Gill morphology of native brown trout *Salmo trutta m. fario* experiencing acute and chronic acidification of a brook in Bavaria, FRG*

Theresia Fischer-Scherl, Rudolf W. Hoffmann

Institut für Zoologie und Hydrobiologie der Universität München, Kaulbachstraße 37, D-8000 München 22,
Federal Republic of Germany

ABSTRACT: Gill morphology of brown trout *Salmo trutta m. fario* inhabiting a periodically acidified brook in the eastern part of Bavaria, Federal Republic of Germany, was studied by light and electron microscope. Gills from brown trout caught at a snowmelt period (pH 4.9; Al 0.8 mg l⁻¹) were characterized by an increase in mucous cells and excessive mucus production which caused the fusion of secondary lamellae. Gills from brown trout sampled from the same brook in a dry period in September (pH 6.2 to 6.5; Al 0.1 mg l⁻¹) showed an increase in chloride cells in the secondary lamellar epithelium and an attachment of opposing cells, epithelial or chloride cells in the respiratory epithelium of neighbouring secondary lamellae.

INTRODUCTION

The acidification of inland waters and soil has been recognized as a serious problem in parts of Canada, the USA, Scandinavia and the UK (Last et al. 1980). In the last few years acid precipitation has also become a primary environmental problem in the Federal Republic of Germany (FRG) (Wieting et al. 1984, Lehmann et al. 1985). How acid precipitation affects an area depends on the vegetation and on the physical and chemical nature of the soils and bedrock (Last et al. 1980, Mason & Seip 1985). In areas such as the Bavarian Forest ('Bayerischer Wald'), with no appreciable amount of carbonate-bearing materials and slow chemical weathering, the neutralizing capacity of soil and water is low and does not allow complete neutralization of acid runoff. The acid input causes the pH of the water to decrease while aluminium and other metals are mobilized (Mason & Seip 1985).

Acidification produces profound changes in the biota of aquatic ecosystems and has far-reaching consequences on all trophic levels of aquatic life (Muniz 1981, Bauer & Fischer-Scherl 1987). One of the consequences, the depletion of fish stocks in brooks, has

recently become increasingly evident in some regions of the FRG (Fischer-Scherl et al. 1986).

In this article we report on the gill morphology of a native brown trout population experiencing chronic and periodically acute acidification of a brook. In contrast to previous studies, which mainly discussed laboratory findings, we present the results of a field investigation, which provides the opportunity to consider, under natural conditions, the synergism of a variety of potentially toxic factors and to evaluate their acute and long-term pathological effects on gills.

MATERIAL AND METHODS

Study area. The study area comprised the basement-complex area of Eastern Bavaria – the Bavarian Forest – near the mountain of Arber (1456 m). The region is very susceptible to acid precipitation because of the geochemistry of surficial sediments and bedrocks which are mainly composed of slow-weathering granites and gneisses. Brooks in this area are oligotrophic, show low conductivity (60 µS cm⁻¹, most of them less than 40 µS cm⁻¹), low buffering capacity and pH levels ranging from more than pH 6 to pH 3.7. The vegetation is typical for the region, consisting mainly of coniferous forest with a mixture of spruces and firs. The

* Dedicated to Professor Dr M. Ruf, University of Munich, on his 60th birthday

Table 1. Water analysis data from acidified and control brook

	Acidified brook		Control brook	
	April snowmelt period. 'Acute acid exposure'	September dry period. 'Chronic acid exposure'	April snowmelt period	September dry period
pH	4.9	6.2–6.5	6.2	6.5
Ca (mg l ⁻¹)	1.75	2.2–3.3	2.25	4.7
Mg (mg l ⁻¹)	0.8	0.9–1.2	0.9	1.1
Al (mg l ⁻¹)	0.8	0.1	0.01	0.01
Conductivity (μS cm ⁻¹)	41	34–36	31	38
Water temperature (°C)	5	5.0–8.5	5.5	8.2

number of deciduous trees is insignificant. The upper part of the brook was investigated, where there is no fishing activity, houses are far off and the brook is not polluted by domestic or agricultural sewage. A dam prevents fish from leaving or entering the upper course of the stream. pH levels vary from 4.9 in spring to 6.5 in summer and autumn (Table 1).

A second brook in the same region was used as a control. This brook has a comparable mineral content (soft water), but does not experience acidification (Table 1).

