Nature and Time Course of Acclimation to Aluminum in Juvenile Brook Trout (*Salvelinus fontinalis*). II. Gill Histology

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Mueller, M. E., D. A. Sanchez, H. L. Bergman, D. G. McDonald, R. G. Rhem, and C. M. Wood. 1991. Nature and time course of acclimation to aluminum in juvenile brook trout (*Salvelinus fontinalis*). II. Gill histology. Can. J. Fish. Aquat. Sci. 48: 2016–2027.

Gill samples from juvenile brook trout (*Salvelinus fontinalis*) acclimated to low-level aluminum at pH 5.2 showed severe damage by day 4, with necrosis and fusion of secondary lamellae and hyperplasia and hypertrophy of mucous cells. Over the following 20 d, there was a continual process of repair with proliferation and hypertrophy of mucous cells. Qualitative analysis of gill samples plus physiology and mortality data collected in a companion study indicated progressive development (by day 10 onward) of increasing acclimation to Al. Quantitative analysis of gill samples on day 13 showed that mucous cell volume density had tripled and mucous cell area had doubled in Al-exposed fish compared with control fish. A lamellar fusion index showed evidence of fusion in Al-exposed fish by day 4 with recovery to nearly control levels by day 13. Physiological disturbances appear to be directly related to the histological changes observed in the gill epithelium. At the cellular level, changes in either mucous cell production and secretion or changes in mucus chemistry contribute, in part, to acclimation to Al.

L'analyse d'échantillons de branchies d'ombles de fontaine juvéniles acclimatés à de faibles teneurs en aluminium dans un milieu de pH 5,2 a révélé d'importantes lésions dès le quatrième jour: nécrose et fusion des lamelles secondaires ainsi que hyperplasie et hypertrophie des cellules muqueuses. Au cours des 20 jours suivants, on a observé une prolifération et une hypertrophie constantes des cellules muqueuses. Les résultats d'une analyse qualitative d'échantillons de branchies ainsi que des données sur la physiologie et le taux de mortalité recueillies dans le cadre d'une étude parallèle ont révélé l'apparition progressive, à partir du dixième jour, d'une acclimatation croissante à l'Al. L'analyse quantitative d'échantillons de branchies prélevés le treizième jour a révélé que la masse volumique des cellules muqueuses avait triplé et que la superficie des cellules muqueuses avait doublé chez les poissons exposés à l'Al par rapport aux poissons témoins. Un indice de fusion des lamelles a permis de noter une fusion dès le quatrième jour chez les poissons exposés à l'Al ainsi que leur rétablissement, dès le treizième jour, presque jusqu'au niveau observé chez les témoins. Les perturbations physiologiques semblent être directement liées aux modifications histologiques observées dans l'épithélium des branchies. Au niveau cellulaire, des modifications dans la production et la secrétion de cellules muqueuses ou de la chimie du mucus contribuent en partie à l'acclimatation à l'Al.

Received November 27, 1989
Accepted August 12, 1990
(JA376)

Reçu le 27 novembre 1989
Accepté le 12 août 1990

Many morphometric and physiological studies indicate that low pH and elevated aluminum primarily cause damage at the gills. Many structural changes associated with these conditions have been described. After acute exposure of brook trout (*Salvelinus fontinalis*) to pH 5.2 or lower, Daye and Garside (1976) reported lifting of the outer epithelium from the pillar cells on the secondary lamellae, as well as some hypertrophy of filamental mucous cells. Fathead minnows (*Pimephales promelas*) chronically exposed to pH 5.5 or 5.0 showed increased numbers of chloride cells and increases in apical pit formations in these cells (Leino and McCormick 1984). In field studies of acidified lakes, Chevalier et al. (1985) found increased numbers of chloride cells in brook trout in addition to hyperplasia of the primary filament.

When Al is added to acid exposures, histological damage in fish gills is similar to that reported from acid exposure alone, yet the effects are often more severe. Changes described with acid/Al exposure include increases in diffusion distances as the epithelium lifts, increases in numbers of chloride cells, the presence of dense cells (a chloride cell variant), and extensive necrosis and degeneration of gill tissue (Tietge et al. 1988; Evans et al. 1988; Ingersoll et al. 1990). In addition to the reported Al effects on gill structure, there have been reports of intracellular and extracellular Al deposits (Karlsson-Norgren et al. 1986; Youson and Neville 1987).

Furthermore, it has been established that low-level chronic exposure to acid and Al results in a reduction in toxicity to Al.

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This phenomenon is described as acclimation (Orr et al. 1986; Wood et al. 1988a, 1988b). Its significance is that fish are often exposed to low-level "background" Al concentrations in acidified waters. This exposure may confer increased resistance to short-term pulses of high levels of Al that may occur during episodes of snowmelt or rainfall (Orr et al. 1986; Siddens et al. 1986; Wood et al. 1988a, 1988b; McDonald and Milligan 1988).

The physiological changes described following acid/Al exposure (McDonald et al. 1991), as well as acid/Al acclimation probably result from structural alterations at the gills. However, no study has simultaneously addressed both physiological and histological changes associated with damage or acclimation. Thus the objectives of this study were to address simultaneously the physiological and histological changes that may occur over the course of acclimation to acid and Al. This paper presents the results of the histological observations.

Methods

Exposure Conditions

All tests were conducted at the Fish Physiology and Toxicology Laboratory, Laramie, WY. Juvenile brook trout were continuously exposed to three acclimation conditions in flowing artificial soft water for up to 42 d. Acclimation conditions included the following: pH 6.5, 0 Al (controls); pH 5.2, 0 Al; and pH 5.2, 150 μg Al/L (reduced to 75 μg/L on day 3). All calcium levels were at 25 μequiv/L. Exposure animals were juvenile brook trout (average weight 5 g) that had been softwater acclimated for at least 60 d. One tank was used for each exposure and each tank began with 400–600 fish. To assess the nature and time course of acclimation, fish were periodically sampled from each group for physiological and histological status or for challenge to lethal Al (1000 μg/L) (Fig. 1). Physiological measurements included body electrolytes, hematology, gill chemistry, and branchial Na^+ fluxes. Resistance to challenge was assessed on the basis of mortality and by the magnitude of physiological disturbances in survivors.

From day 0 to 24 of the acclimation experiment, 52 fish from each acclimation group were removed at each sampling time (days 1, 2, 4, 7, 10, 13, 17, and 24). Sixteen of these fish were used for measurements of Na^+ uptake, of which half were subsequently returned to their respective acclimation tank and the other half sacrificed for measurements of whole-body electrolytes. Another 10 fish were immediately sacrificed and a blood sample obtained by caudal puncture using a modified 100 μL Hamilton gas-tight syringe. Hematocrit and plasma protein were determined on this blood sample. The second gill arch from the right side was taken for histological examination and the remaining gill basket was weighed, frozen, and later digested for gill Al and sialic acid determination. A third group of 10 fish from each acclimation group was transferred to a single challenge tank (pH 5.2, nominal Al = 1000 μg/L) and cumulative mortalities recorded over time until all fish were dead. An estimate of the time to 50% mortality (ET50) was determined by log/probit analysis for each group exposed to the challenge. A fourth group of 16 fish from each acclimation group was challenged simultaneously with the same condition; survivors at 24 h were sacrificed for either (i) determinations of whole-body electrolytes and lactate or (ii) measurements of hematocrit, plasma protein, gill Al and sialic acid content, and gill histology.

A final challenge experiment was begun on day 28 of the acclimation period. Here, a total of 90 fish from each of the 6.5/0 Al and 5.2/Al groups were transferred in groups of 15 to one of six challenge exposures (Fig. 1). The challenge exposures were at pH 4.8/0 μg Al/L, 100 μg/L, or 300 μg/L and at pH 5.2/0 μg Al/L, 100 μg/L, or 300 μg/L. Fish were maintained for 2 wk under these conditions and cumulative mortalities recorded over time. At the end of the 2 wk, up to 10 survivors from each group in each tank were then sacrificed for measurements of whole-body electrolytes, hematology, gill Al, and gill histology. (For additional details on exposure and sampling protocols, see McDonald et al. 1991.)

Tissue Preparation

Five fish from each acclimation and challenge group were sampled for histopathology. Fish were stunned by a blow to the head. The second gill arch from the right side was excised and immediately placed in 2.5% buffered glutaraldehyde solution for at least 24 h. Tissues were then postfixed in buffered 1% osmium tetroxide, dehydrated in a graded series of ethanols, rinsed in propylene oxide, and embedded in an araldite epoxy resin. Longitudinal thin sections (1 μm) were cut using a Sorvall MT-2B microtome, mounted on glass slides, and stained with either 0.1% Azure B or Eriochrome Azurol B Al-specific stain (Denton et al. 1984; Humason 1979).

