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THE TOXIC MIXING ZONE OF NEUTRAL AND ACIDIC RIVER WATER: ACUTE ALUMINIUM TOXICITY IN BROWN TROUT (*Salmo trutta* L.)

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Abstract. Mixing of acid river water containing aluminium (pH 5.1, Al 345 $\mu\text{g.l}^{-1}$) with neutral water of a lake (pH 7.0, Al 73 $\mu\text{g.l}^{-1}$) resulted in water (pH 6.4, Al 245 $\mu\text{g.l}^{-1}$) with a pH (6.4) and Al concentration (245 $\mu\text{g.l}^{-1}$) expected to have low toxicity to fish on the basis of current Al toxicity models. However, under semi-field conditions the freshly mixed water (a few sec. after mixing) proved to be highly toxic to brown trout. The fish were exposed to the water at different places along a 30 m channel. At the beginning of the channel acid and neutral water were continuously mixed; the mixed water left the channel after 340 sec. The cells of the gills showed a highly increased rate of cell death by apoptosis and necrosis. Intercellular spaces were enlarged, and many leucocytes penetrated in these spaces. Mucus release was stimulated to depletion. Plasma chloride levels were hardly affected. There was a clear gradient in the deleterious effects on the fish along the channel. The fish at the beginning of the channel (about 12 sec. after mixing of the water), were severely affected, whereas the fish kept at the end of the channel (340 sec. after mixing) were only mildly affected. In the natural situation fish will relatively quickly pass through a mixing zone. In our study we therefore focused on the effects on fish after a 60 min exposure to a mixing zone (5 sec after mixing), with subsequent recovery in a region downstream of the confluence and in neutral water with low Al. The recovery in the downstream area (at the end of the channel, i.e. 5 min after mixing) was clearly hampered when compared to the recovery in neutral water with low aluminium. Thus, a short exposure to the toxic mixing zone followed by a stay in water downstream of this zone, as may occur in nature, is detrimental to migrating trout. We conclude that freshly mixed acid and neutral water contain toxic components during the first seconds to minutes after mixing, that can not be explained by current models on aluminium toxicity.

Keywords: aluminium toxicity, non-equilibrium chemistry, pH, stress, apoptosis, necrosis, trout.

1. Introduction

Fish mortality has been reported in areas where acid, Al-containing water mixes with neutral (limed water) even though the mixed medium typically has a pH above that known to produce toxic aluminium (Al) species (>pH 5.5; Muniz and Leivestad, 1980; Baker and Schofield, 1982; Howells et al., 1983; Verbost et al. 1992). In field experiments with Atlantic salmon and brown trout, higher mortality has been observed in the mixing zone (0-20 sec after mixing of water of an acid inlet with that of a neutral lake) than in the acid inlet, which has been attributed to transient products of Al-polymerization (Rosseland et al., 1992; Poleo et al., 1994). In this study we aimed to gain more insight in the toxic effects of such mixing zones on brown trout by electron microscopy of the gills in combination with measurements of plasma Cl⁻ levels and blood haematocrit. In an artificial channel neutral water and acid water, coming from two lakes, were mixed and led through the channel with a maximum water residence time of 340 sec. Brown trout were exposed for up to 72 h to the mixed water at different sites along the channel.

Water, Air and Soil Pollution 85: 341–346, 1995.

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In addition to the long term exposure experiments intended to demonstrate the toxicity of the mixing zone, we studied a more natural situation where the fish relatively quickly pass through the toxic mixing zone. The fish are known to try to escape from toxic mixing zones if they can (Åtland and Barlaup, 1995). This field study focused on the effects of a relatively short exposure (60 min) of brown trout to a toxic mixing zone, with and without subsequent recovery in a region down stream of the toxic zone and in neutral water with low Al.

2. Materials and methods

Fish. Brown trout (*Salmo trutta*), 5 to 14 g in weight (8 ± 2 g, n=80), were obtained from the Oslomarkas Fish Administration (OFA) hatchery near Oslo, Norway. Fish were transferred to the experimental site (1 h by road) and were kept in a large tank until being used in the experiment.

