PHYSIOLOGICAL EFFECTS OF ALUMINUM ON RAINBOW TROUT IN ACIDIC SOFT WATER, WITH EMPHASIS ON THE GILL MICRO-ENVIRONMENT

By

RICHARD COLIN PLAYLE, B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

PHYSIOLOGICAL EFFECTS OF ALUMINUM ON RAINBOW TROUT

DOCTOR OF PHILOSOPHY (1989) McMASTER UNIVERSITY

(Biology) Hamilton, Ontario

TITLE: Physiological effects of aluminum on rainbow trout in acidic soft water, with emphasis on the gill micro-environment

AUTHOR: Richard Colin Playle, B.Sc. (McMaster University)
M.Sc. (University of Manitoba)

SUPERVISOR: Professor C.M. Wood

NUMBER OF PAGES: xiv, 249

Abstract

This thesis examined the physiological and toxicological effects of Al (~100 $\mu g.L^{-1}$) in acidic soft water (pH 4.0-6.5) on the rainbow trout (*Salmo gairdneri* = *Oncorhynchus mykiss*), and the mechanisms of Al deposition on the gills. Cannulated trout exposed for 66 h to Al in synthetic soft water (Ca^{2+} = 45 or 410 $\mu equiv.L^{-1}$) showed highest mortality at pH 5.2, intermediate mortality at pH 4.8, and least mortality at pH 4.4. Aluminum caused losses of Na^+ and Cl^- from the plasma at pH 5.2 and 4.8, where there were no ion losses in the absence of Al. Respiratory disturbances (decreases in arterial O_2 tension, increases in CO_2 tension) occurred in fish exposed to Al at pH 5.2 and 4.8.

Experiments on trout fitted with ventilation masks and opercular catheters showed that acidic soft water is rendered more basic as it passes over fish gills, and basic inspired water is made more acidic. A model was generated in which these effects were adequately explained by measured ammonia, base, and CO_2 release at the gills.

Exposure to acidic soft water containing Al resulted in hyperventilation during both short (2-3 h) and longer term (44 h) exposures. Alkalinization of the gill micro-environment was modified but not abolished in the presence of Al. This alkalinization was large enough to exceed the theoretical solubility limit for Al, so that Al precipitation from solution

onto the gills would occur. The alkalinization also altered the theoretical speciation of Al near the gills, resulting in shifts in the direction $Al^{3+} \rightarrow Al(OH)^{2+} \rightarrow Al(OH)_2^{+} \rightarrow Al(OH)_3^{o}$.

Aluminum extraction at the gills, and Al accumulation on the gills, was greatest at higher inspired and expired pH, in agreement with an Al precipitation explanation of Al deposition on fish gills. Aluminum precipitation experiments indicated that precipitation of Al from solution was fast enough to occur during the short (<2 s) residence time of water at the gills. Measured accumulation on trout gills was about 10% of the deposition calculated from measurements of ventilation volume and Al extraction; the difference was explained as extensive sloughing of Al from the gills.

A model of Al interactions at fish gills is presented, in which precipitation of Al onto the gills causes the respiratory effects of Al. A smaller proportion of Al deposition is proposed to be positively charged Al species interacting with the negatively charged gill surfaces, causing the ionoregulatory effects of Al.

Acknowledgements

I thank Steve Munger, Rod Rhem, Greg Goss, M. Kovocevic, and James Curtis for their capable technical help. Drs. Gord McDonald, Mike O'Donnel, and Dave Rollo, thesis committee members, are thanked for their ideas and time. Financial assistance included an NSERC Postgraduate Scholarship, a Hooker Graduate Scholarship, and a Clifton W. Sherman Graduate Scholarship. Dr. Chris Wood, my supervisor, is thanked for his time, effort, and patience during my studies. This work is dedicated to Anne, Muriel, Yvonne, Colin, and Lyle.