Qualitative Examination

Gills were qualitatively examined for general pathology. Special categories of examination included primary filament hyperplasia, structural integrity of secondary lamellar epithelium, evidence of degeneration or necrosis, fusion of primary or secondary lamellae, proliferation of undifferentiated cells, size and distribution of chloride and mucous cells, and infiltration of white blood cells.

Stereology and Morphometry

Point count stereological methods (Weibel 1979) were used to quantify volume density of pillar cells, undifferentiated cells, inner epithelial cells, pavement cells, chloride cells, mucous cells, dense cells, and white blood cells in the secondary lamellae. Volume density of lymphatic spaces and pillar cell-capillary network were also determined by point counting.

Tissues were examined directly on an Olympus BH-2 microscope with a 10 mm square lattice grid (Olympus Corp., Precision Instrument Div., Lake Success, NY) in the ocular. This grid superimposed a regular array of 121 points on the microscope image. The entire gill tissue was first mapped to delineate usable fields and then five random fields per secondary lamella were selected for analysis using a random number generator. The ocular was randomly rotated between each field to assure random placement of the grid over each field. Points falling on parameters of interest were tallied and volume density of each component was calculated by dividing total points for each category by the total number of points in the tally. A minimum of 1000 points were counted.

Mucous cell area and lamellar fusion were measured using a digitizing tablet (Houston Instruments) interfaced to an IBM-XT computer equipped with a morphometric software package (Bioquant, Rand M Biometrics) (Tietge et al. 1988). Lamellar fusion measurements were made by projecting a slide image onto a monitor and tracing lamellar lengths and perimeters using

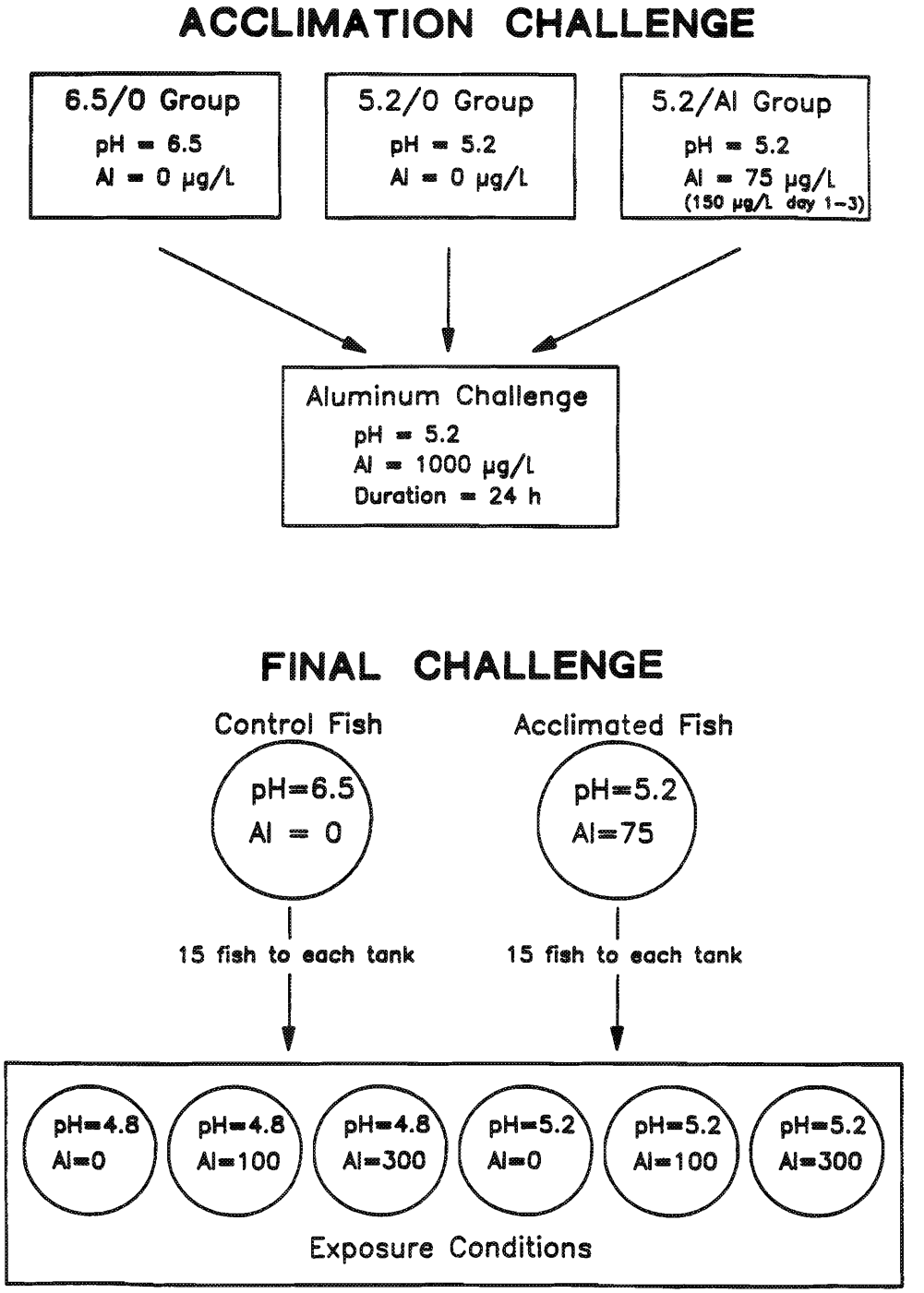


FIG. 1. Flow diagrams illustrating acclimation and final challenge conditions.

a digitizing tablet. Lengths (L1) of a random sample of secondary lamellae were traced down the center of the pillar capillary network from the intersection with the central venous sinus to the marginal channel. Perimeters (L2) of exposed epithelium were traced from the interlamellar midpoint of adjacent lamellae around the entire lamellae to the next adjacent interlamellar midpoint (Fig. 2). A lamellar fusion index was calculated by dividing L2/L1.

Statistical Analysis

Cell volume density (volume fraction of cell of interest relative to the entire gill) means were calculated for each fish (n = 5, except for controls where n = 4) and a final mean was calculated for each group. No less than 1000 points were tallied for each fish. Percents calculated were then transformed using the arc sine square root transformation. Mucous cell area means were not transformed. Summary statistics were generated and tested for homogeneity of variances using both Bartlett's and Hartley's test. All data were analyzed using analysis of variance ($p < 0.05$). If there were significant differences, pairwise comparisons were made using both Dunnett's and Tukey's test for mean comparisons. All analyses were performed using the TOXSTAT statistical computer program (Gulley et al. 1988).

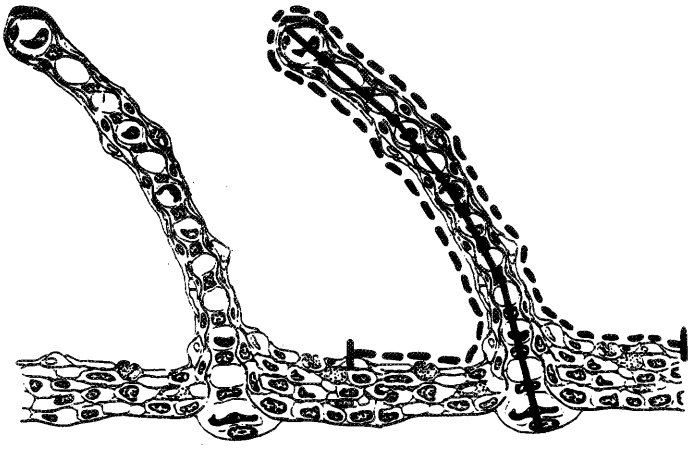


FIG. 2. Diagram illustrating lamellar fusion index measurements. Measurements were taken by tracing a projected slide image using a digitizing tablet. Lamellar lengths (L1) were measured down the center of the pillar cell – capillary network (solid line) from the intersection with the central venous sinus of the marginal channel. Perimeter lengths (L2) of exposed epithelium were measured from the interlamellar midpoint of adjacent lamellae around an entire lamella to the next adjacent interlamellar midpoint (broken line). A lamellar fusion index was calculated by dividing L2/L1. (Diagram from Skidmore and Tovell 1972).

Results

Histology

Controls

Control gills (6.5/0 Al) exhibited normal gill architecture (Morgan and Tovell 1973) for all sampling periods with occasional exceptions (Fig. 3). Gill filaments consisted of a primary filament covered by undifferentiated cells and a surface layer of pavement epithelial cells. Projecting from the primary filament at regular intervals were leaflike structures known as secondary lamellae. These secondary lamellae were composed of a network of blood-filled spaces (capillary network) separated by pillar cells whose cell processes or flanges connected. Covering this pillar–capillary network was a thin basal lamina over which rested an inner and outer layer of epithelial cells. The inner layer of epithelium consisted of relatively undifferentiated cells found primarily with their nuclei above pillar cells. The outer epithelium consisted of a flattened layer of pavement cells, dome-shaped chloride cells, and mucous cells. Chloride cells were primarily located at the bases of the lamellae and were distinguished by prominent staining of mitochondria which gave the cytoplasm a granular appearance. Mucous cells, also found on the primary filament, stained darkly, as the mucopolysaccharide content was extremely basophilic. Also a feature of the normal gill was the presence of lymphatic spaces found primarily adjacent to chloride cells. A few control fish showed minor histological changes such as epithelial lifting and slight chloride cell proliferation, which was probably a result of holding stress (Fig. 3).