Experimental design. The mixing zone experiments were performed in a Y-shaped channel (Poleo et al. 1994) in the respective arms of which water was pumped from lake Nepptjern (pH 5.1, Al $345 \mu\text{g.l}^{-1}$, Ca $20 \mu\text{mol.l}^{-1}$) and lake Gorja (pH 7.0, Al $73 \mu\text{g.l}^{-1}$, Ca $160 \mu\text{mol.l}^{-1}$), located in the Nordmarka area north of Oslo. From the point of confluence (where thorough mixing was assured resulting in water of pH 6.4, Al $245 \mu\text{g.l}^{-1}$, Ca $77 \mu\text{mol.l}^{-1}$) to the end, the channel was 30.5 m long. The channel was 20 cm wide and 15 cm deep, the flow rate was around 9 cm.sec^{-1} corresponding with 340 sec residence time of the water. Fish were kept in cages at various intervals along the channel and protected from direct light by covers. Control groups were put in cages in the two inlet arms in front of the mixing zone. Separate cages, with fish that were not sampled, were used for determining mortality.

For the recovery experiment fish were exposed to the toxic mixing zone (5 sec after mixing) for 60 min and subsequently moved to either the far end of the channel (in water aged for 340 min, cage W) or to separate black tanks with neutral, unmixed water (from lake Gorja) which provided optimal recovery conditions. As controls for the exposure and the handling, fish were held for 60 min in the neutral and acid water inlets and cage W, and subsequently transferred to either cage W or a black tank with water from lake Gorja. Experiments were conducted in June 1994.

Electron Microscopy. Gill samples were obtained from the third gill arch on the left side. Tissues were fixed in 3% glutaraldehyde buffered in sodium cacodylate (0.1 mol.l^{-1} , pH 7.3), and post-fixed in 1% osmium tetroxide in the same buffer. Ethanol-dehydrated tissues were embedded in Spurr's resin. Ultrathin sections, collected on 150 mesh copper grids, were contrasted with uranyl acetate and lead citrate. They were examined in a Jeol 100 CXII transmission electron microscope.

Analytical techniques. Total acid reactive Al (Al_r) was measured after acidifying untreated water samples to pH 1.0 (HCl) for at least 24 h before the Barnes-Driscoll extraction-cation exchange method (Driscoll, 1984) was applied to measure Al.

Blood from samples collected from the caudal vessels after cutting the tails was partly used for determination of haematocrit (heparinized capillaries were filled, centrifuged and read). The other part was centrifuged in heparinized eppendorfs and plasma transferred to clean tubes. Plasma chloride was determined in the field (with a chloride titrator) and checked, with similar results in the laboratory (automated colorimetric method according to Zall et al., 1956).

3. Results

Plasma chloride and blood haematocrit

In fish from cage Ne, receiving water from the acid inlet from Lake Nepptjern, there was a progressive decrease in plasma Cl⁻, apparent already after 1 h. All the other groups showed no significant changes in plasma Cl⁻ during the long exposure experiment except for group E (5 sec after mixing) after 24 h (Table 1).

Table 1

Top: Effects on plasma Cl⁻ and haematocrit of brown trout of exposure for 1 h or 24 h to neutral, unmixed water with low Al (Go; neutral inlet of channel), to acid water with high Al (Ne; acid inlet of channel), to the confluence of both water types (E; beginning of channel, 5 sec after mixing), or to mixed water 340 sec after mixing (W; end of channel).

Bottom: Effects on plasma Cl⁻ and haematocrit of recovery for 24 h (after 1 h exposure to water of the mixing zone, E) in tanks with neutral water from Lake Gorja (GoT) or to mixed water at the end of the channel (W).

