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Water pH and aluminum chemistry in the gill micro-environment of rainbow trout during acid and aluminum exposures

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Summary. Rainbow trout (*Salmo gairdneri*) were exposed to acidic soft water (pH_{in} 4.2–6.3) in the presence (93 $\mu g \cdot l^{-1}$) or absence of Al. Fish were fitted with latex masks and opercular catheters to measure ventilation ($\dot{V}_w$), pH changes at the gills, O_2 consumption ($\dot{M}_{O_2}$), ammonia excretion ($\dot{M}_{amm}$), and Al extraction. During 2–3-h exposures, $\dot{V}_w$ was generally higher in Al-exposed fish over the pH_{in} range 4.7–6.3. Alkalinization of expired water was about 0.3 pH units less in Al-exposed fish than in acid-only exposed fish at pH_{in} 4.5–5.2, an effect attributable to both increased $\dot{V}_w$ and to buffering by Al. During 44-h exposures to pH_{in} 5.2 and 4.8 plus Al, $\dot{V}_w$ increased greatly and expired water pH (pH_{ex}) decreased with time. There was a small increase in $\dot{V}_w$ over 44 h at pH 4.4 plus Al, and no changes in pH_{ex}. In contrast, during 44-h exposures to pH 5.2, 4.8, and 4.4 in the absence of Al, such changes were much smaller or absent. During both short- and longer-term exposures, measured Al accumulation on the gills was only 5–18% of that calculated from cumulative Al extraction from the water, suggesting considerable sloughing of Al. In free-swimming trout, gill Al accumulation was greatest during exposure (2 h) to Al at pH 5.2, lower at pH 4.8, and least at pH 4.4 and 4.0. Our results suggest that Al deposition occurs at the gills, causing respiratory and ionoregulatory toxicity, because the pH in the branchial micro-environment is raised above that in the acidic inspired soft water. Higher pH at fish gills may result in Al precipitation due to loss of solubility, or Al accumulation because of shifts in Al species to Al-hydroxide forms which more readily adsorb to the gills.

Key words: Aluminum – pH – Gills – Trout – Water chemistry

Abbreviations: pH_{ex} expired pH; pH_{in} inspired pH

Introduction

Aluminum is a gill toxicant of fish, and is especially important in acidic soft water where Al leached from soil and rock may add to the toxic effects of acidity alone (for reviews of acidic precipitation and Al see Howells et al. 1983; Dillon et al. 1984; Havas and Jaworski 1986; Schindler 1988). Aluminum can enhance the ion losses at fish gills above that caused by acidity (Muniz and Leivestad 1980; Neville 1985; Witters 1986; Booth et al. 1988), and can cause respiratory disturbances that are not seen in acid exposures alone (Rosseland 1980; Neville 1985; Wood et al. 1988; Playle et al. 1989). In general, ionic and respiratory disturbances owing to Al are more pronounced at moderate to higher pH (i.e., pH 4.8–6.5). At these pHs, Al deposition onto gills could cause gill inflammation and damage, leading to ion losses and gas transfer impairment (Wood and McDonald 1987; Wood et al. 1988; Playle et al. 1989).

Inspired soft water of $pH < 6$ is rendered more basic as it passes across the gills of the trout. Conversely, inspired soft water of $pH > 6$ is rendered more acidic as it passes across the gills (Randall and Wright 1989; Playle and Wood, in press). The changes occurring between inspired and expired water are adequately explained by carbon dioxide, base, and ammonia release at the gills, which tend to acidify or alkalinize the water passing over the gills (Playle and Wood, in press). Such changes in water pH may be particularly important for gill contaminants whose toxicities vary with pH: higher or lower pH near the gills may change toxicant solubility or speciation in the branchial micro-environment compared to the bulk, inspired water. Aluminum is of particular interest in this regard because of its prevalence in acidified soft waters, and its pH-dependent solubility and speciation, both of which vary considerably over a fairly nar-

row range of acidic pH (i.e., pH 4.0–6.5). Furthermore, changes in Al speciation (i.e., $Al(OH)_3 + 3\ H^+ \rightleftharpoons Al^{3+} + 3\ H_2O$) can buffer pH.

The first objective in the present study was to determine whether the pH changes in soft water passing over fish gills are altered in the presence of Al. The second goal was to determine whether the pH changes in the branchial micro-environment are large enough to cause loss of Al solubility, or shifts in Al speciation, resulting in Al deposition onto the gills. The Rainbow trout was selected as a model fish species because of its sensitivity both to acidity and to Al (Neville 1985; Playle et al. 1989), and soft water of defined composition was used to approximate natural soft waters most susceptible to acidification. The pHs examined (pH 4.2–6.3) represent very acidic to circumneutral conditions, and the Al concentration used (93 $\mu g \cdot l^{-1}$) is representative of concentrations commonly found in acidic soft water in the field (Dillon et al. 1984). It is concluded that pH changes at trout gills are large enough to affect Al solubility and speciation, and that the resultant deposition of aluminum hydroxides onto the gills can explain the toxic effects of Al.

Materials and methods

Experimental animals and water. Rainbow trout (*Salmo gairdneri*) were purchased from Spring Valley Trout Farm, New Dundee, Ont. Mean weight of the 40 trout used for opercular catheter and ventilation experiments was 282 ± 7 g (± 1 SEM). Fish were held in dechlorinated Hamilton city tapwater, then acclimated for at least two weeks to synthetic soft water before all experiments; details are given in Playle and Wood (in press). Soft water acclimation conditions were $Ca^{2+} \sim 47$ $\mu equiv \cdot l^{-1}$, $Na^+ \sim 68$ $\mu equiv \cdot l^{-1}$, $Cl^- \sim 95$ $\mu equiv \cdot l^{-1}$, titratable alkalinity to pH 4.0 ~ 135 $\mu equiv \cdot l^{-1}$, pH ~ 6.7, at 15° C.

Latex surgical gloves minus thumbs and fingers were sewn around each fish's mouth to serve as ventilation masks (see Cameron and Davis (1970) and Wright et al. (1986) for details), and the fish were fitted with opercular catheters made from Clay-Adams PE-190 polyethylene tubing (Playle and Wood, in press). Fish were then placed in one of 5 ventilation collection boxes of the design described by Cameron and Davis (1970). Well-aerated water flowed from a head tank into the anterior chamber of each fish box (flow > fish ventilatory demand), passed over the gills into the posterior chamber as a result of the fish's ventilation, then overflowed to waste. Opercular catheters were tested to ensure they were siphoning water from a site which provided representative O_2 transfer (i.e., not drawing water from an anatomical dead space; cf. Davis and Watters 1970). If O_2 transfer (the difference between inspired and expired O_2 concentrations) was less than about 20 μM, the catheter was repositioned on the operculum. Recovery time was ~ 48 h after the initial operations and ~ 24 h after catheter repositioning.

Water in the head tank was acidified using 0.5 *M* H_2SO_4, delivered by a magnetic valve controlled by a Radiometer PHM82 pH meter and Radiometer GK2401C combination electrode. The headtank was vigorously aerated to keep P_{O_2} high (~ 150 torr) and P_{CO_2} low (~ 1 torr). Aluminum was added by peristaltic pump as a concentrated solution ($AlCl_3 \cdot 6\ H_2O$ (Sigma); 0.39 $g \cdot l^{-1}$; pH ~ 4.0) to water leaving the head tank. The standard Al concentration used was 93 ± 2 $\mu g \cdot l^{-1}$ (mean ± 1 SEM, $n = 67$); Al concentrations in the absence of added Al were about 5 $\mu g \cdot l^{-1}$. Water flowed directly to the inspired chambers of the fish ventilation boxes, then to waste. This one-pass, flow-through system was used instead of a static system to minimise Al complexation with organic material.