Exposure to pH 5.2/0 Al

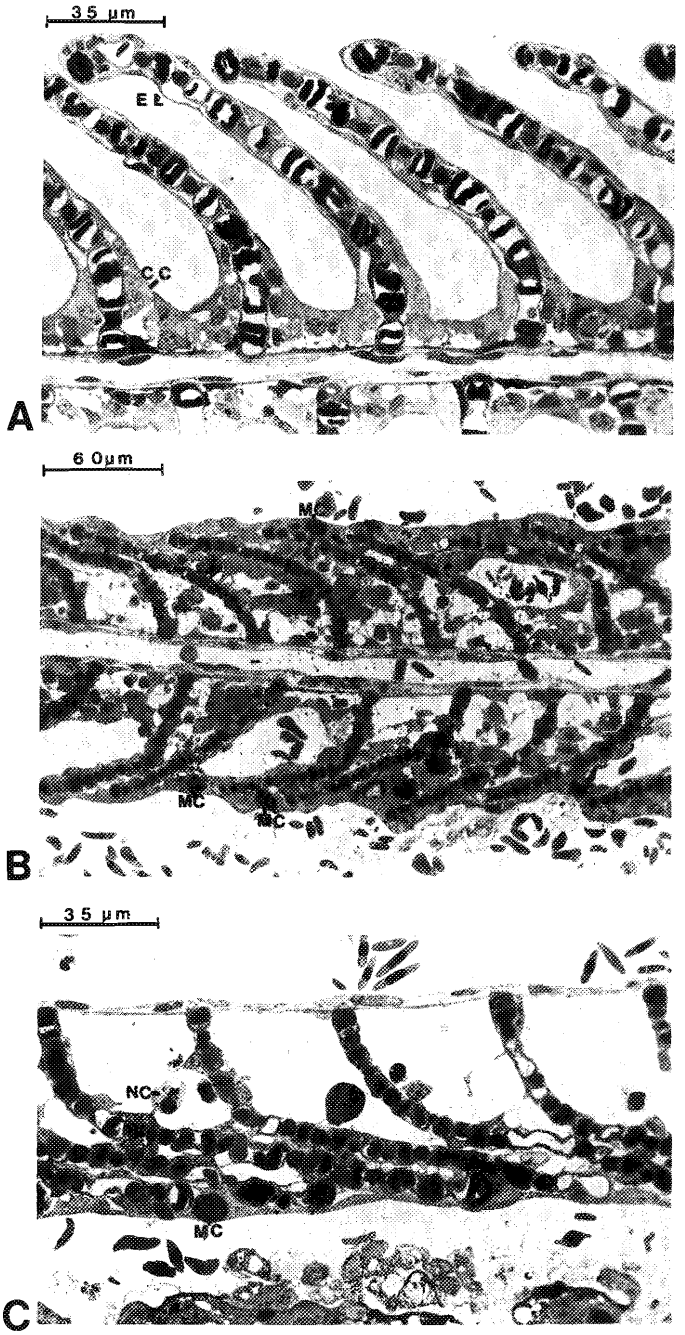
Fish exposed to low pH in the absence of Al had primary filaments with slight hyperplasia of undifferentiated cells and some proliferation and hypertrophy of chloride cells by day 4 (Fig. 4A). The most prominent feature of the secondary lamellae was a lifting of the epithelium away from the basal lamina accompanied by an influx of white blood cells in the lymphatic spaces. Pavement epithelial cells were vacuolated and degenerating (Fig. 4A).

FIG. 3. Gills from control brook trout (6.5/0 Al group) illustrating (A) normal gill architecture observed in most sections as well as (B) minor pathology, probably indicative of holding stress, observed in occasional sections. In Fig. 3B, hypertrophic chloride cells have proliferated, occurring not only in the basal areas of the secondary lamellae but more distally as well. In some areas the outer epithelium is lifted away from the basal lamina. Note the size and location of a mucous cell. CN = capillary network, PC = pillar cell, PV = pavement cell, CC = chloride cell, EL = epithelial lifting, MC = mucous cell.

Exposure to pH 5.2/Al

To induce acclimation, fish were initially exposed to 150 µg Al/L. On day 3, the Al concentration was reduced to 75 µg/L due to unacceptably high mortality rates. The first samples examined histologically were taken on day 4, and these samples reflected damage of gills due to Al. Lesions in these gills were extensive and severe (Fig. 4B, 4C).

The primary filament and secondary lamellae were hyperplastic, with the proliferating cells being completely detached (desquamating) from the basal lamina and extended distally towards the outermost tips of the secondary lamellae (Fig. 4B). Although cells had lost their attachments to the basal lamina, some cells had maintained lateral connections which gave the appearance of an outer membrane or epithelium covering the tips of adjoining secondary lamellae (Fig. 4B, 4C). The only remaining discrete structure was the pillar cell – capillary net-



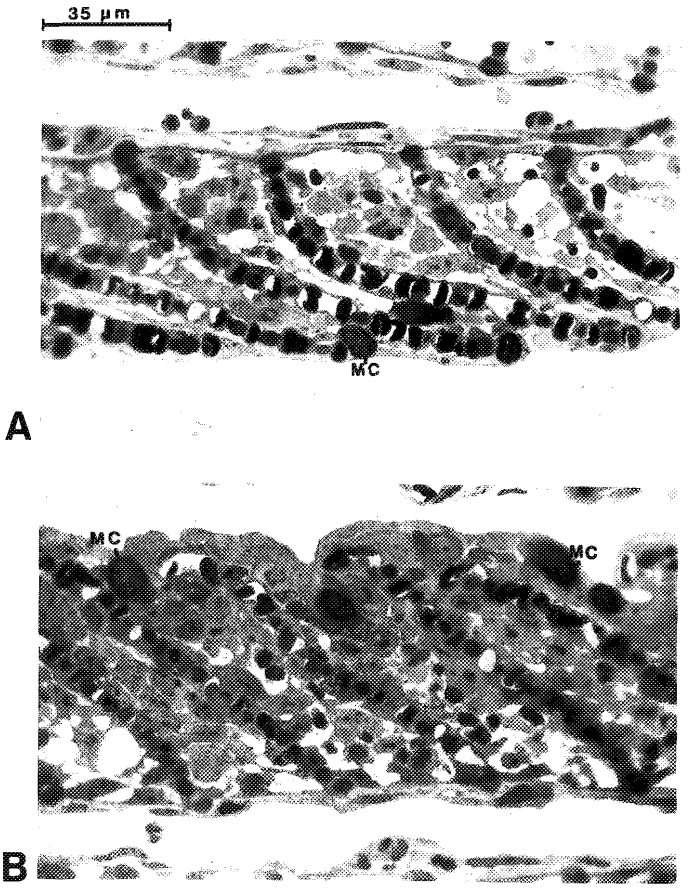


FIG. 5. Gills from brook trout exposed to low pH and Al (pH 5.2/Al) from the acclimation experiment sampled on days 7 and 10. (A) Gill sampled on day 7 showing severe damage to the secondary lamellae. Loose aggregates of degenerate and necrotic cells fill the interlamellar regions. Remaining epithelial cells are distally located and appear to cover the tips of the secondary lamellae. Mucous cells (MC) are hyperplastic and hypertrophic. (B) Gill from fish sampled on day 10. Proliferating undifferentiated cells fill the interlamellar regions resulting in fused secondary lamellae. Hyperplastic mucous cells are extremely hypertrophic.

FIG. 4. Gills from brook trout acclimated for 4 d to (A) low pH (pH 5.2) and (B and C) low pH plus Al (pH 5.2/Al). In Fig. 4A, note chloride cell proliferation (CC) and epithelial lifting (EL) often associated with acid stress. In Fig. 4B, the primary filament is extensively hyperplastic and the proliferating cells are detached from the basal lamina and located at the outermost tips of the secondary lamellae. Note the appearance of an "outer membrane" or epithelium covering the tips of adjacent secondary lamellae. There is extensive hyperplasia and hypertrophy of mucous cells (MC). Necrotic and degenerating aggregates of cells fill the interlamellar regions. In Fig. 4C, the pillar–capillary network, surrounded by the basal lamina, is the only structure remaining intact on the secondary lamellae. Mucous cells are extremely hypertrophic and have proliferated to the tips of the secondary lamellae. Necrotic cells (NC) are seen in the interlamellar regions.

work surrounded by a basal lamina (Fig. 4C). Necrotic and degenerating aggregates of cells, undifferentiated proliferating cells, and fluid filled the interlamellar spaces (Fig. 4B, 4C).