Methods. Brown trout *Salmo trutta m. fario* were caught by electrofishing in April (10 individuals) and in September (13). The examined fish were aged between 2 and 4 yr. They were killed with 0.007 % tricaine methane-sulfonate (MS 222; Sandoz). Gills were immediately taken, fixed in 5 % buffered formalin and embedded in Paraplast®. Sections 5 μm thick were stained with hematoxylin eosin (H & E), methylene blue, Giemsa's technique and periodic acid-Schiff (PAS) reaction. The plane of section through the gills

Tissue samples for transmission electron microscopy (TEM) were fixed in 6.25 % phosphate-buffered glutaraldehyde (pH 7.4) for 2 h, postfixed in 1 % buffered OsO₄ for 2 h, dehydrated in a graded acetone series and finally embedded in Epon 812®. Semi-thin sections were stained with toluidine blue; ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss transmission electron microscope (EM 109) at 80 kV.

Trout (15 in April, 15 in September) from the control brook served as reference fish.

Total aluminium content (Al) of the water samples was determined by atomic absorption spectroscopy. To study the Al accumulation in gills, tissue samples were dissolved in concentrated HNO₃ at room temperature and analysed by an ICP-AES analyser (965 Jarell-Ash).

Additionally, samples of all fish were routinely examined by virological, bacteriological and parasitological standard methods.

RESULTS

Gill morphology of reference fish

Macroscopic lesions were seen neither in the gills of brown trout from the acidic nor in those from the non-acidic control brook. Gills of controls (Fig. 2) exhibited fairly normal microscopic features comparable to those of other fish species, described by Morgan & Tovell (1973), Karlsson (1983) and Fischer (1984). Each gill arch bears a double row of primary lamellae (holobranch) positioned alternately. Each primary lamella carries secondary lamellae consisting of a network of interconnecting blood lacunae, which are lined by pillar cell flanges. A distinct basement membrane separates pillar cells from the double-layered respiratory epithelium. Extracellular spaces between the 2 layers of the respiratory epithelium were hardly detectable. Occasionally 2 or 3 mucous cells could be

Fig. 1. *Salmo trutta*. Plane of section through the gills

detected in the respiratory epithelium, as well as the 'light' and 'dark' form of chloride cells. The majority of mucous cells in the gills of control brown trout were localized in the interlamellar crypts, at the anterior dorsal and posterior ventral edges and at the distal end of primary lamellae. Gills from reference brown trout caught in April did not differ from those gills of reference brown trout caught in September. Fusion of lamellae was never seen in the gills of control brown trout.

Morphology of gills sampled in April at acute acid exposure

The most remarkable feature of gills from brown trout caught in April (pH 4.9, Al 0.8 mg l⁻¹) during a snowmelt episode, was the increase in number of mucous cells (Fig. 3) and the excessive mucus production (Fig. 4) in the respiratory epithelium. In contrast to reference gills and to those sampled in the same brook in September at the dry period, mucous cells regularly occurred in the respiratory epithelium of gills sampled at pH 4.9. PAS-stained sections revealed clogging of the interlamellar spaces, either due to mucous hypersecretion or through the attachment of mucous cells of neighbouring secondary lamellae (Figs. 4 and 5). Layers of mucous substances covered the cells of the respiratory epithelium. Additionally the interlamellar spaces between the 2-layered respiratory epithelium were extended and/or the respiratory epithelium was lifted from the basement membrane (Fig. 6). In electron micrographs chloride cells exhibited malformed aggregated mitochondria with increased electron density and cristae placed to the periphery. Aggregations of apparently degenerating mitochondria and endoplasmic reticulum (ER) were limited by a membrane (phagolysosome) (Fig. 7). TEM also revealed that the cytoplasm of some chloride cells was highly vacuolated and contained distended fragments of ER (Fig. 8). In a few respiratory epithelial and chloride cells the nucleus was undergoing karyorhexis followed by karyolysis.

Morphology of gills sampled in September at chronic acid exposure

The morphological features of gills sampled in September (pH 6.2 to 6.5, Al 0.1 mg l⁻¹) essentially differed from those studied during the snowmelt period at pH 4.9. Mucous cells mainly resided in interlamellar crypts of the primary lamellae with only 1 to 2 mucous cells occasionally appearing in the respiratory epithelium. No fusion due to mucus secretion occurred. The most striking feature however, was the increase of chloride

cells, in number as well as in size, particularly in the respiratory epithelium (Figs. 9a, b and 10). Up to 70 % of the cells in the respiratory epithelium were represented by chloride cells. They were situated side by side, usually in direct contact with the external medium. Apical surfaces of chloride cells were large with many microvilli-like cytoplasmic extensions. Flanges of respiratory epithelial cells passing between neighbouring chloride cells were in contact with the external medium covering parts of the apical surfaces of the chloride cells as a small rim (Fig. 10).