Experimental protocols. In the first set of experiments, five fish at a time were exposed to one of seven acidic pHs between pH 6.3 and 4.2 (no Al) for 2–3 h. Measurements were taken, then the fish were exposed to the same pHs *plus* Al for a further 2–3 h, and measurements taken again. The cycle was then repeated at a different pH. Parameters measured in these experiments were ventilation volume ($\dot{V}_w$), water pH, oxygen tension (P_{O_2}), ammonia concentrations, and Al concentrations, for both inspired and expired samples (see below). In general, fish recovered from acid or acid plus Al exposures quickly, but as a precaution exposures to extreme pHs in the presence of Al (< 4.5, > 6.0) were made at the end of the day, followed by a return to circumneutral pH overnight. In a second set of experiments five fish at a time were exposed to pH 5.2, 4.8, or 4.4 (no Al) for ~ 44 h, or were exposed to pH 5.2, 4.8, or 4.4 for 2–3 h, then Al was added for the remaining 44 h. Measurements of $\dot{V}_w$ and inspired and expired parameters were taken as in the preceding set of experiments. At the end of some of the 44-h exposures, gills were removed for analysis of accumulated Al (see below). Over the course of both sets of experiments, water Ca^{2+} and Na^+ concentrations averaged 54 ± 1 and 63 ± 1 $\mu equiv \cdot l^{-1}$ ($n = 97$), respectively, and experimental temperatures were 15–16 °C. Fluoride concentrations were < 1 $\mu equiv \cdot l^{-1}$, measured by HPLC (Waters 510 pump, 430 conductivity meter, and IC-Pak anion exchange column).

A third set of experiments was run to test hypotheses regarding Al deposition onto gills of free-swimming, relatively unrestrained trout (316 ± 7 g, $n = 31$). Groups of three trout were placed in a single 33-L container, through which flowed acidified soft water, or acidified soft water plus Al, at about 520 $ml \cdot min^{-1}$. Exposure conditions used were: pH 4.8 and 5.2 (no Al), and pH 4.0, 4.4, 4.8, and 5.2 (98 ± 2 $\mu g \cdot l^{-1}$ Al, $n = 16$), 14–15 °C, for 2 h. Inflowing water Ca^{2+} and Na^+ concentrations were 54 ± 1 and 83 ± 2 $\mu equiv \cdot l^{-1}$ ($n = 3$), respectively. Each exposure was run twice, using three fish each time, in random order. At the end of 2 h, fish were quickly netted, killed, and gill portions removed for Al determinations (see below). One fish from the acclimation tank was also sampled for gill Al.

Analytical methods. Alternate expired and inspired water pH readings were taken by siphoning water through a polyethylene vial (water volume ~ 4.5 ml) into which was sealed a Radiometer GK2401C combination pH electrode, connected to a Radiometer PHM82 pH meter (Playle and Wood, in press). The pH electrode was conditioned to the soft water, and was calibrated using BDH pH 4.00 and 7.00 buffers. Ionic strength of the buffers was 0.05 *M*, much greater than that of the soft water ($\sim 10^{-4}$ *M*), but the measured differences in pH between soft water samples and the same samples brought to 0.05 *M* with 5 *M* KCl were only 0.05 to 0.15 pH units (i.e., junction potential effects, Jones et al. 1987). Maximum effect on the differences between expired and inspired pH was only about 0.05 pH units, a negligible amount.

Ventilation volume ($\dot{V}_w$) was the volume of water overflowing the posterior chamber of the ventilation collection boxes in 1 min. Oxygen tension was measured immediately on expired and inspired samples drawn anaerobically from the pH vial,

using a Radiometer E5046 micro-electrode unit (15 °C) and Radiometer PHM72 acid-base analyzer. Water P_{O_2} (torr) was converted to O_2 concentration (μM) using O_2 solubility in water of 0% salinity (Boutilier et al. 1984). Inspired water was always close to O_2 saturation (~ 300 μM). Oxygen consumption ($\dot{M}_{O_2}$) was calculated as (inspired $[O_2]$ minus expired $[O_2]$)$\cdot \dot{V}_w$. Ammonia and Al samples were collected by filling 7.5-ml polyethylene vials from the inspired and expired siphons. Samples for ammonia were frozen and later analysed using the salicylate-hypochlorite method (Verdouw et al. 1978). Ammonia excretion ($\dot{M}_{amm}$) was calculated as (expired [ammonia] – inspired [ammonia])$\cdot \dot{V}_w$. Total aqueous Al concentrations were determined using the pyrocathechol violet method (Dougan and Wilson 1974). In this study the difference between expired and inspired values are referred to as "transfer" or "Δ", e.g., ammonia transfer, ΔpH.

In some of the 44-h exposures, surviving fish were killed with a blow to the head, and a section of the third right gill arch was removed for Al determinations. Each gill portion was placed for 1 min in 7 ml deionised water (in an attempt to remove excess, loosely-bound Al), then frozen. Filaments were later cut from the frozen gill portions, weighed, and then digested in five times their weight of 0.05 M reagent-grade H_2SO_4 for 8 h at 80 °C. Samples were vortexed, centrifuged for 1 min, and the supernatant diluted 100× before analysis on a Varian AA-1275 atomic absorption spectrophotometer with GTA-95 graphite tube atomizer. Standard additions were used to show there was no interference of Al measurements by the dilute solutions of gill digest. 10-µl samples were introduced into the graphite tube with 20 µl deionized water or Al standard; water was evaporated at ~100 °C for 50 s, a total of 12 s was spent at 1200° C, and Al was volatalized at 2500 °C for a total of 2.7 s.

For the free-swimming fish exposed to acidic conditions, in the presence or absence of Al, fish were killed after 2 h. A section of the third right gill arch was removed, as above, except that the gill portions were held by forceps and gently agitated for 20 s in each of three, 250-ml deionised water rinses, in an attempt to remove *all* interlamellar Al. The rest of the Al analysis was identical, except that 20 µl sample and 10 µl deionised water or Al standard were injected into the graphite tube, because the diluted gill digests had much lower Al concentrations than those measured in the 44-h exposures (see Results).

Experimental data are usually presented as means ±1 standard error (n). Statistical comparisons were done using Student's two-tailed t-test, paired or unpaired design, as appropriate. For the 44-h exposures, paired t-tests were used to compare experimental values (crosses) with initial values obtained at circumneutral pH. To assess the additional effects of Al, relative to those of acidity alone, paired t-tests were used to compare experimental values (asterisks) with those at 2–3 h in acid alone, after which the Al exposures began (see Experimental protocols). Analysis of variance followed by Duncan's Multiple Range test was used to compare gill Al accumulations. Unless stated otherwise, the level of significance was $P<0.05$. In comparisons using the theoretical solubility of Al at the gills, 95% confidence limits were used, to give more information regarding variability in the estimates.

Results

Short-term exposures to acidity and Al

Rainbow trout fitted with opercular catheters and ventilation masks were exposed to acidic soft water (seven different pHs between 4.2 and 6.3) for 2–3 h in the presence or absence of Al. The goal was to determine short-term effects of acidity and Al on water pH near the gills, on ventilation volume, and on O_2 consumption and ammonia excretion. Soft water was more basic after it passed the gills if the inspired water pH (pH_{in}) was <6, and was more acidic if pH_{in} was >6 (Fig. 1A). Trout exposed to 93 µg·l^{-1} Al for 2–3 h also alkalinized the water if pH_{in} was <6, but the rise in pH at the gills was not as great in the pH_{in} range 4.5–5.2 as it was in the absence of Al (Fig. 1A). Ventilation volume ($\dot{V}_w$) of trout exposed to acidity alone was about 0.33 l·kg^{-1}·min^{-1} if inspired water pH was between pH 4.7 and pH 6.6 (Fig. 1B); $\dot{V}_w$ increased to about 0.5 l·kg^{-1}·min^{-1} at pH_{in} <4.5. In trout exposed to Al, $\dot{V}_w$ was generally higher than $\dot{V}_w$ of trout exposed to acidic pH alone if the inspired water pH was >4.5 (Fig. 1B). Fish exposed to Al had especially high $\dot{V}_w$ at $pH_{in}=6.3$.