Mucous cells had proliferated throughout the gill filament and the secondary lamellae. These cells were extremely hypertrophic and they were commonly located on the outer layer of remaining cells that had formed at the distal ends of the secondary lamellae (Fig. 4B, 4C). Control gills had few mucous cells on the gill filaments and rarely any on the secondary lamellae.

Identifiable chloride cells did not appear to proliferate but were hypertrophic and often degenerate. However, undifferentiated cells were proliferating, and some of these may have been developing chloride cells. Pillar cells seemed least affected, although they appeared to be slightly swollen.

Subsequent samples taken on days 7, 10, 13, and 24 revealed a continuing process of repair (Fig. 5, 6). Desquamation of proliferated cells continued to abate, with masses of connected cells gradually replacing what had been isolated masses of aggregated cells (Fig. 5, 6). Lamellae that had been fused by these masses gradually became separated as the proliferation

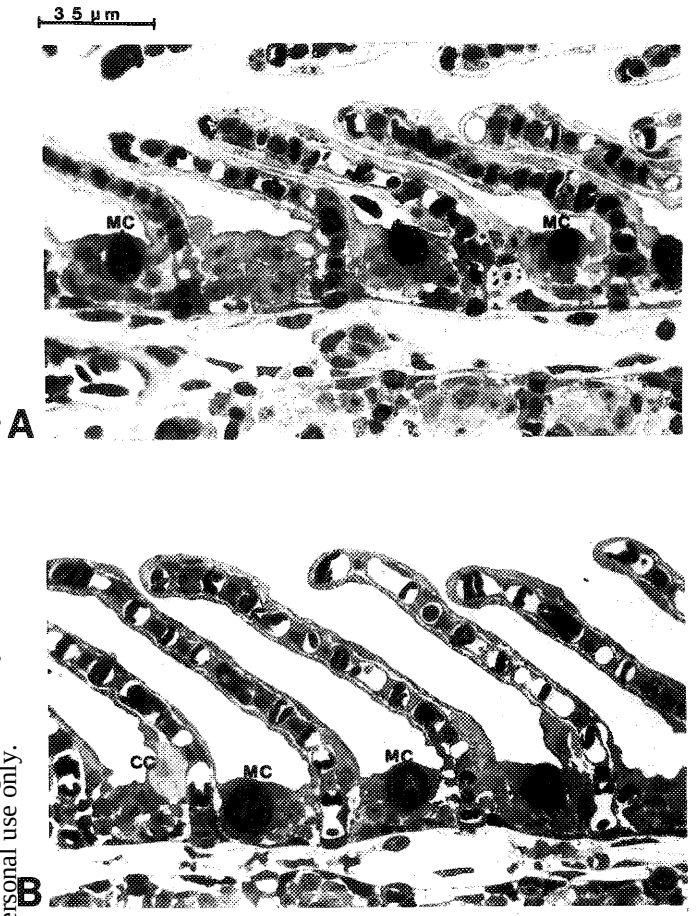


FIG. 6. Gills from brook trout exposed to low pH and Al (pH 5.2/Al) from acclimation experiment sampled on day 13 and 24. (A) Gill from fish sampled on day 13. Proliferation of undifferentiated cells has subsided with hyperplasia restricted to the primary filament. Mucous cells (MC) remain hyperplastic and hypertrophic. There is slight lifting of the epithelium. (B) Gill from fish sampled on day 24. The secondary lamellae show evidence of extensive repair. Mucous cells remain hypertrophic but are confined to the primary filament. Hypertrophic chloride cells (CC) are apparent.

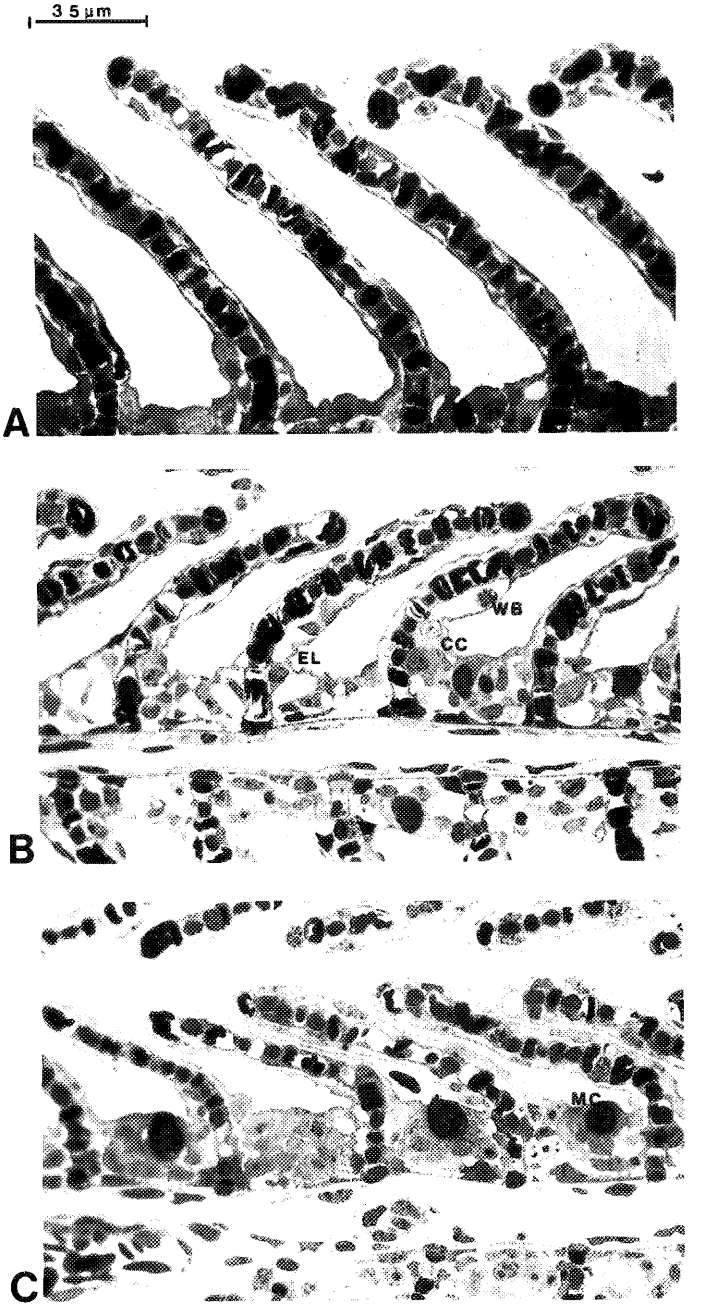


FIG. 7. Comparison of brook trout gills from control (pH 6.5/0 Al), low pH (pH 5.2/0 Al), and low pH plusAl (pH 5.2/Al) exposure sampled on day 13 of the acclimation experiment. (A) Secondary lamellae from control gill showing a flat epithelium surrounding a pillar–capillary network. Note the absence of hypertrophic mucous cells or epithelial lifting. (B) Primary filament from low pH exposed fish showing extensive hyperplasia. The epithelium of the secondary lamellae is lifted away from the basal lamina (EL). Chloride cells (CC) are hypertrophic and white blood cells (WB) are present in the lymphatic spaces. (C) The primary filament and secondary lamellae from a low pH plus Al exposed fish shows proliferation of undifferentiated cells and hypertrophy and hyperplasia of mucous cells (MC). Epithelium of the secondary lamellae is thickened.

subsided (Fig. 6A). By the end of the acclimation experiment (day 24), undifferentiated cells of the primary filament as well as some cells in the proximal portions of the secondary lamellae remained hyperplastic (Fig. 6B). The distal portions of the secondary lamellae had relatively normal epithelium with the exception of mucous cells described below. Necrotic and degenerating cells decreased over time.

Mucous cells remained a prominent and persistent feature throughout the acclimation period in the Al-exposed fish (5.2/Al group). Although these cells appeared to be less hypertrophic at the end of the acclimation period, they were still proliferating. They occurred throughout the primary filament (Fig. 6B) and secondary lamellae.

Near the end of the acclimation period, chloride cells appeared to increase in number, suggesting that many of the undifferentiated proliferating cells, observed on day 4, may have been developing chloride cells. In addition, these and the surrounding cells had highly folded margins. There was a noticeable increase in apical pit formation as well as chloride cell epithelial margins (Fig. 6B). The folding may have been the result of displacement and/or pressure from the surrounding hyperplastic cells.

