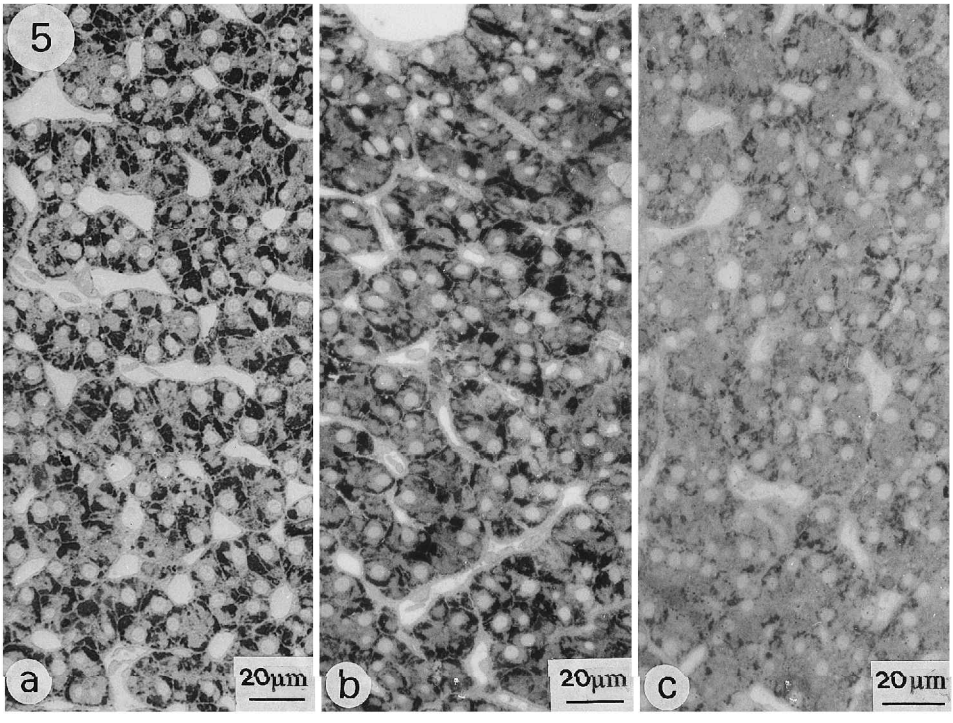

Figure 5. Hepatocytes of trout. Glycogen areas are dark-stained. a) Control, overview of several hepatocytes. b) Fish exposed to the lightly polluted water (Krähenbach), overview. c) Fish exposed to highly polluted water (Körsch), overview.

changes (Couch 1975, Meyer and Hendricks 1982, Arias *et al.* 1988), the cytopathological alterations found in liver ultrastructure of exposed trout in this study are discussed with respect to the analytical results mentioned above, and in relation to functional adaption/alterations of the metabolism.

The loss of cellular compartmentation in hepatocytes of exposed trout is probably a non-specific reaction to a wide variety of environmental changes which might be due to chemical attack of the cytoskeleton (Réz 1986). Bucher *et al.* (1992) describe a loss of cellular compartmentation in hepatocytes of fish after exposure to treated paper mill effluents.

An increase in the number of mitochondria is also a common cell reaction to environmental toxicants (Phillips *et al.* 1987), which has also been described by Arnold *et al.* (1995) for rainbow trout after exposure to various pesticides. There is evidence that this effect is related to an increased consumption of energy, which might be caused by intensified biotransformation processes. Also, the observed dilation of Golgi cisternae and of cisternae of the rough endoplasmic reticulum (RER) might be related to this metabolic adaptation to polluted conditions. Similar effects have been described by Köhler (1989, 1990) and by Heining and Hoffmann (1993). Klaunig *et al.* (1979) directly correlate alterations of the ER with induction of biotransformation.