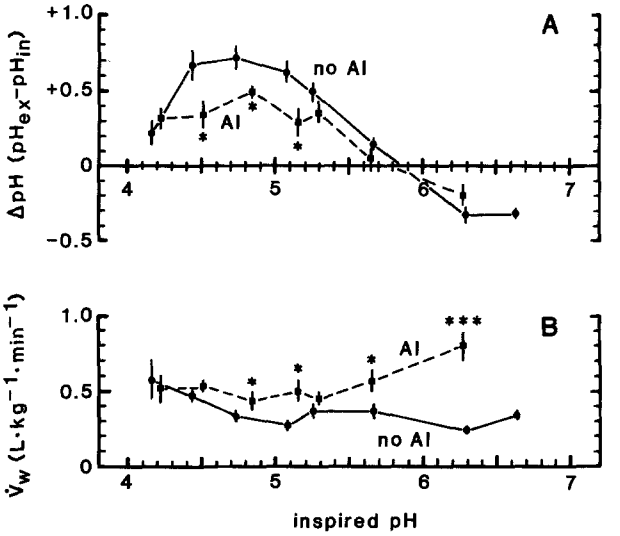

Fig. 1. A The difference between pH of expired water (pH_{ex}) and inspired water (pH_{in}) plotted against inspired soft water pH for rainbow trout fitted with opercular catheters and latex masks. Fish were exposed for 2–3 h to acidity in the absence (n=29) or presence of Al (93 µg·l^{-1} Al; n=22). Positive ΔpH: expired water is more basic than inspired water; negative ΔpH: expired water is more acidic. ΔpH was lower for the Al-exposed fish in the pH_{in} range 4.5–5.2. Means ±1 SEM are indicated; significant differences between ΔpH of Al-exposed fish and ΔpH of fish exposed to acidity alone are indicated by * ($P<0.05$, unpaired t-test). For the acid only exposures, the mean number of fish represented at each point is 13, minimum number is 6 ($pH_{in}=4.2$). For the fish exposed to Al, mean number of fish represented at each point is 7; minimum number is 3 ($pH_{in}=6.3$), and the maximum number is 10 ($pH_{in}=4.5$, 4.8). **B** Ventilation volume ($\dot{V}_w$) of rainbow trout fitted with opercular catheters and latex masks after 2–3 h exposures to acidity or acidity plus Al, in soft water. Above $pH_{in}=4.8$ the $\dot{V}_w$ of Al-exposed fish was usually higher than in fish exposed to acidity alone (unpaired t-test; *$=P<0.05$; ***$=P<0.001$). Other details as given in Fig. 1A

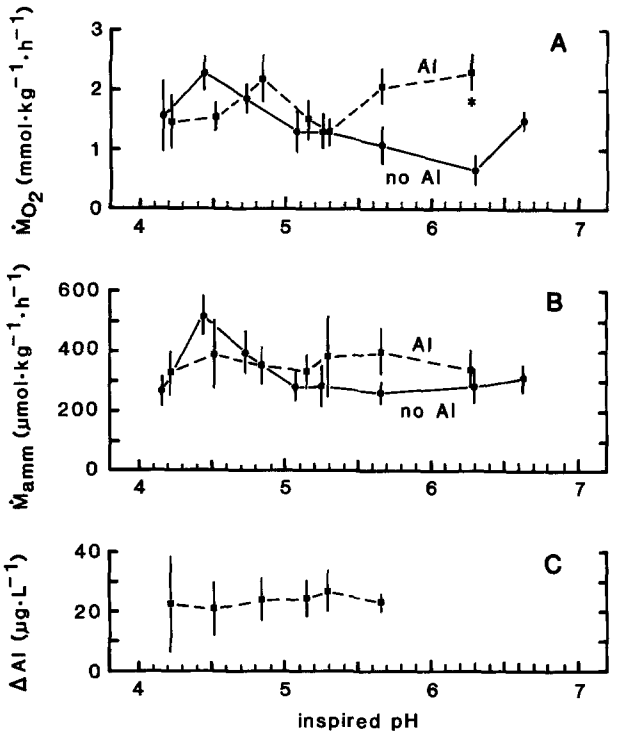

Fig. 2. **A** Oxygen consumption ($\dot{M}_{O_2}$) of rainbow trout fitted with opercular catheters and latex masks, after 2–3 h exposures to acidity or acidity plus 93 $\mu g \cdot l^{-1}$ Al. Generally there were no significant differences in $\dot{M}_{O_2}$ between Al-exposed fish and fish exposed to acidity alone (unpaired *t*-test). Numbers of fish as given in Fig. 1A, except for $pH_{in} = 6.3$ (acidity alone), where $n = 3$. **B** Ammonia excretion ($\dot{M}_{amm}$) of rainbow trout fitted with opercular catheters and latex masks, after 2–3 h exposures to acidity or acidity plus Al. There were no significant differences in $\dot{M}_{amm}$ between fish exposed to acidity alone and Al-exposed fish (unpaired *t*-test). Numbers of fish as given in Fig. 1A. **C** The differences between inspired and expired Al concentrations (ΔAl) for some of the fish exposed to 93 $\mu g \cdot l^{-1}$ Al for 2–3 h. A technical problem with one set of Al analyses precluded the use of those data. For this figure, from left to right, $n = 3$, 7, 8, 4, 7, and 3

Oxygen consumption ($\dot{M}_{O_2}$) in fish exposed to acidity alone for 2–3 h was variable, but averaged about 1.6 $mmol \cdot kg^{-1} \cdot h^{-1}$ (Fig. 2A). In spite of often higher $\dot{V}_w$, mean $\dot{M}_{O_2}$ values in Al-exposed fish were not generally different than in fish exposed to acidity alone (Fig. 2A). Only at $pH_{in} =$ 6.3, the point of maximum elevation in $\dot{V}_w$ in the presence of Al (Fig. 1B), was $\dot{M}_{O_2}$ significantly elevated. Ammonia excretion ($\dot{M}_{amm}$) was also variable, but averaged about 350 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ for both treatments (Fig. 2B). For the Al-exposed trout, Al removed from water passing over the gills was approximately constant ($\Delta Al = 20$–30 $\mu g \cdot l^{-1}$) between $pH_{in} = 4.2$ and 5.7 (Fig. 2C).

Longer-term exposures to acidity and Al

The effects of prolonged (44-h) exposures to acidity in the presence or absence of Al were also assessed using rainbow trout fitted with opercular catheters and latex dams. Mildly acidic conditions alone ($pH_{in} = 5.2$) had no effect on $\dot{V}_w$ (Fig. 3A). For $pH_{in} = 4.8$, $\dot{V}_w$ increased gradually, and by the end of 44 h was significantly higher than at 2–3 h exposure. For the $pH_{in} = 4.4$ treatment, $\dot{V}_w$ increased quickly over the first few hours, stabilising at about twice the initial value. Expired pH was stable (after the acid additions started) throughout the 44-h exposures to $pH_{in} = 5.2$ and 4.4, but decreased significantly (by 20 h) in the $pH_{in} = 4.8$ treatment (Fig. 3B). Expired pH was *always* significantly lower after acid additions started than before the acid additions (usually $P < 0.01$, paired *t*-tests), but because pH_{ex} is dependent on pH_{in} (i.e., Fig. 1A) this is not surprising. It is the change in the new pH_{ex} with time (asterisks on Fig. 3B) which is of interest.