Comparison of gills from the three acclimation groups on day 13 illustrated distinct differences (Fig. 7). Control groups

(6.5/0 Al) (Fig. 7A) appeared normal, while the low pH groups (5.2/0 Al) showed evidence of acid stress (Fig. 7B). In the low-pH group, lifting of the epithelium was common as were white blood cells in the lymphatic spaces. Mucous cells were scarce. In contrast, the low pH plus Al groups (5.2/Al) (Fig. 7C) had many hypertrophic mucous cells along the primary filament and occasionally on the secondary lamellae. In addition, undifferentiated cells had proliferated and some pavement cells of the secondary lamellae were thickened and vacuolated.

Challenge gills

Gill samples were taken from surviving fish after each Al challenge. By day 13, mortality and physiological observations indicated a reduced response to the challenge in fish previously exposed to low-level Al (75 μg/L) (McDonald et al. 1991). Fish from the control (6.5/0 Al) and 5.2/0 Al groups showed desquamation of the epithelium from both the primary filament and the secondary lamellae with massive necrosis and extracellular edema after exposure to pH 5.2, 1000 μg Al/L (Fig. 8A, 8B). Often only a few epithelial cells remained attached at the most distal portions of the secondary lamellae near the marginal channels. Any remaining epithelial cells were degenerate or necrotic. Only the pillar–capillary network remained intact (Fig. 8A, 8B).

Conversely, gills from acclimated fish, those continuously exposed for 13 d to pH 5.2, 75 μg Al/L, were dramatically different. These gills still showed evidence of the damage induced by initial low-level Al, but the effects of Al challenge were minimal (Fig. 8C). There were areas of fusion and proliferation of undifferentiated cells with hyperplastic mucous and chloride cells. Epithelial cells, although swollen and vacuolated, remained attached to the basal lamina (Fig. 8C).

Fish examined after challenge on day 24 were similar to those seen on day 13, especially the control (6.5/0 Al) and low-pH (5.2/0 Al) groups (Fig. 9A, 9B). Acclimated fish (those previously exposed to low-level Al for 24 d), although affected by the challenge, showed lamellae still hyperplastic but with few areas of fusion (Fig. 9C). Hyperplasia of the primary filament and a preponderance of mucous cells were the most noticeable features. There was some proliferation and hypertrophy of chloride cells, but this was mostly confined to the proximal portions of the secondary lamellae. Distal portions of the lamellae appeared nearly normal with the exception of some areas of epithelial lifting and dilation of lymphatic spaces.

Final challenge groups

The only fish with sufficient survivors for histology sampling were those challenged at pH 5.2 at 0 and 100 μg Al/L for both controls (6.5/0 Al) and acclimated (5.2/Al) fish. At pH 5.2, 300 μg Al/L, only previously acclimated fish were sampled, as there were no survivors in the control group.

Gills from acclimated 5.2/Al fish in Al challenge appeared structurally similar to those gills taken at the end of the acclimation period, but with evidence of more repair. Mucous cells were prominent but not hypertrophic. They were evident on both the primary filament and the secondary lamellae (Fig. 10B). Hyperplasia of the primary filament was present but reduced. Chloride cell proliferation was slight and there appeared to be an increase in the number of phagocytic white cells. In some areas, the epithelium was lifted and the lymphatic spaces were dilated. Gills from the control group (6.5/0 Al) after the 5.2/0 Al challenge showed evidence of acid stress with epithelial lifting as the primary feature (Fig. 10A).

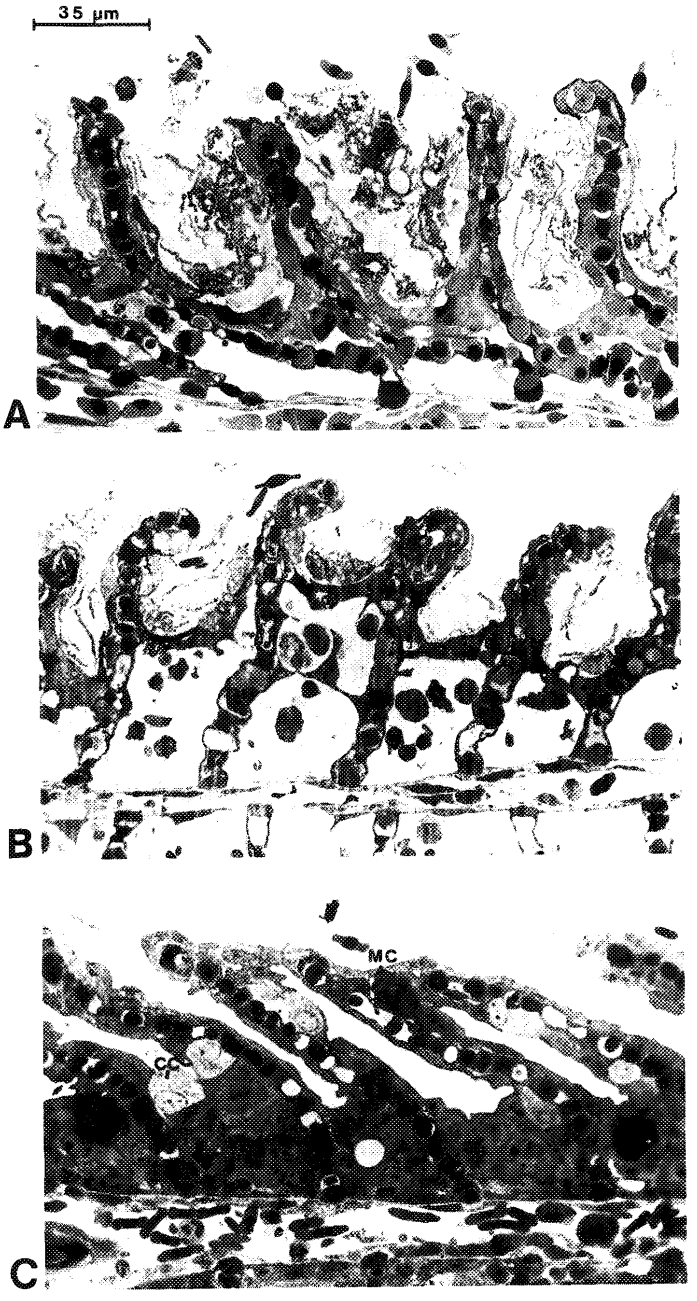


FIG. 8. Comparison of brook trout gills from control (pH 6.5/0 Al), low pH (pH 5.2/0 Al), and low pH plus Al (pH 5.2/Al) exposure after challenge with 1000 μg Al/L for 24 h on day 13. (A) Control gill (6.5/0 Al) showing necrosis of the lamellar epithelium. Only the pillar–capillary network is identifiable. (B) Low pH gill (5.2/0 Al) also showing necrosis of the lamellar epithelium. Blood spaces are distended and necrotic cells and debris fill the interlamellar spaces. (C) Low pH plus Al (5.2/Al) gill showing considerably less damage than control or low pH fish (Fig. 8A, 8B). There is proliferation of undifferentiated cells and some areas of lamellar fusion. Mucous cells (MC) and chloride cells (CC) are hypertrophic.

In the pH 5.2/100 Al final challenge, there were distinct differences between gills from control fish and those previously acclimated to pH 5.2 and 75 μg Al/L. Control fish showed hyperplasia of the primary filament (Fig. 11A) with occasional areas of fusion at the tips of the secondary lamellae. Mucous cells had proliferated and were hypertrophic and generally