In gills sampled at pH 4.9, 3 or more secondary lamellae were fused caused by the excessive mucus production and hyperplasia of mucous cells. Gills sampled at pH 6.2 to 6.5 also showed fusions, but this process occurred by simple apposition of adjacent lamellae to each other due to the attachment of long microvilli-like cytoplasmic extensions either to chloride cells or to epithelial cells (Figs. 11 and 12). These cytoplasmic protrusions met in a tight-junction-like contact (Fig. 13). Fusion of 1 or 2 cells in the middle part or distal ends of 2 adjacent secondary lamellae was a dominant feature. In secondary lamellae, displaying these tight contacts, widened extracellular spaces appeared beneath the cells involved. Distended tubules, lysosomes and residual bodies with myelin figures (Fig. 14) were most frequently seen in chloride cells and respiratory epithelial cells, which participated in contacts between adjacent secondary lamellae, while most other chloride cells exhibited fairly normal structures (Fig. 15).

DISCUSSION

This study clearly demonstrates that gill morphology varies according to changes in water chemistry. All examined fish were free of bacterial and viral diseases, and parasites that could damage gill epithelium were not detected.

Gills, the organs involved in the physiological process of gas transfer and acid-base regulation (Randall et al. 1982), as well as osmotic and ionic regulation (Eddy 1982), are considered to be the primary target organ in cases of fish deaths in acidified water (Muniz & Leivestad 1980a). The described gill lesions at pH 4.9 and Al 0.8 mg l⁻¹ can be attributed to a synergistic effect of low pH, low mineral content and the accumulation of metal ions in the water.

In gill tissue of brown trout caught at pH 4.9 a higher amount of Al was found compared to gills sampled at pH 6.2 to 6.5 (Fischer-Scherl et al. unpubl.). Thus it can be concluded that, in addition to the low pH, the high amount of Al is probably responsible for the signs of degeneration observed in the respiratory epithelium.

Figs. 2 to 4. (For Fig. 5, see legend opposite.) *Salmo trutta*. Fig. 2. Light micrograph of longitudinal Epon section of secondary lamellae of gills of brown trout from control brook (pH 6.2, Al 0.01 mg l^{-1}) showing normal appearance. Chloride cells (cc) are mainly restricted to the interlamellar epithelium. Toluidine blue, $\times 240$. Figs. 3 and 4. Light micrographs of gills of brown trout caught at a snowmelt period (acute acid stress; pH 4.9, Al 0.8 mg l^{-1}). Fig. 3. Mucous cells (mc; long arrows) proliferate in the respiratory epithelium. Secondary lamellae are fused and respiratory epithelium is detached from the basement membrane (short arrows). PAS, $\times 280$. Fig. 4. Fusion of secondary lamellae caused by the excessive production and accumulation of mucous substances (arrows). PAS, $\times 180$

Figs. 5 to 8. *Salmo trutta*. Transmission electron micrographs of gills of brown trout from the acidified brook at pH 4.9 and Al 0.8 mg l^{-1} . Fig. 5 (opposite). A mucous cell. Mucus attaches to an epithelial cell of the adjacent lamella. $\times 5000$. Fig. 6. Note extended intercellular spaces between epithelial cell (ec) and basement membrane (bm) and between epithelial cells of the respiratory epithelium. bl: blood lacuna; arrows: dilation of endoplasmic reticulum (ER). $\times 8300$. Fig. 7. Detail of an apparently degenerating chloride cell with aggregation of electron-dense mitochondria (m) segregated by a membrane. $\times 33\,000$. Fig. 8. Chloride cells showing vacuolated cytoplasm (va), lytic mitochondria (long arrows) and dilation of ER (short arrows). $\times 8200$

Figs. 9 to 11. *Salmo trutta*. Gills of trout sampled in September (chronic acid stress; pH 6.2 to 6.5, Al 0.1 mg l^{-1}). Fig. 9 (a, b). Light micrograph showing (a) enormous proliferation of dome-shaped chloride cells in the respiratory epithelium (semithin section; toluidine blue, $\times 600$); (b) light (short arrows) and dark (long arrows) form of chloride cells are easily distinguishable (semithin section; toluidine blue, $\times 480$). Fig. 10. Transmission electron micrograph (TEM), showing increase in chloride cells (cc) in the secondary lamellae, epithelial cells (ec) with picnotic masses and chloride cells with large apical surfaces characterized by many microvilli-like cell extensions (arrows). $\times 1500$. Fig. 11. TEM showing chloride cell (cc) with its cytoplasmic extensions closely adhering to the epithelial cell (ec) in the region of the marginal channel (mc) of adjacent secondary lamellae. $\times 10\,750$