Oxygen consumption ($\dot{M}_{O_2}$) stayed more or less constant throughout 44-h exposures to $pH_{in} = 5.2$ and 4.8, but doubled by 10 h in the $pH_{in} = 4.4$ exposure (Fig. 4A). This pattern follows that of $\dot{V}_w$ (Fig. 3A). Ammonia excretion ($\dot{M}_{amm}$) was also fairly constant throughout 44-h exposures to $pH_{in} = 5.2$ and 4.8 (Fig. 4B), actually decreasing slightly in the latter treatment for unknown reasons. As with $\dot{V}_w$ and $\dot{M}_{O_2}$, $\dot{M}_{amm}$ increased significantly after 2–3 h at $pH_{in} = 4.4$. In general, acidity alone had little effect over 44 h on these four parameters at $pH_{in} = 5.2$, mild effects on $\dot{V}_w$ and pH_{ex} at $pH_{in} = 4.8$, and largest effects on $\dot{V}_w$, $\dot{M}_{O_2}$, and $\dot{M}_{amm}$ at $pH_{in} = 4.4$.

In contrast, mild acidity ($pH_{in} = 5.2$) in the presence of 93 $\mu g \cdot l^{-1}$ Al caused large increases in fish ventilation (almost quadrupling by 20 h, Fig. 3C), and 4 of 5 fish died by 40 h (Fig. 3D, crosses in circles). For $pH_{in} = 4.8$ plus Al, $\dot{V}_w$ increased to the same extent, but only 1 fish died. As before, $\dot{V}_w$ increased at $pH_{in} = 4.4$ due to acidity alone (crosses, Fig. 3C), but there was an additive effect of Al on $\dot{V}_w$ (asterisks), significant after 20 h exposure to Al. However, this hyperventilation was less than half that seen at the two higher pHs in the presence of Al, and there were no fish deaths. In accord with increased $\dot{V}_w$, expired pH decreased slightly during exposure to $pH_{in} = 5.2$ plus Al, and decreased substantially during the $pH_{in} = 4.8$ plus Al treatment (Fig. 3D). There had been no decrease in pH_{ex} in the acid-only exposure to $pH_{in} =$ 5.2, and a smaller decrease in pH_{ex} in the acid-only exposure to $pH_{in} = 4.8$ (Fig. 3B). In the $pH_{in} = 4.4$ plus Al exposure there were no significant changes in pH_{ex} over time, similar behaviour to the $pH_{in} =$ 4.4 treatment without Al.

Despite the large increases in $\dot{V}_w$ seen in Al-exposed fish at pH_{in} 5.2 and 4.8 (Fig. 3C), there were only small increases in $\dot{M}_{O_2}$ (<2-fold), signifi-

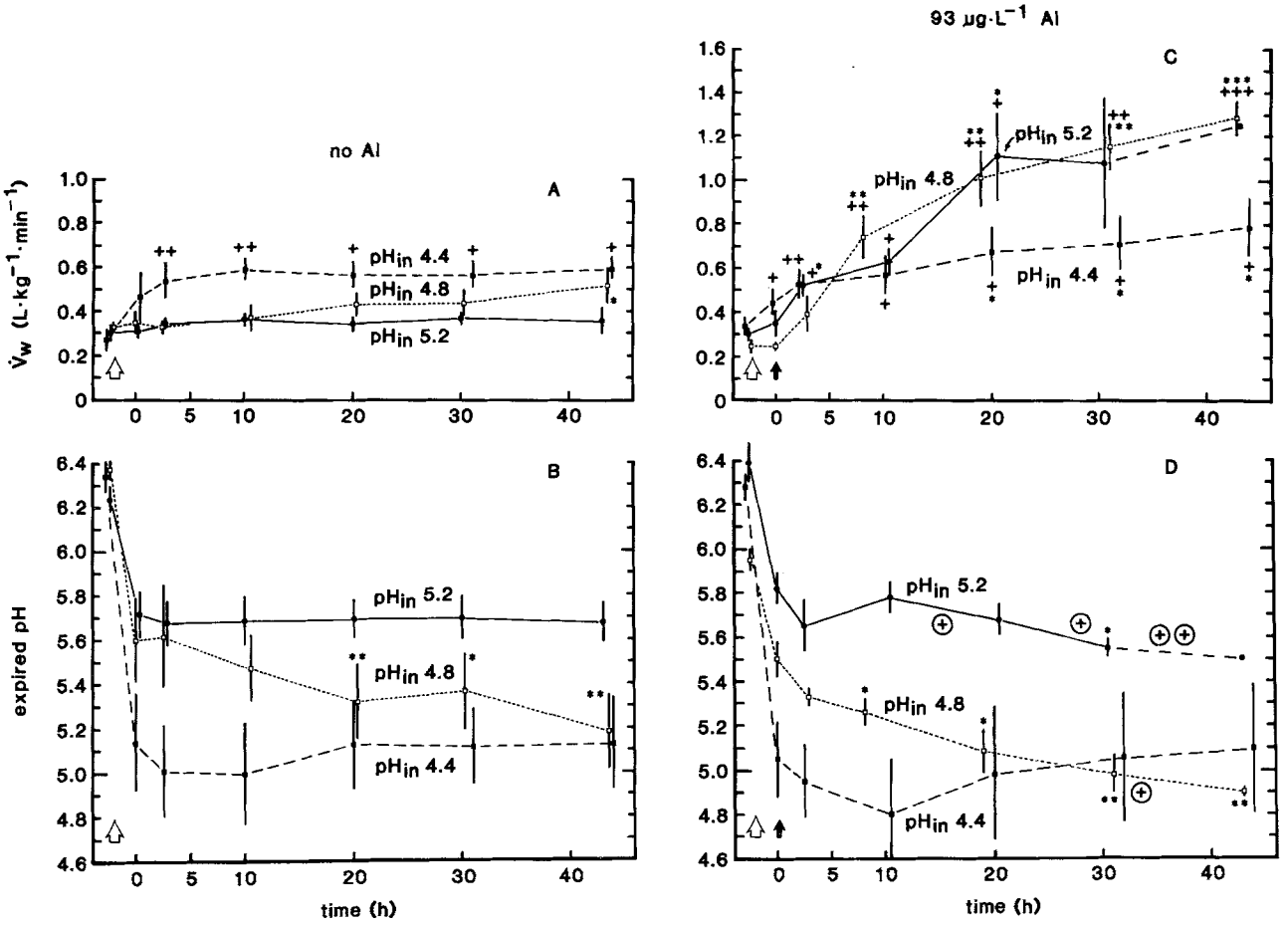

Fig. 3. **A** Ventilation volumes ($\dot{V}_w$) of rainbow trout exposed to pH_{in} 5.2, 4.8, and 4.4 soft water for 44 h. White arrow represents start of acid exposures. Five trout per treatment. Trout exposed to $pH_{in}=4.4$ showed most increase in $\dot{V}_w$. Crosses: significant difference (paired *t*-test) when compared to initial value, before acid addition started ($+=P<0.05$, $++=P<0.01$, $+++=P<0.001$). Asterisks: significant difference (paired *t*-test) when compared to value after 2–3 h exposure to acidity ($*=P<0.05$, $**=P<0.01$, $***=P<0.001$). **B** Expired pH of the same 15 fish presented in Fig. 3A. Mean $pH_{in}=6.62\pm0.01$ (15) before start of acid exposures (white arrow). Trout exposed to $pH_{in}=4.8$ showed significant decreases in expired pH, related to their slow increase in $\dot{V}_w$, from about 20 h. Expired pH was *always* lower after acid additions started than before the additions (usually $P<0.01$); crosses have been left off all points (Fig. 3B, D) for clarity. **C** $\dot{V}_w$ of rainbow trout exposed to pH_{in} 5.2, 4.8, and 4.4 soft water, in the presence of Al, for 44 h. White arrow indicates start of acid exposures; black arrow indicates start of Al addition (time 0). Five trout per treatment. Increases in $\dot{V}_w$ in response to Al were greatest at $pH_{in}=5.2$ and 4.8. **D** Expired pH of the same 15 fish presented in Fig. 3C. In accord with the large increases in $\dot{V}_w$ in fish exposed to $pH_{in}=5.2$ and 4.8 in the presence of Al, expired pH of these fish decreased significantly by about 30 h and 8 h, respectively. The smaller increases in $\dot{V}_w$ in the $pH_{in}=4.4$ plus Al treatment did not result in significant depression of expired pH. Mean $pH_{in}=6.50\pm0.03$ (15) before start of acid exposures (white arrow). Crosses in circles indicate fish deaths; four of five fish died in the $pH_{in}=5.2$ plus Al treatment starting by 16 h, and one fish died at 32 h in the $pH_{in}=4.8$ plus Al treatment