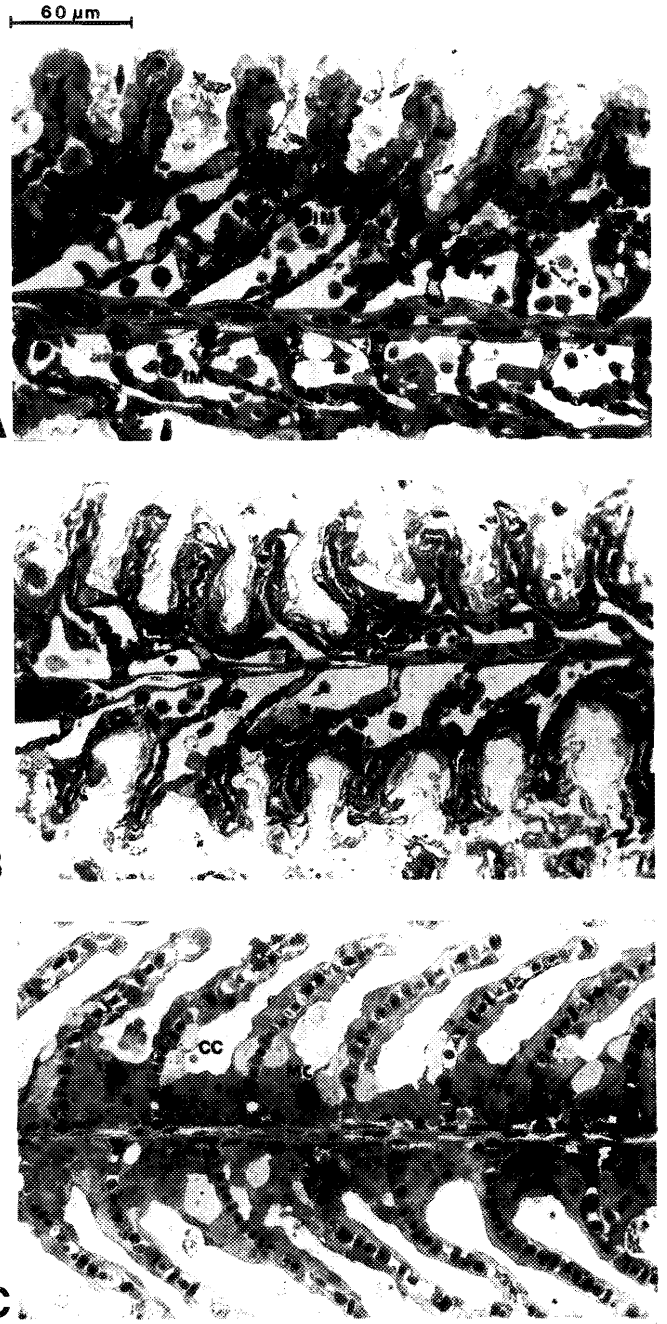


FIG. 9. Comparison of brook trout gills from control (pH 6.5/0 Al), low pH (pH 5.2/0 Al), and low pH plus Al (pH 5.2/Al) exposure after challenge with 1000 µg Al/L for 24 h on day 24 of the acclimation period. (A) Gill from control fish (pH 6.5/0 Al) showing desquamation of the epithelium from both the primary filament and the secondary lamellae. White blood cells, immature mucous cells (IM), and necrotic debris fill the fluid-filled interlamellar regions. (B) Gill from low pH fish (pH 5.2/0 Al) showing cellular necrosis. Necrotic debris fills the interlamellar spaces. (C) Gill from low pH plus Al (pH 5.2/Al) fish is dramatically less damaged than controls. There is hyperplasia of the primary filament and proliferation and hypertrophy of chloride cells (CC) and mucous cells (MC). Although most portions of the secondary lamellar are nearly normal, epithelial lifting is evident.

located at the bases of the secondary lamellae. Chloride cells were extremely hypertrophic and had proliferated throughout the secondary lamellae. The epithelium of the secondary lamellae appeared lifted, and pavement cells appeared swollen.

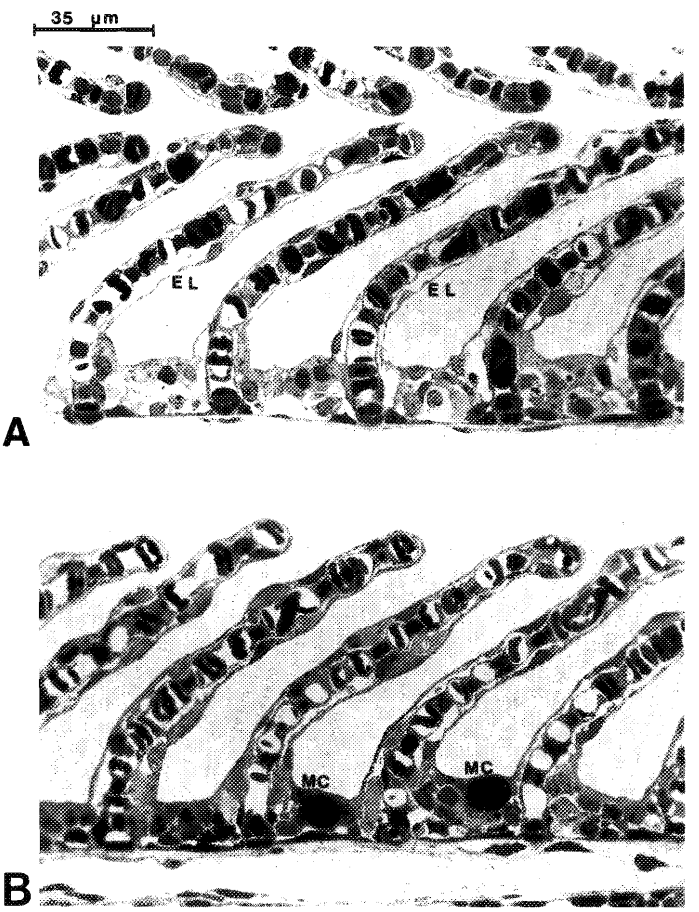


FIG. 10. Comparison of brook trout gills from control fish (pH 6.5/0 Al) with gills from "acclimated" fish (those previously exposed to pH 5.2, 75 µg Al/L for 24 d) when challenged with low pH (5.2/0 Al) for 14 d. (A) Gill from unacclimated fish (6.5/0 Al) showing evidence of acid stress with prominent epithelial lifting (EL). There is slight hyperplasia and edema of the primary filament. (B) Gills from previously acclimated fish (pH 5.2/Al) showing hyperplasia of the primary filament. Mucous cells (MC) are present but less hypertrophic.

In contrast, fish previously acclimated to pH 5.2 and 75 µg Al/L showed minimal effects. Again, the most prominent feature was the hypertrophic and proliferated mucous cells, although in this group they were confined to the primary filament and basal portions of the secondary lamellae. The distal portions of the secondary lamellae were nearly normal, although slightly thickened with numerous phagocytic white cells in the lymphatic spaces (Fig. 11B).

Gills from the acclimated fish that survived the final pH 5.2/300 Al challenge showed definite lesions, although these were not nearly as severe as those from fish examined (initially) during the acclimation experiment (Fig. 4B, 4C). In the gills from acclimated fish after the 14-d challenge (Fig. 11C), extreme proliferation and hypertrophy of chloride cells was a dominant feature with increased apical pit formation. Hyperplasia of the primary filament and areas of degeneration were common. Mucous cells were hypertrophic but were not as numerous as in other experiments. The epithelium of the secondary lamellae was degenerate with many vacuolated and swollen pavement cells.

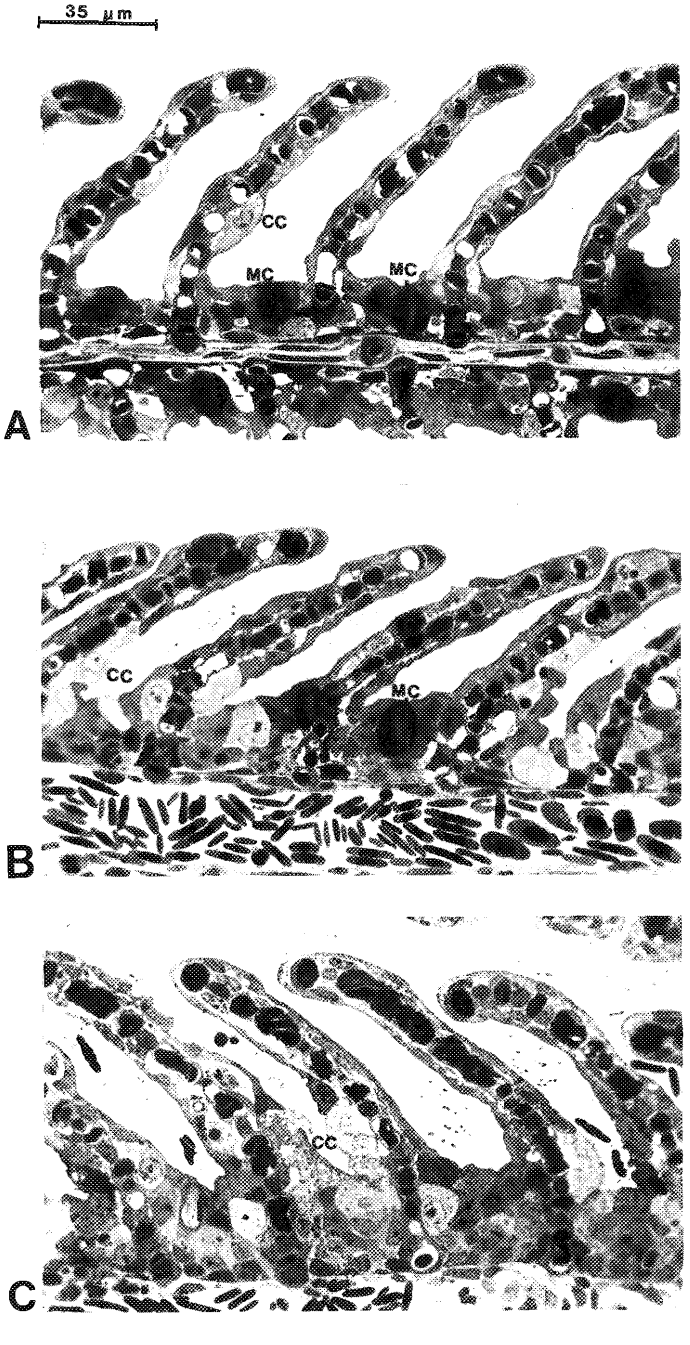


FIG. 11. Comparison of brook trout gills from control fish (pH 6.5/0 Al) with gills from "acclimated" fish (those previously exposed to pH 5.2, 75 µg Al/L for 24 d) when challenged with low pH and Al (5.2, 100 µg Al/L) for 14 d and with gills from "acclimated" fish challenged with 5.2, 300 µg Al/L for 14 d. (A) Gill of challenged unacclimated (6.5/0 Al) fish showing hyperplasia of the primary filament and proliferation and hypertrophy of mucous cells (MC) and chloride cells (CC). The epithelium of the secondary lamellae is lifted. (B) Gill of challenged previously acclimated (5.2/Al) fish showing minor hyperplasia of the primary filament. Secondary lamellar epithelium is nearly normal. Mucous cells remain hypertrophic. (C) Gill of challenged acclimated (5.2/Al) fish showing hyperplasia of the primary filament and hypertrophy and hyperplasia of chloride cells. The epithelium of the secondary lamellae is thickened and white blood cells are present in the lymphatic spaces.