	Go	Ne	E	W
plasma Cl ⁻ 1 h	121.0 ± 1.9	114.4 ± 0.7*	119.8 ± 0.9	122.2 ± 1.0
plasma Cl ⁻ 24 h	126.5 ± 3.2	99.0 ± 1.6*	114.2 ± 3.5*	125.0 ± 4.1
haematocrit 1 h	37.3 ± 1.3	42.2 ± 1.9	39.3 ± 2.2	37.3 ± 1.5
haematocrit 24 h	42.8 ± 2.2	60.6 ± 1.9*	46.0 ± 3.3	37.8 ± 1.4
plasma Cl ⁻ 24 h recov. in GoT	122.9 ± 3.0	116.9 ± 3.2	128.9 ± 2.7	125.9 ± 2.9
24 h recov. in W	113.9 ± 4.9	118.6 ± 3.1	117.3 ± 4.3#	114.1 ± 4.1#
haematocrit 24 h recov. in GoT	36.0 ± 2.2	37.3 ± 1.5	34.2 ± 1.3	33.5 ± 3.8
24 h recov. in W	38.8 ± 0.9	34.3 ± 2.2	36.8 ± 1.9	34.2 ± 2.7

* : significantly different ($P < 0.05$) from 'Go group'

: significantly different ($P < 0.05$) from '24 h recov. in GoT group'

n = 6, means ± SEM

Haematocrit increased in fish held in acid water for 24 h (cage Ne). In the recovery experiment fish were placed for 1 h in the toxic mixing zone (cage E), the end of the mixing channel (cage W), the neutral (Go) or acid (Ne) inlet. Subsequently they were transferred to either cage W or to separate tanks with neutral water (GoT) for 24 h. There was no mortality in the recovery experiments. Fish exposed to cage E restored their

Cl⁻ levels well in GoT but not in cage W. Fish exposed to cage W maintained their plasma Cl⁻ in GoT but showed a significant drop in cage W. Control fish kept in cage Go reacted similarly to the W group but the decrease in Cl⁻ was just not significant. Fish kept in cage Ne for 60 min maintained their low plasma Cl⁻ levels in cage W and did not recover very well in GoT (neither after 72 h, results not shown) in contrast to the Cl⁻ levels of the other groups. In the recovery experiment there were no significant differences in haematocrit between the different groups.

Electron microscopy of the gills.

In fish exposed for 24 h to water from lake Gorja (cage Go) the gills showed the normal ultrastructure known for brown trout, and no differences were observed with fish sampled at the hatchery (results not shown). Only few chloride cells (branchial cells specialized for ion exchange) showed signs of degeneration by necrosis or apoptosis (necrosis: accidental cell death, characterized by rupture of membranes and swelling of cell compartments; apoptosis: physiologically controlled cell death, characterized by cellular shrinkage and densification of nuclei, mitochondria and cytoplasm; Wendelaar Bonga et al., 1990). However, in fish from acid water (NE) and from the mixing zone (E) Al deposits on the branchial filaments and severe damage of the branchial epithelium were found. After 24 h the percentage of necrotic and apoptotic chloride cells was significantly increased (Fig. 1; $P < 0.01$) when compared to the fish from water of lake Gorja. Also many respiratory cells, which form the epithelium covering the respiratory lamellae, showed highly increased percentages of necrotic and apoptotic cells. The intercellular spaces of the branchial epithelia were enlarged in many places, and many leucocytes (many macrophages, lymphocytes and some neutrophilic granulocytes) had left the blood and penetrated into these spaces. In mixed water 340 sec after mixing (W), hardly any Al deposits nor substantial damage were observed, and the percentages of necrotic and apoptotic chloride cells were not different from control levels (Fig. 1). After 1 h of exposure, the branchial damage of groups NE and E already was substantial, and hardly less than after 24 h. The percentage of necrotic cells was similar, although the percentage of apoptotic cells was 30-40% of that observed after 24 h in these groups. Al deposits were as dense as after 24 h, and enlargement of intercellular spaces and presence of leucocytes in these spaces were hardly less than after 24 h.