cant at only a few exposure times in the various groups (Fig. 4C). The Al-induced hyperventilation did not result in a proportional increase in $\dot{M}_{O_2}$ because ΔO_2 (i.e., O_2 extraction efficiency) dropped. Ammonia excretion was approximately constant during the $pH_{in}=5.2$ and 4.8 plus Al exposures (Fig. 4D). An increase in $\dot{M}_{amm}$ was seen after 2–3 h exposure to $pH_{in}=4.4$ alone, with no significant increase in $\dot{M}_{amm}$ after Al addition started.

In summary, the effects of prolonged exposure (44 h) to 93 $\mu g \cdot l^{-1}$ Al, over and above those due to acidity alone, were progressive hyperventilation which was most marked at pH_{in} 5.2 and 4.8, and some mortality. Accompanying the increased ventilation were decreases in ΔpH across the gills, gradually decreasing pH_{ex} closer to pH_{in}. There were only small increases in $\dot{M}_{O_2}$ and $\dot{M}_{amm}$, because ΔO_2 and Δamm decreased during the Al-induced hyperventilation.

Deposition of Al onto the gills

Aluminum removed from solution by rainbow trout gills during the three 44-h Al treatments is given in Table 1. Usually ΔAl was between 5 and 20 $\mu g \cdot l^{-1}$, slightly lower than the ΔAl values

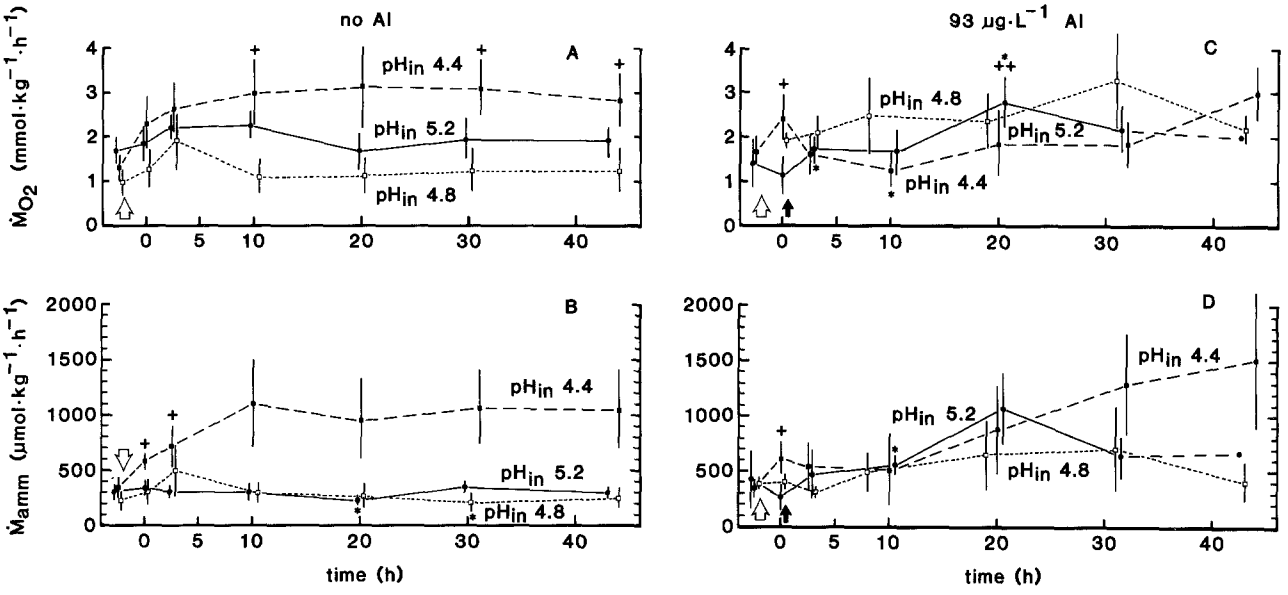

Fig. 4. **A** Oxygen consumption ($\dot{M}_{O_2}$) of rainbow trout exposed to pH_{in} 5.2, 4.8, and 4.4 soft water for 44 h. Same 15 fish as presented in Fig. 3A, B. White arrow = start of acid exposures. $\dot{M}_{O_2}$ increased only during the pH_{in} = 4.4 treatment, in accord with increased $\dot{V}_w$ (Fig. 3A). See Fig. 3A for details of statistics used. **B** Ammonia excretion ($\dot{M}_{amm}$) of the same 15 fish presented in Fig. 4A. $\dot{M}_{amm}$ increased upon exposure to pH_{in} = 4.4. $\dot{M}_{amm}$ for pH_{in} = 5.2 and 4.8 treatments stayed steady or even decreased during the exposures. **C** $\dot{M}_{O_2}$ of rainbow trout exposed to pH_{in} 5.2, 4.8, and 4.4 soft water, in the presence of Al, over 44 h. Same 15 fish presented in Fig. 3C, D. White arrow represents start of acid addition; black arrow, start of Al addition (Time 0). There were only small increases in $\dot{M}_{O_2}$ in the pH_{in} 5.2 and 4.8 plus Al treatments, in spite of the large increases in $\dot{V}_w$. There were no increases in $\dot{M}_{O_2}$ in the pH_{in} = 4.4 plus Al treatment, whereas increased $\dot{M}_{O_2}$ was seen for the pH_{in} = 4.4, acid only treatment (Fig. 4A). There are no $\dot{M}_{O_2}$ values for the pH_{in} = 4.8 treatment before acid addition, because of a faulty O_2 electrode at that time. **D** $\dot{M}_{amm}$ for Al-exposed fish. The only significant increases in $\dot{M}_{amm}$ were after 2–3 h exposure to acidity alone (pH_{in} = 4.4), related to increased $\dot{V}_w$, and at 10 h for pH_{in} = 5.2 plus Al

(~20 μg·l^{-1}) measured during the 2–3 h exposures (Fig. 2C). Occasional negative values seen at later times during some exposures were presumably due to release of Al from the gills, perhaps bound by mucus, which was drawn down the opercular catheters. A rough estimate of Al deposition at the gills was made by multiplying the ΔAl values by the volume of water passed over the gills (l·min^{-1}, i.e., $\dot{V}_w$·fish weight), and this value by the time elapsed from the previous sample. Average cumulative deposition of Al onto one set of gills (i.e., total divided by 2) of a fish exposed to 93 μg·l^{-1} Al for 44 h at pH_{in} = 5.2, 4.8, and 4.4, was estimated to be about 2.0, 1.6, and 4.2 mg, respectively.