Morphometry

By day 13 of the acclimation period, physiological and mortality data indicated that fish acclimated to pH 5.2, 75 µg Al/L exhibited a reduced response to an Al challenge over controls and pH 5.2/0 Al fish. Therefore, samples taken from all three acclimation groups on this day were selected for a quantitative morphometric analysis.

Exposure to pH 5.2/Al

Gills from fish acclimated to pH 5.2 and 75 µg Al/L had significantly higher ($p < 0.05$) volume densities for undifferentiated cells and mucous cells than did those from fish from other groups (Fig. 12). Volume density of mucous cells had nearly doubled from 0.09 to 1.8%, while that of undifferentiated cells, an indication of the proliferative response, tripled from 7.5 to 23.3%. Mucous cell area, a measure of hypertrophy, was significantly increased, being nearly twice that of controls or 5.2/0 Al fish (Fig. 13). Volume densities of chloride pillar cells were significantly decreased (Fig. 12). There were no significant differences seen in any other cell categories (capillary space, pavement cells, basal cells, empty lymphatic space (ELS), white blood cells, dense cells, degenerated or necrotic cells, and unknown cells).

Direct measurements of diffusion distance and lamellar thickness could not be made on these tissues because of extensive fusion of the lamellae in Al-exposed fish. In these cases, a lamellar fusion index was calculated as a relative measure of damage reflecting some deterioration in respiratory ability. In control fish with little or no hyperplasia or fusion, the expected ratio of exposed perimeter to length of lamellae in a cross-sectional plane would be nearly 2:1 (or 2). Therefore, in fish with areas of extensive fusion, this ratio would be less than 2 , indicating a reduced amount of epithelium exposed to the water and available for gas exchange. At the height of damage (gills sampled at day 4), this index ranged from 0.408 to 0.912, indicating massive fusion and loss of respiratory capacity. By the time the fish had acclimated (by day 13), this index had increased to that of control fish with no significant differences among groups on day 13 (one-way ANOVA, Tukey's multiple comparison, $P > 0.05$) (Table 1).

Exposure to 5.2/0 Al

In most categories, pH 5.2/0 Al fish did not differ from controls. The exceptions were the greater amount of empty lymphatic space in this group over controls and that of Al-exposed fish, and significantly fewer pillar cells than controls (Fig. 12). The increase in empty lymphatic space is misleading because dilation of the lymphatic space appeared more severe in Al-exposed fish but was often filled with degenerating aggregates of cells, leukocytes, and necrotic debris, and "hits" in these areas rarely encountered actual "empty" space. There were also significantly fewer undifferentiated cells in this group relative to Al-exposed fish (5.2/Al).

Al Staining

Al staining was used to confirm the presence of Al on Al-exposed gills. Al was often seen in a mucoid microlayer on the surface of the epithelium as well as in dense accumulations of mucus in the interlamellar regions. As expected, challenge fish showed qualitatively greater amounts of mucus and Al than prechallenge fish, but no attempt was made to quantify differences between groups.

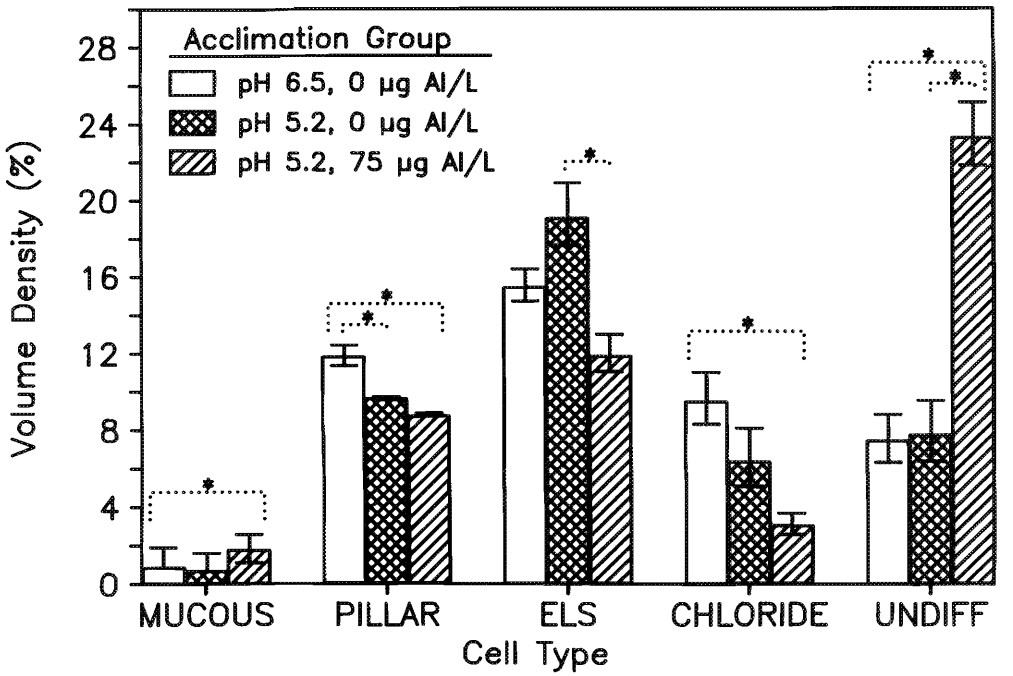


FIG. 12. Histogram comparing cell volume densities of mucous, pillar, chloride, undifferentiated cells, and empty lymphatic space (ELS) among acclimation groups on day 13. Bars represent mean values expressed as percents. Lines at the top of each bar show ± SEM; asterisks indicate significance difference at $p < 0.05$.

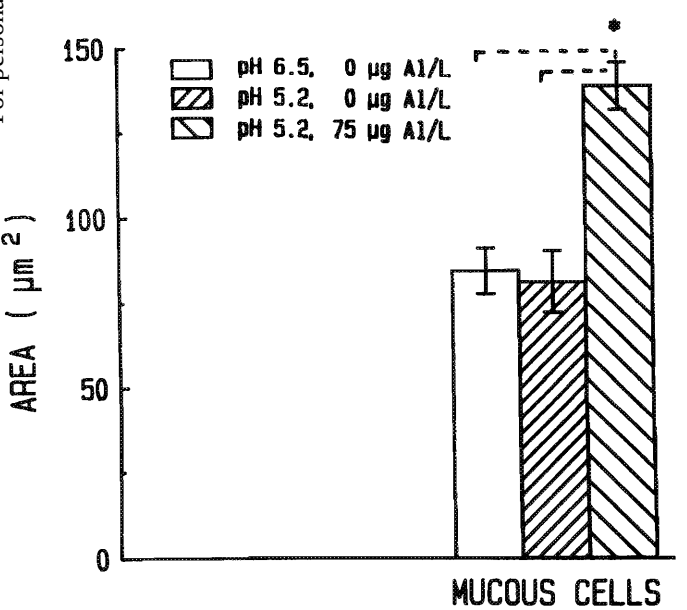


FIG. 13. Histogram comparing mucous cell area among acclimation groups on day 13. Bars represent mean values for mucous cell areas. Lines at the top of each bar show ± SEM; asterisks indicate significant difference at $p < 0.05$.

TABLE 1. Mean values for lamellar fusion indices (L2/L1). n = sample size; SD = standard deviation. *Significantly different from other groups (Tukey's multiple comparison, $p = 0.05$).

Exposure conditions	Day	n	L2/L1	SD
6.5, 0 Al	13	5	2.00	0.033
5.2, 0 Al	13	5	2.01	0.074
5.2, 75 Al	13	5	1.67	0.463
5.2, 75 Al	4	4	0.62*	0.216

Discussion

Exposure of juvenile brook trout to sublethal Al concentrations at low pH provokes profound changes in gill morphology, changes that are much more marked than those provoked by the same low pH alone. These morphological changes can be characterized as a two-phase response: rapid damage (reaching a peak at or before 4 d) followed by gradual repair. The repair phase was almost complete by 24 d of Al exposure. While the repair process was obvious, there were subtler acclimation-related changes as well, for fish challenged after acclimation to low levels of Al showed much less morphological damage than did unacclimated fish.