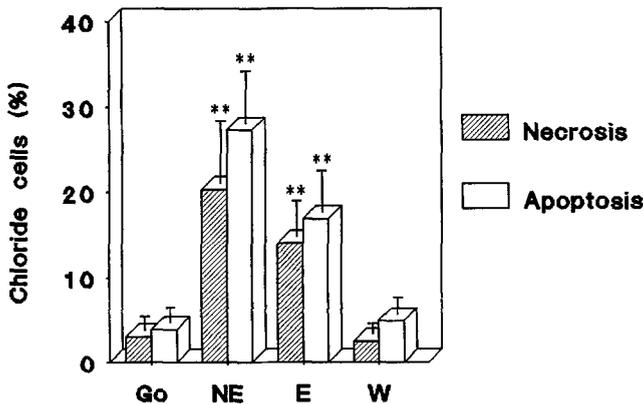


Fig. 1. Percentage of apoptotic and necrotic chloride cells in the gills of trout exposed for 24 h to neutral water (Go), acid water with Al (NE), freshly mixed water (E) and mixed water 340 sec after mixing (W); means \pm SD; $n = 6$. (** $P < 0.01$)

After 24 h recovery following 1 h of exposure to freshly mixed water (E), the structure of the branchial epithelium was substantially improved in fish that recovered in neutral lake Gorja water, and slightly improved in fish that recovered in cage W. Improvement was observed for all ultrastructural parameters mentioned.

4. Discussion

Our results show that freshly mixed acid and neutral waters are toxic to brown trout especially during the first minutes after mixing and this toxic effect can not be explained on the basis of current models on Al toxicity that predict no toxic effect of the metal above pH 6. The acidification was very mild in the mixed water (pH 6.4) compared to that in the acid inlet (pH 5.1). The reduction in plasma Cl⁻ levels is more drastic in fish kept in the acid inlet (cage Ne) than in the first meter of the mixing zone (cage E). However, fish in cages further downstream experienced the same pH as fish in cage E but did not show any reduction in plasma Cl⁻. This observation and the microscopical results show that toxicity decreases rapidly with time after mixing. The EM analysis clearly showed that the structural damage to the gill epithelium (necrosis and apoptosis of chloride cells) and infiltration of leucocytes in fish kept in cage E (beginning of the mixing zone) were comparable to that seen in fish from the acid inlet (Ne).

The artificial channel that was built for this experiment (as in Poleo et al. 1994) simulated a mixing zone as they occur in the field very closely because natural water sources were used. The length of the channel and the flow rate dictated that the time after mixing was maximally 340 sec. In other words, the last cage (cage W) was 340 sec away from the confluence. A really un-natural situation is created, however, by keeping the fish at one spot for 24 h (Table 1, top). When the fish were kept in the toxic mixing zone (cage E) for 1 h and then moved to a more down stream area (cage W, which is far less toxic than cage E), thus mimicking a more natural situation of a relatively short encounter with the toxic mixing zone, there was clearly a reduced capacity to restore the damaged gill tissue and plasma Cl⁻ levels when compared to fish recovering in neutral, unmixed water (GoT).

The mechanism for the toxicity of the mixing zone is unknown at present. A wide spectrum of metals and ions was determined (Fe, Mn, Zn, Ni, Cu, Pb, Ca, Mg, Na, K, Cl, SO₄, NO₃, F) but only Al levels were in the toxic range. In previous articles of our group reporting on the mixing zone phenomenon a causal relationship between the toxicity and the process of Al-polymerization has been suggested (Rosseland et al., 1992; Salbu et al., 1995). The proposed mechanism was a combination of suffocation by Al-precipitates on the gills and loss of plasma Na⁺ and Cl⁻ by the acidification (Poleo et al., 1994). The essential point made by this kind of studies is that liming of water or mixing of acidic Al-rich water with neutral (limed) water leads to a transient toxicity of the water. In this study we found further evidence for toxic effects of these so-called mixing zones that may have negative effects on the survival of migrating trout as indicated by the physiological

disturbances following a relatively short exposure to the mixing zone. When the fish migrate seaward in spring they will repeatedly encounter mixing zones. It is likely that the young trout will reach the ocean in a weakened state when they have to pass several toxic mixing zones in a river system.

Acknowledgements

The study was supported by a grant from the EU (EV5V-CT92-0073). P.M. Verboost was supported by a grant from the Dutch Science Foundation (project NBI 22.2832).

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