These cumulative estimates were compared with Al accumulation directly measured on the gills at the end of the 44-h exposures to 93 μg·l^{-1} Al at pH 4.4 and 4.8 (Table 1). Background filament Al concentration (from the pH 4.4, no Al

Table 1. Gill Al concentrations and Al extraction (ΔAl; inspired [Al] minus expired [Al]) at gills of rainbow trout exposed for 44 h to 93 μg·l^{-1} Al in soft water at pH 5.2, 4.8, and 4.4. Means ±1 SEM (n). Cumulative Al deposition after 44 h onto one set of gills was estimated from ΔAl, $\dot{V}_w$, and fish weight (see text for details). Actual Al accumulations per set of gills after 44 h were calculated directly from measured gill Al concentrations

Exposure pH	Measured gill Al concentration (μg·g^{-1} wet tissue)	ΔAl (μg·l^{-1})					Estimated cumulative Al deposition (mg)	Measured gill Al deposition (mg)
		2–3 h	10 h	20 h	31 h	44 h		
5.2	–	16±6 (5)	17±7 (5)	12±7 (4)	12± 6 (3)	−9 (1)	2.0	–
4.8	97±10 (3)	14±1 (5)	6±4 (5)	6±5 (5)	−2±11 (4)	9±9 (4)	1.6	0.29
4.4	73± 9 (5)	11±9 (5)	11±4 (5)	21±7 (5)	15±10 (5)	17±9 (5)	4.2	0.22

exposure and from fish from the acclimation tank) was 1.8 ± 0.3 μg Al·g^{-1} (wet tissue; n = 9). Gill filaments from the pH 4.4 and 4.8 plus Al treatments had significantly higher Al concentrations (73, 97 μg·g^{-1} respectively) compared to filaments not exposed to Al ($P < 0.01$), and were significantly different from one another ($P < 0.05$). Total gill filament weight from our fish, for one set of gills, was about 3 g, so total Al accumulation for the pH 4.4 and 4.8 Al exposures was about 0.22 and 0.29 mg, respectively, for one set of gills (Table 1). Measured Al accumulation over 44 h was only 5–18% of Al accumulation estimated from Al extraction across the gills. This suggests that the majority of Al removed from the water was not retained on the gills, but was sloughed off (see Discussion). In addition, some of the Al may have been only loosely bound to the gills and therefore removed during the 1-min gill rinse.

The solubility of Al in water is minimal near pH 6, and increases exponentially in more acidic or more basic conditions (Roberson and Hem 1969). In acidic water saturated with Al, any increase in pH near the gills would theoretically cause Al to come out of solution and precipitate onto the gills. This scenario is illustrated in Fig. 5, where the observed ΔpHs at the gills of fish exposed to Al for 2–3 h (from Fig. 1 A) were used to estimate Al solubility in the gill micro-environment. If the concentration of Al in the bulk water is near saturation, Al will precipitate onto the gills when the inspired water pH is below about pH 5.7, because of the more basic conditions near the gills. According to the bulk water solubility curve, 93 μg·l^{-1} Al should start to precipitate from water at pH ~5.3, the pH where 93 μg·l^{-1} Al intersects the bulk water solubility curve. However, because of the more basic conditions at the gills relative to inspired water, 93 μg·l^{-1} Al will begin to precipitate onto gill epithelia when the inspired soft water is just pH 4.8 or greater (Fig. 5). It should be noted that the Al solubility curve for microcrystalline gibbsite (Roberson and Hem 1969) was used in our calculations: any Al solubility curve will give the same trends, but perhaps with different absolute solubility values.

The preceding analysis indicated that aluminum solubility was lower at trout gills than in the bulk inspired water because of the changes in water pH occurring at the gills. Highest Al solubility would occur at lowest inspired pH, and lowest solubility would occur near $pH_{in} = 5.2$–6.0 (Fig. 5). If Al precipitation resulting from these pH changes were the cause of Al deposition on the gills, one could predict maximum deposition of Al at highest

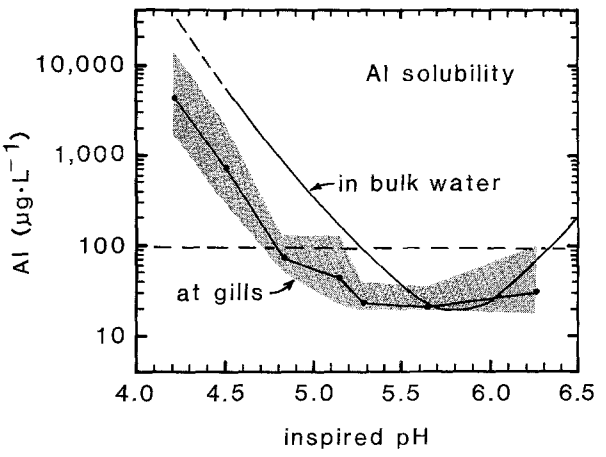

Fig. 5. Aluminum solubility in bulk water and Al solubility predicted at rainbow trout gills, using ΔpH from 22 trout in soft water exposed for 2–3 h to 93 μg·l^{-1} Al (Fig. 1 A). From these curves, Al is predicted to precipitate onto the gills from near saturated solutions when the inspired soft water is below about pH 5.7 or above pH 6.0, because the gill micro-environment is more basic or acidic, respectively, than is the inspired water. 95% confidence limits are indicated about the mean Al solubility near the gills. The dotted horizontal line represents 93 μg·l^{-1} Al. Solubility curve used is for microcrystalline gibbsite (from Roberson and Hem 1969, in Burrows 1977). Note that the Al solubility scale is logarithmic. See text for more details

pH (5.2), and lowest deposition (theoretically none) at lowest pH (<4.4). In order to test these predictions a separate experiment was performed in which free-swimming trout were exposed for 2 h to various acidities (pH 4.0, 4.4, 4.8, and 5.2) plus 98 μg·l^{-1} Al. Short-term exposures were used because the previous measurements of long-term gill Al accumulation, in comparison with Al extraction from the water (Table 1), indicated that most of the Al deposited would be removed over the longer term, confounding interpretation.

In accord with predictions, gill Al accumulations were indeed highest at the pH 5.2 exposure, intermediate at pH 4.8, and lowest at pH 4.4 and 4.0 (Fig. 6). There was significant Al accumulation in the pH 4.4 and 4.0 plus Al exposures relative to background levels (pH 4.4 and 4.0 data grouped and compared to background; unpaired t-test, $P < 0.01$), whereas the above theory would predict no Al deposition at all under these conditions. Measured Al accumulations over 2 h (after rinsing away loosely-bound interlamellar Al) were again only a small fraction (1–12%) of those predicted from the earlier measurements of $\dot{V}_w$ and ΔAl in the short-term exposures (Fig. 1 B, Fig. 2 C, Table 1), indicating that even during relatively short exposures most of the deposited Al was removed from the gills.

