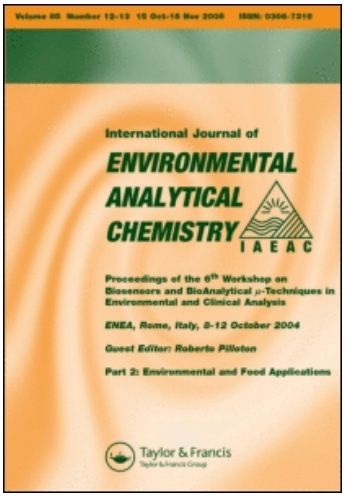

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A Microanalytical Study of the Gills of Aluminium-Exposed Rainbow Trout (*Salmo Gairdneri*)

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A Microanalytical Study of the Gills of Aluminium-Exposed Rainbow Trout (*Salmo Gairdneri*)

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Laser Microprobe Mass Analysis was used to localize aluminium at the subcellular level in gills of rainbow trout (*Salmo gairdneri*), exposed to an Al-concentration of 200 μg/l for 3 days. The pH of the medium was 5. This technique is based on the perforation (diameter: 1–2 μm) of a microtome section of the tissue, by a high-power laser pulse.

Aluminium was mostly found on the surface of the gill lamellae, but also in some epithelial cells. No aluminium was measured in chloride cells. Bulk analysis of the gills gave at least a 15 times higher Al-concentration in the Al-exposed fish, compared to the control fish.

Transmission electron microscopy revealed fused lamellae, swollen and collapsed chloride cells and epithelial cells separated from the underlying tissue.

KEY WORDS: Rainbow trout, gills, Cellular Analytical Chemistry, Laser Microprobe Mass Analysis, aluminium, acidification.

INTRODUCTION

The acidification of the environment has been studied intensely since the first alarming reports on the direct and indirect effects of acid deposition on forest and freshwater ecosystems.[1,2] One of the consequences of the acidification is a higher mobility in the soil of some minerals and metals, which are washed out into the groundwater, and transported to rivers and lakes. Especially aluminium has been put forward as being toxic for roots of trees and gills of fish.[3,4]

Physiological studies have pointed out that low pH results in a disturbance of the ionoregulatory system.[5] Adding aluminium to the low-pH medium exacerbates this toxic effect and also influences the respiratory mechanism.[6–8]

Several authors have examined the morphology of the gills of freshwater fish with Al-intoxication and low-pH stress[9–13] and several deformities were observed. Bulk analyses of the gills revealed at least a 15 times higher Al-concentration in or on the gills of Al-exposed fish.[7,14–16]

To have an idea of the toxic mechanism of aluminium at the level of the gills the subcellular distribution of aluminium must be studied, and thus, microanalysis is inevitable. Jagoe *et al.*[17] used a histochemical staining on light microscopic sections to show Al-accumulations in epithelial cells and Karlsson-Norrgren *et al.*[12] found aluminium in dark precipitates in chloride cells and on the gill surface, using X-ray microanalysis. We applied Laser Microprobe

Mass Analysis (LAMMA) to study the Al-distribution on a subcellular scale in the gills of Al-exposed rainbow trout.

MATERIALS AND METHODS

Treatment of the fish

The experiments were performed with adult rainbow trout, *Salmo gairdneri* Richardson (180–350 g). The fish were kept for some days in running tapwater outside the laboratory and were fed with trout pellets. During the subsequent acclimation and experimental period, the fish were starved and kept in a cold room (±8 °C) with a constant day/night rhythm (12 hr/12 hr). The animals were stored in a 150 l tank, while the water was continuously recirculated and renewed once in 24 hours. Prior to the treatment with elevated Al-concentrations, the fish were kept for 12 days in control water of pH 6.8 (ionic composition given in Table 1) and for another 7 days in acid water of pH 5.0 (Table 1). Demineralized water with some salts ($NaCl$, $NaHCO_3$, KCl, $CaCl_2$, $MgSO_4$) was prepared as control water while a mixture of H_2SO_4 and HNO_3 (2 vol./1 vol.) was used to acidify the water. An automatic device, pH-stat, was used to keep the pH-level at 5.0.