Since we have extensive physiological data for the same fish that were examined histologically (McDonald et al. 1991), it is now possible to put these profound structural changes at the gills into a physiological context and vice versa.

Damage Phase

Earlier studies on brook trout (Booth et al. 1988; McDonald and Milligan 1988; Wood et al. 1988a, 1988b) indicated that the physiological response to Al exposure was a two-phase response: an initial shock phase and a recovery phase. The "shock" phase of Al exposure is typically characterized by large diffusive ion losses plus an inhibition of active ion uptake and interference with oxygen and carbon dioxide movement across the gills (Booth et al. 1988; Wood et al. 1988a, 1988b). The net effect is a marked net loss of electrolytes across the

gills, an associated contraction of the blood volume, and internal hypoxemia and acidosis, the combination of which may lead to death. Fish that die of Al exposure never escape from this phase, but surviving fish show some recovery in which ion balance is reestablished and may, in fact, show some net ion gain. For the fish in the present study, the depression in body Na^+ reached a peak at day 4 (fig. 2A in McDonald et al. 1991) and then began to recover gradually. Hence the gills examined at day 4 probably represent the maximum gill damage experienced by surviving fish.

Histologically, the damage at day 4 consisted of extensive necrosis, desquamation of the gill epithelium, and lamellar fusion (Fig. 4B, 4C). The lamellar fusion was so severe that lamellar surface area could have been reduced by as much as 75%. This would impair oxygen delivery to the tissues as was indicated by an increase in body lactate levels relative to controls (Fig. 2E in McDonald et al. 1991). The increase in lactate was about 80% of the increase seen following exhaustive exercise in rainbow trout (*Oncorhynchus mykiss*) of similar size (M. Scarabello and C. M. Wood, McMaster University, Hamilton, Ont., unpubl. data). It is, therefore, likely that these fish had very little reserve to increase their metabolism or activity level and that their body glycogen reserves would have been depleted by anaerobic metabolism.

Other physiological correlates of the Al damage were the accumulation of Al on the gills and corresponding reduction in gill sialic acid (McDonald et al. 1991). Sialic acid is the major cationic component of gill mucus (Harris et al. 1973). Staining indicated that Al was primarily confined to the mucus layer and mucous cells, thus indicating probable Al binding to sialic acid residues. The corresponding histological response was extensive proliferation and stimulation of mucous cells which were fully evident in fish first examined at day 4 (Fig. 4B, 4C). These proliferated cells probably became functional and hypersecretory before this time, however, as gill Al peaked at day 1 of exposure and subsequently declined (fig. 2F in McDonald et al. 1991). By day 4, gill Al levels had fallen to half of that reported after 24 h. Hypertrophy of mucous cells thus appeares to be the first defense against Al exposure, and the combination of hypertrophy with the decline in sialic acid content of the tissue (McDonald et al. 1991) suggests either a change in the chemical composition of mucus with time or a considerable increase in mucus turnover in the gills. In any case, as gill Al levels fell, sialic acid content returned to preexposure levels and mucous cell hypertrophy persisted. The fact that gill Al levels thereafter remained low, despite the presence of Al in the water, suggests that elevated mucus secretion and sloughing was acting as a mechanism to prevent Al damage or accumulation.

Repair Phase

By Day 13, Al-exposed fish had nearly achieved physiological homeostasis. While they had not recovered completely, they showed no further deterioration in body electrolytes or in hematocrit (fig. 2 in McDonald et al. 1991). By this time, a remarkable degree of repair of the gill damage had taken place (Fig. 7C). There was considerable decrease in fused lamellae, and a corresponding decline in body lactate levels, indicating a return in gas transfer capacity towards normal levels. Necrosis and degeneration of cells had become rare, and the significant increases in mucous cell and undifferentiated cell densities relative to controls (Fig. 7C) were suggestive of a highly dynamic, rapidly changing epithelium. In previous studies, fish gills have been shown to respond to Al exposure with a rapid hypertrophy and hyperplasia of chloride cells (Evans et al. 1988; Youson and Neville 1987; Karlsson-Norgren et al. 1986; Tietge et al. 1988). The apparently delayed response in the present study may have occurred because the chloride cell proliferation was masked by the severity of the initial damage and subsequent repair. As pointed out earlier, many of the proliferating cells were not identifiable but may have been developing chloride cells. By day 24, chloride cells had increased in number (visual estimate) and appeared more folded with increased apical pit formations (Fig. 6B). These changes are thought to increase surface area for ion uptake to compensate for ion loss (Chevalier et al. 1985; Leino and McCormick 1984; Youson and Neville 1987). We did not see the appearance of "dense cells," a chloride cell variant reported by Tietge et al. (1988). This may have been because our study was 24 d in duration as compared with 147 d in that study. The apparently slow chloride cell response, in fact, correlated well with the physiological response (McDonald et al. 1991). Gill Na^+ transport was significantly impaired by sublethal Al exposure (fig. 5A in McDonald et al. 1991); it was maximally depressed by day 4 and then gradually recovered reaching almost normal levels by day 13.

Acclimation to Al

By day 13, the Al-exposed fish had not only achieved physiological homeostasis, but even more importantly had developed significant resistance to otherwise lethal Al concentrations (fig. 6 in McDonald et al. 1991). Fish were challenged with 1000 μg Al/L on three occasions between day 13 and 24, and in each case, the Al-acclimated fish exhibited longer survival times (i.e. increased ET50) and much smaller depressions in body electrolytes (fig. 2 in McDonald et al. 1991). Structural correlates of the increased resistance of acclimated fish were clearly seen in the histological results of the present study (Fig. 8, 9) where acclimated animals showed much attenuated gill morphological response to an otherwise lethal Al challenge. The implication of this finding is that Al is, in some way, chemically complexed before it is able to react with binding sites on the gills. The hypertrophy of mucous cells and proliferation of undifferentiated cells may be of key importance here, for these are the major histological differences between acclimated and unacclimated animals. However, the protective effect of preexposure did not arise from an elevated gill mucous content, for as pointed out earlier, the hypertrophy of mucous cells was not accompanied by an increase in sialic acid content; acclimated and unacclimated animals were virtually identical in this respect by day 7. Rather, it seemed more likely that there was a change in the chemistry of the mucus such that sialic acid content was not representative of mucus content and/or an increased turnover and sloughing rate of the mucus.

In a related study in rainbow trout, Reid et al. (1991) established that sublethal Al acclimation led to significant changes in the cation-binding properties of the gills. Specifically, these consisted of an increased binding affinity for Ca^{2+}, a decreased binding affinity for Al, and a decline in the potency of Al at displacing Ca^{2+}. These changes would contribute to reducing the reactivity of Al at the gill surface. These changes may result entirely from mucous cell hypertrophy and increased mucus production but there may also be changes at the outer epithelial membrane which are below the resolution of histological tech-

niques used in this study. Clearly some of the responses of the gills are too subtle to be resolved by the physiological and morphological techniques employed here. Therefore, a detailed examination of the ultrastructural changes involved in damage and repair processes should also be included in future analyses of branchial responses to environmental stress.

Whatever the mechanistic explanation for acclimation to Al, neither physiological measures (McDonald et al. 1991) nor histological observations provide definite markers for the exact time during exposure when increased resistance first occurs. Acclimation is likely a graded rather than a sudden process. There appears to be some threshold level, not detected morphologically, that must be reached in the compromise between protective measures such as increased production and secretion of mucus versus fusion of lamellae and the decreased ability for gas exchange. Histological observations suggest that this continuum of repair and alteration is gradual and dependent on initial damage due to the toxicant.

In addition, one must be careful in defining "benefit" in terms of the life of the fish. Surely in waters where spring and fall pulses of low pH and Al occur, there is an advantage for a fish with altered gill morphology such as that seen in this study. Our studies have demonstrated that acclimation to low pH and Al increases resistance to an otherwise lethal Al challenge. However, between these Al pulses, or in areas where no pulses occur, the morphological changes described for acclimated fish would inhibit normal activity. Increased diffusion distances and fused lamellae restrict oxygen transfer ability which would limit aerobic activity. It has yet to be determined how much and for how long the changes resulting in acclimation persist.

Acknowledgements

This work was supported by a contract ("Lake Acidification and Fisheries," RP-2346-01) from the Electric Power Research Institute, Environmental Assessment Department, through a subcontract from the University of Wyoming. We thank Dr. J. Mattice, EPRI project manager, for his advice and encouragement. We thank Dr. Scott Reid for his technical assistance.

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