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Chapter 1

Introduction to Al chemistry and toxicity in acidic water

Aluminum mobilization by environmental acidification

Very acidic water (pH 4.0-4.6) entering streams and lakes during snowmelt has long been known to kill fish, by impairing ionoregulatory mechanisms at the gills (eg. Leivestad and Muniz 1976; Hesthagen 1989). However, laboratory tests with acidity alone were unable to explain all mortality in the field, especially in moderately acidic water (pH>4.6) where acute toxicity was not expected (eg. Muniz and Leivestad 1980; Baker and Schofield 1982). Cronan and Schofield (1979) demonstrated that Al is leached by acidic precipitation into natural waters at concentrations toxic to fish, which focussed attention on Al as the possible "missing link" in fish mortality at more moderate pH. Interest in the effects of Al on fish has increased steadily over the past ten years, and a large body of evidence in favour of the toxic role of Al has accumulated (eg. Cronan and Schofield 1979; Muniz and Leivestad 1980; Baker and Schofield 1982; Neville 1985; Witters 1986; Malte and Weber 1988; Booth et al. 1988; Wood et al.

1988a; Playle et al. 1989). For reviews of the effects of acidic precipitation and Al on fish and other organisms see Howells et al. (1983), Dillon et al. (1984), Havas and Jaworski (1986), and Schindler (1988).

Low ionic strength lakes ("soft waters" (1)) in the Canadian shield and Scandinavia are particularly vulnerable to acidification, because of their low acid neutralising capacities. Acidified water usually contains elevated concentrations of Al because Al is leached from soil and rock during partial neutralisation of H^+ ions (Dickson 1978; Cronan and Schofield 1979; Johnson et al. 1981; Bache 1986). The solubility of Al increases exponentially from a minimum of about 20 $\mu g.L^{-1}$ (2) near pH 5.8 as water becomes more acidic (Fig. 1).

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(1) "Soft water" is formally defined as water containing <1000 $\mu equiv.L^{-1}$ Ca^{2+} (<20 $mg.L^{-1}$; Wetzel 1975). In practice, most soft water lakes in this category in the Canadian shield and Europe contain <400 $\mu equiv.L^{-1}$ (8 $mg.L^{-1}$).

(2) In this thesis Al concentrations will be referred to by weight of Al ($\mu g.L^{-1}$) without associated hydroxyl ions. This form is common usage and avoids the problem of charge changes and molecular weight changes with pH, which would arise using $\mu equiv.L^{-1}$ or $\mu mol.L^{-1}$. To convert μg $Al.L^{-1}$ to $\mu mol.L^{-1}$ divide by 27, the approximate atomic weight of Al.

Fig. 1. The solubility of Al in water, log Al concentration *versus* pH. From the solubility of microcrystalline gibbsite at 25°C, re-drawn from Roberson and Hem (1969). Similar Al solubility diagrams are found in May et al. (1979), Dyrssen (1984), and Bache (1986).