The fish were exposed to 200 μg Al/l at pH 5.0 for about 3 days. An open recirculation system with a flow-through rate of 25 l/hr was used to keep the Al-concentration within certain limits (160–200 μg Al/l). Water samples were regularly taken and the total Al-concentration was determined by plasma emission spectrometry (Table 1).

Table 1 The pH and the measured ionic composition (mg/l) in the different water types. Data are mean values ± 95% confidence limits

Water type	*Control*	*Acid*	*Acid + Al*
pH	6.8 ± 0.1	5.0 ± 0.1	5.0 ± 0.1
Al_{total}	< 0.030	< 0.030	0.19 ± 0.03
Ca^{2+}	1.0 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
Na^{+}	9.4 ± 0.3	9.3 ± 0.1	9.7 ± 0.3
Cl^{-}	8.6 ± 0.4	8.6 ± 0.4	10.3 ± 0.2
SO_4^{2-}	1.7 ± 0.3	22.6 ± 1.8	21.3 ± 2.1

Bulk analysis

At the end of the exposure period fish were killed with a blow on the head. Within less than 30 minutes the second gill arch was dissected from the fish.

The wet weight of the gill tissues was determined immediately after dissection. Then they were dried at 105 °C, ashed in a furnace at 500 °C and the ashes were redissolved in a solution of nitric acid and bidistilled water. The total Al-content in the gill preparations was determined by plasma emission spectrometry (Jarrell Ash, Atomcomp Model 750).

Preparation of the gills for transmission electron microscopy and microanalysis

The gills were dissected from the fish and cut in small pieces (1 mm^3), fixed in a buffered 4% paraformaldehyde and 5% glutaraldehyde solution, postfixed in a 1% osmiumtetroxide solution, dehydrated in a graded ethanol series, passed through propylene oxide and embedded in Spurr's low viscosity resin.[18] Sections with a thickness of 0.25 μm were made with glass knives using an LKB ultramicrotome. Before and after LAMMA-measurements, electron micrographs were made to recognize the areas of analytical interest in the section. A Jeol JEM 100-B electron microscope was used.

Microanalysis

For microanalysis, Laser Microprobe Mass Analysis (LAMMA) was applied, using the LAMMA-500 instrument (Leybold-Heraeus, Cologne, F.R.G.). The principle of this technique is based on the evaporation and excitation of a microvolume of the sample (TEM-sections for biological material) by a single pulse of a high-power laser. The formed ions are analysed by a mass spectrometer.

The sample is mounted in vacuum on a movable x-y stage and can be observed through a light microscope. A He-Ne pilot laser beam can be seen as a red spot on the sample. A Nd-YAG high-power laser is collinear with the pilot laser and both lasers are focused onto the sample through the objective lens of the light microscope. By moving the sample, specific areas can be selected for analysis. One single pulse ($\tau = 15$ ns) of the high-power laser perfor-

ates the sample (diameter of the perforation: 1–2 μm). The evaporated and excited material is accelerated through an Einzel-type ion lens into the drift tube of a time-of-flight mass spectrometer. At the end of the tube, the ions are detected by a secondary electron multiplier. Postive or negative ions can be detected just by switching the polarity of the electric fields. A review of applications in the life sciences has been given by Verbueken *et al.*[19]

RESULTS

Bulk analysis

The results of the measurement of the Al-content in gill tissue of control and Al-exposed fish is presented in Table 2. It is found that the aluminium is accumulated in or on the gills when fish are exposed to acid water with elevated Al-levels.

Transmission electron microscopy

Figure 1a shows a part of a secondary lamella of a control fish. The different cell types are clearly visible and show no sign of deformation or degeneration. Figure 2 shows some micrographs of gills of Al-treated fish. Epithelial cells have separated from the underlying tissue (Figure 2a), cell material of collapsed cells is present between the lamellae (Figure 2b). Also, an increased number of chloride cells is observed, together with a very irregular cell surface, fused lamellae and swollen and degenerated chloride cells.