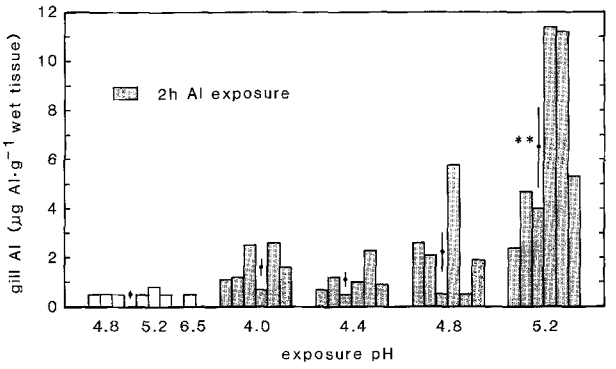

Fig. 6. Aluminum concentrations on gills of rainbow trout held for 2 h at the indicated pHs, in the absence (clear bars) or presence (98 $\mu g \cdot l^{-1}$; grey bars) of Al. The pH 5.2, Al-exposed gills had significantly higher Al concentrations than did gills from all other treatments (** = $P < 0.01$). Means ±1 SEM for each exposure group are also indicated

Discussion

By means of opercular catheters and latex ventilation masks, it is demonstrated that the pH of expired water of rainbow trout is more basic than inspired soft water of pH 4–6, and is more acidic than inspired water of pH > 6 (Fig. 1 A). While the latter trend was studied only up to pH 6.6 in this study, it is known to continue through pH 10 (Playle and Wood, in press). These observations are adequately explained by measured base (including ammonia) and CO_2 transfers at the gills, which tend to alkalinize and acidify the gill micro-environment, respectively, depending on water pH (Randall and Wright 1989; Playle and Wood, in press). Below about pH 5 the acidifying effect of CO_2 released at the gills is negligible; it is solely the alkalinization due to ammonia and base releases at the gills which is important at these acidities. It should be noted that measurements of expired pH probably do not represent conditions throughout the gill micro-environment, because of anatomical deadspace and heterogeneity along the exchange pathway. Water pH is likely to be close to inspired pH at the leading edges of gill lamellae, and change progressively to a value more extreme than measured expired pH at the trailing edges of the lamellae, as CO_2 and base are released into the water (D.J. Randall, pers. comm.). Expired pH may well approximate the overall mean pH near the gill surface.

In the pH_{in} range 4.5–5.2 in the presence of 93 $\mu g \cdot l^{-1}$ Al, the observed ΔpHs near the gills were lower than observed in the absence of Al (Fig. 1 A). Lower ΔpH of the Al-exposed trout can be partly explained by their higher ventilation at pH_{in} = 4.8–6.3 (Fig. 1 B), because approximately the same amount of base, ammonia (Fig. 2 B), and CO_2 were released at the gills, but into a larger volume (higher flow) of water (Playle and Wood, in press). In addition, chemical species of Al change with pH, from mostly Al^{3+} at pH < 4.5 to mostly $Al(OH)_3$ near pH 6.0 (Dyrssen 1984), which buffers pH by consuming OH^- ions. 93 $\mu g \cdot l^{-1}$ Al^{3+} represents about 10.5 $\mu equiv \cdot l^{-1}$ of consumed base if the Al^{3+} is converted completely to $Al(OH)_3$; over the observed ΔpHs, actual base consumed was only about 3 $\mu equiv \cdot l^{-1}$. In our soft water, 3 $\mu equiv \cdot l^{-1}$ represents a ΔpH of about 0.05, 0.1, and 0.2 pH units near pH_{in} = 4.5, 4.8, and 5.2, respectively (estimated using titration curves in Playle and Wood, in press). Thus the buffering action of Al could explain 15–60% of the measured difference in ΔpH between fish exposed to Al and fish not exposed to Al, the greatest contribution occurring at higher inspired pH.

In longer-term exposures of rainbow trout to acidity alone in soft water, there were few significant changes in $\dot{V}_w$, expired pH, $\dot{M}_{O_2}$, and $\dot{M}_{amm}$, and no mortality (Fig. 3 A, B; 4 A, B). Largest effects on $\dot{V}_w$, $\dot{M}_{O_2}$, and $\dot{M}_{amm}$ were seen at pH_{in} = 4.4. In contrast, in the longer-term exposures to pH_{in} = 5.2 and 4.8 plus Al, there were large increases in $\dot{V}_w$ with time, significant decreases in expired pH (in accord with increased $\dot{V}_w$), and fish deaths (Fig. 3 C, D); Al had little added effect in the pH_{in} = 4.4 treatment. Ventilation increased because the fish undoubtedly developed very low arterial oxygen tensions, as did rainbow trout fitted with arterial catheters in our earlier study at comparable pHs and Al concentrations (Playle et al. 1989). The continuous increase in $\dot{V}_w$ over time at pH_{in} = 4.8 contrasts with the results of Walker et al. (1988) where brook trout exposed to 330 $\mu g \cdot l^{-1}$ Al at pH_{in} = 4.8 showed an initial increase in $\dot{V}_w$, but then a decrease in $\dot{V}_w$ to pre-exposure levels within 6 h. Walker et al. (1988) attributed the latter decrease in $\dot{V}_w$ to mucus clogging of the gills, or fatigue of the ventilatory mechanism, which presumably did not occur in the present study with lower Al concentrations. The large increases in ventilation resulted in only small increases in $\dot{M}_{O_2}$ in the present study (Fig. 4 C), probably because of Al-induced increases in the branchial diffusion barrier, or greater shunting of water past the gills. The small toxic effects of Al over 44 h at pH_{in} = 4.4, compared with pH_{in} = 4.8, and especially pH_{in} = 5.2, where four out of five fish died, corresponds exactly with the results of our previous cannulation study on Al-exposed rainbow trout (Playle et al. 1989).

The simplest explanation for low toxicity of Al

in very acidic water (pH < 4.5) and high Al toxicity in less acidic water (pH 5.0–6.0) is that toxicity is caused by Al precipitation onto the gills. In the presence of 93 $\mu g \cdot l^{-1}$ Al, the rainbow trout gill micro-environment is up to 0.5 pH units more basic than is the inspired water if the pH of inspired soft water is less than 6 (Fig. 1A). The increase in pH near the gills may cause Al to precipitate onto the gills, because the solubility of Al in the more basic water near the gills is less than in the more acidic inspired water (Fig. 5). Precipitation of Al from an Al solution of 93 $\mu g \cdot l^{-1}$ onto the gills was predicted from Fig. 5 to be minimal in very acidic conditions, to begin at $pH_{in} = 4.8$, and to be highest at $pH_{in} = 5.2$. These predictions were confirmed in a simple 2-h exposure of trout to 98 $\mu g \cdot l^{-1}$ Al (Fig. 6). Over longer exposures, expired pH decreased enough (as $\dot{V}_w$ increased) to alleviate Al precipitation at the gills in only the $pH_{in} = 4.8$ plus Al exposure: a decrease in expired pH from 5.3 to 4.9 (Fig. 3D) theoretically increased the amount of Al which could be held in solution, from about 70 to about 500 $\mu g \cdot l^{-1}$ (from Fig. 5). Otherwise, calculations of Al solubility after 2–3 h exposures were reasonable approximations of longer-term conditions at the gills. Once precipitated onto the gill epithelia, Al likely causes irritation, inflammation, oedema, cell deformation, and excess mucus production, all of which have been described in morphological studies (Karlsson-Norrgren et al. 1986a, b; Youson and Neville 1987; Tietge et al. 1988). In turn, gill damage would result in ionoregulatory and respiratory disturbances (Neville 1985; Wood et al. 1988; Playle et al. 1989), which ultimately kill the fish.

Differences in Al solubilities between inspired water and the water near fish gills (Fig. 5) help explain previous results on the toxic effects of Al. There is general agreement, in a variety of fish species, that Al is most toxic at $pH_{in} = 5.0$–6.0 (Driscoll et al. 1980; Muniz and Leivestad 1980; Baker and Schofield 1982; Neville 1985; Karlsson-Norrgren et al. 1986b; Kane and Rabeni 1987; Ormerod et al. 1987; Sadler and Lynam 1987; Reader et al. 1988; Wood et al. 1988; Playle et al. 1989). In this pH_{in} range, Al solubility in the inspired water is already low and would be lower still at the gills if $pH_{in} < 5.7$ (Fig. 5). At $pH_{in} > 6$, Al may still theoretically precipitate onto fish gills even though Al solubility in the bulk water increases above pH 6: the more acidic conditions near the gills (Fig. 1A) would now keep Al solubility low. This precipitation of Al likely explains the very high ventilation of trout exposed to Al at pH_{in} 6.3 (Fig. 1B).