Figs. 12 to 15. *Salmo trutta*. Transmission electron micrographs of gills of trout sampled in September (pH 6.2 to 6.5, Al 0.1 mg l^{-1}). Fig. 12. Adhesion of epithelial cells (ec) at the marginal channels (mc) of adjacent secondary lamellae. $\times 13\,900$. Fig. 13. Attachment of 2 chloride cells (cc) exhibiting close tight-junction-like cell connections (arrows). $\times 25\,900$. Fig. 14. Detail of chloride cell participating in an attachment. Lysosomal structures, myelin figures and dilated tubules (t) can be seen. $\times 33\,000$. Fig. 15. Essentially normal appearance of chloride cell; numerous mitochondria (m) are intermingled with cytoplasmic tubular network (t). The cell's surface is characterized by long microvilli-like cytoplasmic extensions. $\times 10\,750$

Similar observations and conclusions were also made by Karlsson-Norrgrén et al. (1986) in laboratory experiments after exposing brown trout to water at pH 5.5 and an Al concentration of $500 \mu\text{g l}^{-1}$ for 3 wk.

In a laboratory experiment Daye & Garside (1976) noticed hypertrophied mucous cells and excessive mucus production after they had exposed brook trout *Salvelinus fontinalis* to water of pH 5.2. Muniz & Leivestad (1980a) observed macroscopically visible mucus secretion and clogging of gills of brown trout at a pH of 4.5 to 4.9, after they had added $\text{Al}_2(\text{SO}_4)_3$ to soft brook water. They attributed this phenomenon to the presence of Al. According to Mallat (1985) hypersecretion of branchial mucous cells is a lesion most frequently associated with heavy metals. Mucins, which are often polyanions, may trap metal cations and prevent toxicants from crossing the gill epithelium (Lock & van Overbeeke 1981, Pärt & Lock 1983). A similar mechanism may exist in the case of Al. But fused secondary lamellae, thick layers of mucus covering the respiratory lamellae and extended intercellular spaces reduce gas exchange at the gills (Hughes & Morgan 1973, Ultsch & Gros 1979). These pathological alterations, as well as degenerative features of the cells in the respiratory epithelium, probably represent the morphological base for pathological effects or failure in electrolyte regulation and gas exchange described in various fish species exposed to acidified water (Packer 1979, Muniz & Leivestad 1980b, Rosseland 1980, Ultsch et al. 1981, McKeown et al. 1985).

The increase of chloride cells in number as well as in size was the most obvious and constant characteristic in gills sampled at pH 6.2 to 6.5 (chronic acid stress). This phenomenon is well documented and has been described in various fish species under different conditions: adaptations to low pH levels (Leino & McCormick 1984), influence of stress (Peters & Hong 1985), elevated Al concentration (Chevalier et al. 1985, Karlsson-Norrgrén et al. 1986) and ionic challenge in hard and soft water (Karnaky 1980, Fischer 1984, Laurent et al. 1985). According to Mallat (1985) the increase of chloride cells has to be considered largely non-specific in nature and may primarily represent stereotyped physiological reactions. As several synergistic factors have to be considered in our field study, the effects observed on chloride cells could be interpreted here as a result of chronic stress from both elevated concentration of Al and radically fluctuating H^+ ion concentration during heavy rain.

Daoust et al. (1984) observed similar close contacts between adjacent lamellae when they exposed rainbow trout *Salmo gairdneri* to acutely lethal aqueous concentrations of inorganic mercury and copper. They suggested that the presence of heavy metal ions may result in an alteration of the charge of glycoproteins

coating the lamellae and thus favors attractions between cells of adjacent lamellae. Additionally, low pH, as in our case, could intensify the attraction between secondary lamellae.

Telangiectasias in the gills was not observed in our investigation. Chevalier et al. (1985) described a severe congestion of blood spaces by erythrocytes in the secondary lamellae of brook trout from acidified lakes. They attributed these lesions to circulatory anomalies. Unfortunately they did not mention how they killed the fish, as telangiectasias is well documented after killing fish with a blow on the head.

In this study we demonstrated that gills exhibit distinct morphological features according to different water regimes in an acidified brook at different times. It was the purpose of this study to provide field observations in natural running waters, because so far most papers on this subject have described experiments under laboratory conditions or under more stagnant water conditions in lakes. Muniz & Leivestad (1980a) have however shown that low pH is less toxic for fish in laboratory experiments and the same pH is far more toxic for fish when they are in the field.

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