Microanalysis

Electron micrographs are made before and after LAMMA-measurements to select areas of analytical interest, and to identify the analysed areas after the perforation of the sample by the high-

Table 2 Al-content of the gills of rainbow trout, expressed as μg Al/g wet weight

	Control (12 days)	*Al-exposure (3 days)*
Mean value $\pm 95\%$ confidence limits	1.0 ± 0.5	31 ± 15
Level of significance	$t = 5.23$, d.f. $= 10$, $P < 0.001$	

power laser. Figure 1 shows a secondary lamella of the control fish, before and after LAMMA-analysis. The perforations can be seen as the white spots in Figure 1b.

Spectra obtained from the LAMMA-instrument are given in Figure 3 Figure 3a shows a spectrum of a secondary lamella of a control fish. The spectrum in Figure 3b is obtained from the gill surface of an Al-exposed fish. A high Al-signal is observed.

Due to the fact that the LAMMA-technique is only semi-quantitative, no concentrations can be given, but the intensity of the signal of aluminium can be compared between the control fish and the Al-exposed fish. Generally, no Al-signal or a very small one appeared in the spectra of the control fish, while in Al-exposed fish, the peak of aluminium was in overflow in most cases (see Figure 3b). Aluminium was found most frequently on the surface of the lamellae. Areas analysed just below the surface did, in most cases, not contain aluminium. In some epithelial cells however, an Al-signal was observed. High Al-concentrations were also measured in the cell material of collapsed cells between the lamellae.

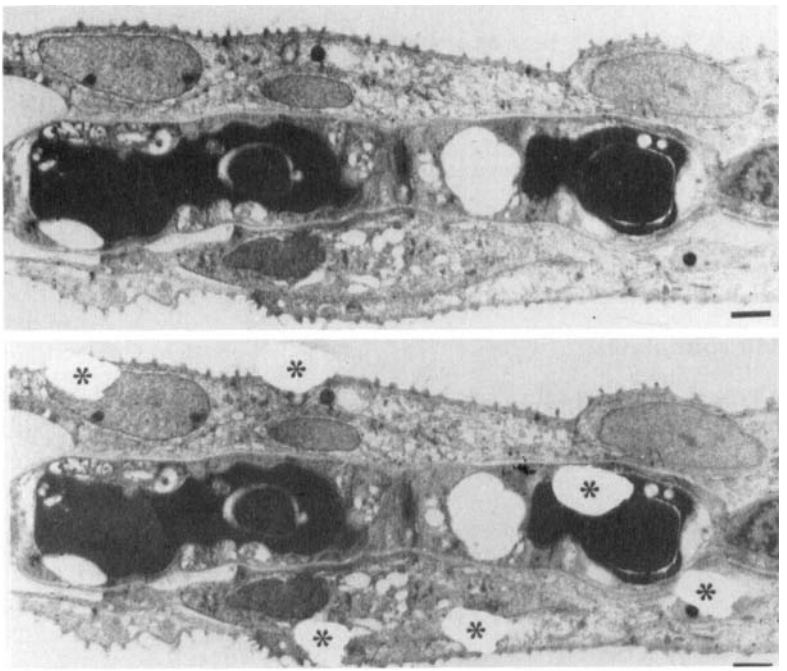

Figure 1 Secondary lamellae of control fish, (a) before and (b) after LAMMA-measurements. * = laser perforation; bar is 1 μm.