Until now the toxic effects of Al at fish gills have been interpreted as a precipitation phenomenon. Although this interpretation generally explains the currently available data, it may well be an oversimplification. For example, explanations are needed as to why Al is accumulated on gills of fish exposed to Al for 2 h at $pH_{in} = 4.0$ and 4.4 (Fig. 6), or 44 h at $pH_{in} = 4.4$ (Table 1): in these instances, measured pH near the gills was so low that no Al should have precipitated from solution (Fig. 5). Three plausible explanations are (i) alkalinization near the gills has been underestimated, thereby underestimating Al precipitation, (ii) changes in Al speciation are important with regard to Al deposition onto fish gills, and, related to this possibility, (iii) gill mucus and Al interactions are likely important in gill Al accumulations.

In favour of the first explanation, it is probable that the opercular catheter method underestimates pH changes towards the trailing edges of the lamellae, as outlined earlier. The alkalinization of acidic inspired water, and the resultant Al precipitation, would be higher at the trailing edges than indicated by measured expired pH. It would be interesting to localise Al deposition along the lamellar surface to see if Al is concentrated on these downstream edges.

To address the second possibility, theoretical speciation of Al in inspired and expired water has been calculated (Fig. 7) on the basis of the measured ΔpHs of Al-exposed fish (Fig. 1A), and the Al speciation scheme of Dyrrsen (1984). Note that, as with Al solubility curves, there is no consensus regarding Al speciation schemes (discussed by Sadler and Lynam 1987), but most schemes will give similar trends. Complexation of Al by fluoride and organic material was ignored in these calculations, in view of the low fluoride concentrations in our soft water (< 1 μM) and the use of a one-pass, flow-through system. In the absence of data regarding rates of Al speciation and precipitation, we assumed that these reactions can occur during water transit time at the gills; clearly it would be useful to determine these reaction rates.

Four important conclusions arise from Fig. 7. First, below $pH_{in} \sim 5.2$, changes in Al solubility are much larger than changes in Al speciation as water passes over the gills. For example, at $pH_{in} = 4.8$, Al solubility is theoretically about 10-fold lower at the gills than in the inspired water, but the contributions of the various aluminum hydroxide species increase only 1–4-fold. Second, changes in Al solubility and speciation are of similar relative magnitude between $pH_{in} \sim 5.2$ and 6.3, so the two effects could be of equal importance here.

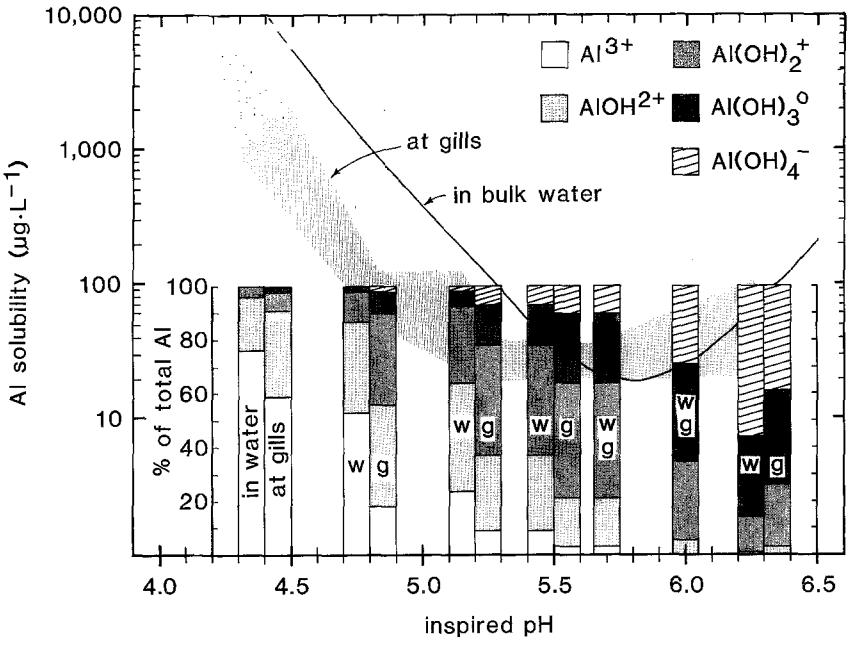

Fig. 7. Chemical species of Al in the bulk water at selected inspired pHs (pH_{in} = 4.4, 4.8, 5.2, 5.5, 5.7, 6.0, and 6.3), and Al species predicted in the gill micro-environment (i.e., from expired pH) at those pHs (bars in foreground). Also illustrated behind the bars are Al solubilities in the bulk water and solubilities predicted to exist near the gills (from Fig. 5). Aluminum species are given as per cent of total Al, taken from Dyrssen (1984). Here, total Al = 93 $\mu g \cdot l^{-1}$ (= height of bars on solubility scale). Note that the speciation bars use a linear scale (inner scale) whereas the solubility scale is logarithmic (outer scale). w and g refer to speciation bars for bulk soft water and for the gill micro-environment, respectively

Note however that Al solubility at these pHs is so low that supersaturated conditions existed in both inspired and expired water in our tests. Thirdly, changes in Al species help explain Al accumulation onto gills under conditions where Al should theoretically have stayed in solution (i.e., pH 4.0, 4.4; Fig. 6). Aluminum hydroxides are generally thought to be the more toxic forms of Al; presumably these species adsorb to the gill surface and polymerize (Baker and Schofield 1982). Conversion of Al^{3+} to the various hydroxides in the gill micro-environment would be greatest at the lowest inspired pHs (Fig. 7). Finally, whatever the relative importance of the different Al species, our analysis illustrates that previous efforts to determine which Al species is most toxic at fish gills (Sadler and Lynam 1987) (Neville and Campbell 1988) should be reconsidered. Due to pH changes in the gill micro-environment, the Al species in contact with the branchial surface are undoubtedly different than the species of Al calculated to exist in the bulk water.

The final alternative explanation for Al toxicity at the gills is that of interactions with mucus. Even during short term exposures only a fraction (<18%) of Al extracted from water actually accumulated on the gills. There is no evidence that Al enters a fish's body during shorter term exposures (i.e., days; Neville 1985; Booth et al. 1988). The difference between Al extraction and Al accumulation can be attributed to the rapid sloughing of mucus and Al complexes from the gills. Their density, viscosity, or size would preclude their being regularly sampled by the opercular catheters. Rinsing of gill samples (see Methods) might further remove mucus-bound Al from gill surfaces. Additional evidence for sloughing of mucus-bound Al is provided by the observation that brook trout can clean the Al burden from their gills during chronic sublethal exposures (McDonald et al., pers. comm.). Binding and rapid sloughing of Al by mucus is likely an important defence against Al accumulation and therefore toxicity at the gills. Whether Al-mucus complexation also contributes to toxicity, i.e., by increasing the branchial diffusion barrier for O_2 and CO_2, is impossible to determine at present. However, it is possible that the small proportion of Al which is not sloughed off with mucus, but rather persists on the gill epithelia, is central to toxicity.

To summarise, by using opercular catheters and ventilation masks it has been shown that in soft water the pH near rainbow trout gills is higher than in acidic inspired water. In the presence of Al the difference between expired pH and acidic inspired pH was reduced (i.e., the gill micro-environment is less basic), as a consequence of increased ventilation and pH buffering by Al itself. Over longer exposures (44-h versus 2–3-h) there were few changes in expired pH or ventilation volume in fish exposed to soft water of pH 5.2, 4.8, or 4.4, but in exposures to Al at the same pHs

there were progressive increases in ventilation which were greatest at higher pH. Expired pH decreased with time in the pH 5.2 and 4.8 plus Al treatments. Aluminum accumulation on the gills was a small fraction of that removed from the water, suggesting extensive sloughing of Al bound by mucus. It is proposed that the toxic effects of Al are a consequence of Al deposition onto fish gills, as acidic water containing Al becomes more basic in the gill micro-environment, resulting in loss of solubility and changes in speciation.

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