Biochemical investigations of 12 metabolic enzymes, performed in parallel with fish exposed under the same conditions as fish investigated in the present study, revealed greater differences in fish exposed to the Körsch tha

compared to controls (Konradt *et al.* 1996). A significant increase in the activity of cytochromec-oxidase in the liver of fish exposed to the Körsch indicates an increased energy turnover. This could also be true for the observed increase in number of mitochondria, which might directly be correlated to the observed increase of succinate dehydrogenase activity (Konradt and Braunbeck 1997). These results may indicate an activation of reactions related to the citrate cycle, which provide energy equivalents for metabolic processes. This impact on energy metabolism might be caused by effects on catecholamines and corticosteroids as a result of an activation of the hypothalamic pituitary-inter-renal axis (Pottinger and Mosuwe 1994, Roberts, 1985, Servizi *et al.* 1993, Waring *et al.* 1996). Both hormones are responsible for the mobilization of energy reserves as the fish attempts to avoid, or to overcome, the immediate threat (Donaldson, 1981, Mazeaud and Mazeaud 1981).

The observed proliferation of the ER in the liver of trout exposed to the Körsch could not only be correlated to this activation of the metabolism, but also to an induction of biotransformation enzymes. A proliferation of the RER in hepatocytes caused by environmental toxicants was described by Hacking *et al.* (1978), Salas *et al.* (1980) and Gingerich (1982). The connection between ER proliferation and induction of phase 1 biotransformation via cytochrome P450, which is located in the RER, has often been described (Achazi 1989, Braunbeck and Segner 1992; Arnold and Braunbeck 1993). Biotransformation enzymes were studied in fish exposed under the same conditions to the two streams as the trout used for ultrastructural investigations. In these studies, there was a significant increase of EROD (7-ethoxyresorufin-deethylase) activity (Segner and Behrens 1997). The EROD activity in the liver of fish exposed to the Krähenbach was also raised in comparison to the controls, but to a lower extent than in trout exposed to the Körsch (Segner and Behrens 1997). Investigations concerning the activity of phase 2 biotransformation enzymes did not reveal this tendency. Nevertheless, in fish exposed to the Körsch an increase in number of apical vesicles located close to bile canaliculi and a reduction of microvilli connected to the bile canaliculi was observed. Similar symptoms were described by Pentreath (1976), Phillips *et al.* (1987) and Köhler (1990) who related these effects to the elimination of PAHs and metals via the bile.

The presence of large amounts of lipid, occasionally found in the liver of fish exposed to the Körsch, might be correlated with an increased activity of malate enzyme (Konradt *et al.* 1996, Braunbeck *et al.* 1997), which points to an increased lipid synthesis (Klaunig *et al.* 1979, Braunbeck *et al.* 1990b, Arnold *et al.* 1995). The malate enzyme activity in fish exposed to the Krähenbach does not differ from that in controls, which corresponds to results of ultrastructural investigations. An increase in number of peroxisomes as observed in trout exposed to the Körsch was also noted by Bucher *et al.* (1992) as an effect of an exposure to treated paper mill effluents. Arnold *et al.* (1996) described similar symptoms in trout after exposure to disulfoton. De Craemer *et al.* (1994) found a positive correlation between peroxisomal enzyme activities and the number of the peroxisomes. The increased number of macrophages and granulocytes in the bile canaliculi and liver sinusoids in fish exposed to the Körsch is similar to the responses of fish exposed to other chemicals, e.g. atrazine (Braunbeck *et al.* 1992, Negele and Hoffmann 1991). Intensified macrophage aggregations in livers of fish after exposure to a polluted stream was shown by Rice (1996). This increased invasion of macrophages is probably

symptom. The same is true for the loss of the cellular compartmentation and the decrease of hepatic glycogen (Negele and Hoffmann 1991).

To summarize, the present study clearly indicates that brown trout display distinct hepatocellular response patterns after exposure to complex environmental pollution as found in the two streams. Fish exposed to highly polluted stream water (Körsch) show more drastic alterations to liver ultrastructure than fish exposed to lightly polluted water (Krähenbach). The results of the semiquantitative evaluation and validation of the cytopathological alterations point to a correlation between water pollution and biomarker reponses. Furthermore, it is noticeable that the biomarker responses do not vary much over the year. This seasonal stability is an important advantage of this biomarker for field application compared with biochemical markers, e.g. stress proteins, which are highly depend on temperature (Triebskorn *et al.* 1997). The present study suggests that ultrastructural alterations in hepatocytes of brown trout may constitute suitable biomarkers for an assessment of small stream pollution caused by complex mixtures of harmful substances.

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