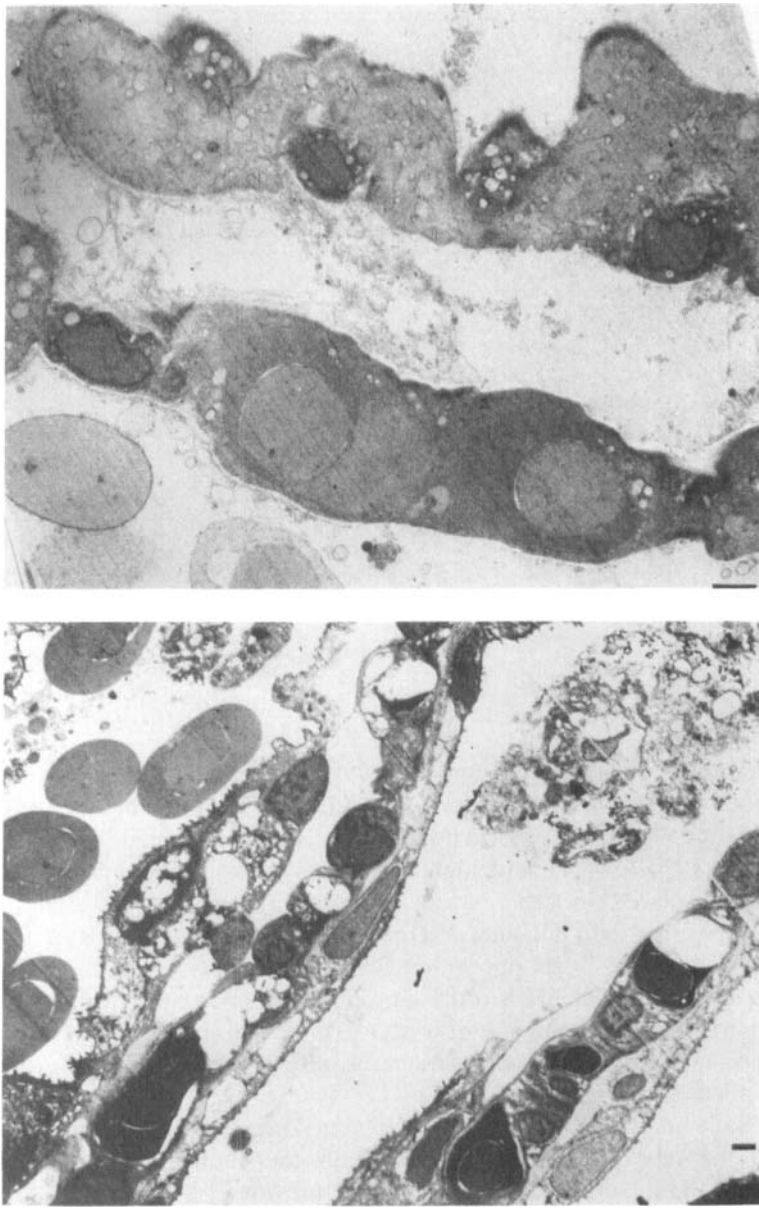

Figure 2 Secondary lamellae of Al-exposed fish. (a) epithelial cells have separated from the underlying tissue. (b) cells have collapsed and cell material of destructed cells is present between the lamellae. Bar is 1 μm.

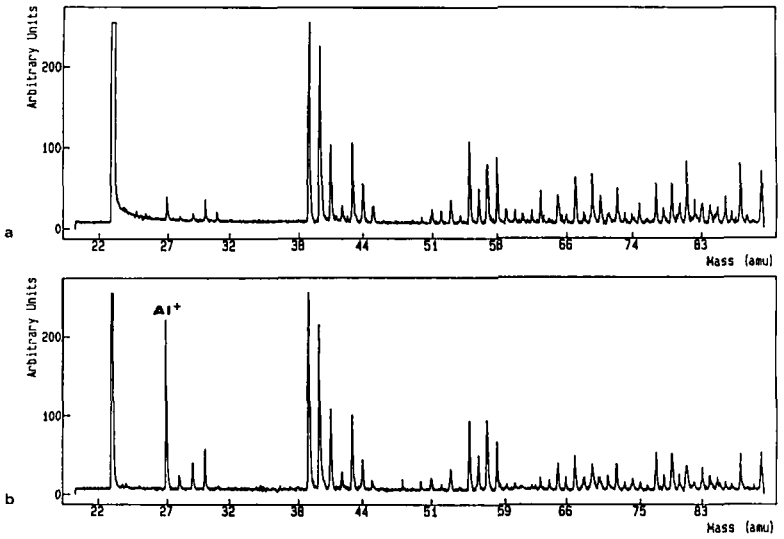

Figure 3 LAMMA-spectra of (a) gill of control fish and (b) surface of gill of Al-treated fish.

DISCUSSION

Examination of the morphology of Al-intoxicated gills, using TEM (Figure 2), shows significant gill damage because of the stress situation. Similar observations have also been made by other authors for low pH and high aluminium concentrations.[4,9,14] Gills of fish placed in other stress situations (heavy metals, high pH, phosphorus, light oil and detergents) also show symptoms of toxicity, e.g. Baker[20] has put winter flounder (*Pseudopleuronectes americanus*) in water of pH 8 containing a high copper concentration and observed similar gill alterations. An increased number of chloride cells is also observed when bacteria, nitrite or deionised water are applied.

Bulk analyses gave at least a 15 times higher concentration in or on the Al-exposed gills, compared to the control gills (Table 2). These data are in agreement with previous[16] or other[7,8,14–15] literature data. Other organs did not accumulate aluminium after 5 to 10 days exposure.[7,15] Karlsson-Norrgren *et al.*[14] found an

Al-accumulation also in the kidneys after a prolonged Al-exposure.

The real impact of aluminium on the gills is not yet known. Wood and McDonald[8] speculate that the increased distance between water and blood, caused by the separation of the gill epithelium from the underlying tissue, the swelling of the gills, the precipitation of Al-complexes on the gill surface and the accumulation of mucus on the gills, are probably the main causes of the blockade of the respiration, while the high H^+-concentration is most responsible for the disturbed ionoregulatory system. It is suggested that the increased number of chloride cells that we, as well as other authors[9,13,14], observed, compensates the disturbance of the ionoregulatory system.

Bulk analyses give only an idea of the amount of aluminium accumulated in or on the lamellae, but no conclusions can be made about the accumulation site. For this purpose, microanalysis has become a valuable tool in recent years to examine the elemental distribution at the subcellular level. Lack of essential or excess of toxic elements can be located at specific sites or organels within the cells of biological material. Several techniques have been developed and optimized over the last ten years. LAMMA is a very sensitive, multielemental detection technique, with a very short analysis time period. But it suffers of severe limitations: it is destructive, it has a rather large spatial resolution (1–2 μm) and the obtained spectra are semi-quantitative: no exact concentrations can be given. The biological tissue that has to be analysed, must be prepared as for electron microscopy. This involves chemical fixation methods which cause a loss of elements, mostly light elements, from the tissue. We measured total aluminium by bulk analysis at different stages of the preparation procedure. It was established that at least 30 to 40% of the total aluminium was lost from the gills during the fixation and dehydration. Most of this lost aluminium was probably adsorbed to the mucus layer, which dissolves during the dehydration step in the sample preparation. The aluminium present within the cells of the epithelial tissue is probably less affected by leaching effects. But since LAMMA only provides semiquantitative data, the relative distribution of the elements is of more importance, not the exact concentration. For this reason, we decided to use the chemical fixation technique, in view of its simplicity and the good structural preservation of the gill tissue.

Karlsson-Norrgren *et al.*[12] also applied chemical fixation tech-

niques and used X-ray microanalysis to analyse the dark precipitates they observed in the chloride cells and on the gill surface, and detected aluminium. We never observed any precipitates in the chloride cells, and also, no aluminium was ever detected by the more sensitive LAMMA in chloride cells. This difference is probably due to the fact that we subjected our fish for only three days to an Al-concentration of 200 μg/l while Karlsson-Norrgren *et al.*[12] used 500 μg Al/l during 6 weeks. Jagoe *et al.*[17] observed metallic deposits in epithelial cells and found aluminium there, using histochemical stains. We also found aluminium in the epithelial layer, but mostly at the outer surface of the cells. Wood and McDonald[8] suggest that the aluminium on the surface irritates the gills, resulting in increased mucus production and an inflammatory response, while the surface coating inhibits ion exchange by affecting carrier systems in the membranes. Using the LAMMA-technique, it is not possible to detect in what chemical form aluminium is present on the lamella surface.

The material of collapsed cells, which is present between the lamellae, also contained high concentrations of aluminium. It is not certain however that these cells collapsed because of the high Al-accumulation; the aluminium may have been adsorbed on the destructed cell material after the cell collapsed.

Freshwater fish suffer and even die from high Al-concentrations at low pH. Microanalytical research gives possibilities to link electron microscopical observations with physiological measurements. More research in this direction is certainly needed to get a better view on the toxicological impact of aluminium on the gills of freshwater fish.

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