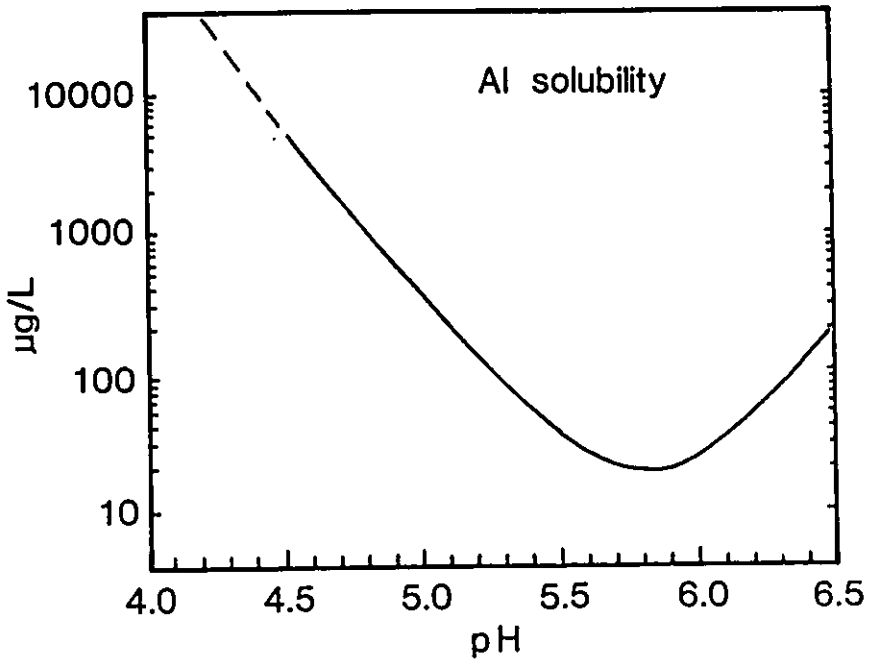
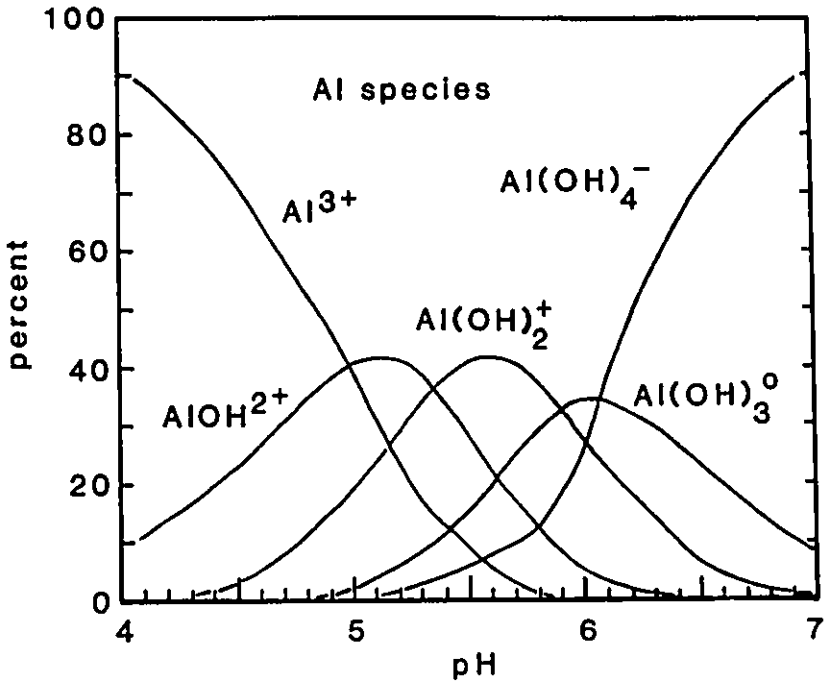

Al solubility
10000
1000
100
10
µg/L
4.0
4.5
5.0
5.5
6.0
6.5
pH

Fig. 2. A speciation scheme for Al, in terms of percent composition of total Al in solution *versus* pH. Re-drawn from Dyrssen (1984).


Al species
Al^{3+}
$AlOH^{2+}$
$Al(OH)_2^+$
$Al(OH)_4^-$
$Al(OH)_3^o$
percent
100
80
60
40
20
0
4
5
6
7
pH

Chemical forms of Al also vary with pH, from the Al^{3+} cation in very acidic water, to the various Al-hydroxides between pH 5 and 6, and the $Al(OH)_4^-$ anion in more basic water (Fig. 2; after Dyrssen 1984). Other speciation schemes, which may include different Al species or exclude $Al(OH)_3^o$, are given by May et al. (1979), Helliwell et al. (1983), and Bache (1986). Besides these inorganic monomers, other forms of Al expected in water include inorganic polymers, Al fluorides and sulphate, amorphous Al, microcrystalline Al, and organically-bound Al (LaZerte 1984).

Aluminum toxicity to fish

Studies of toxicological and physiological effects of Al on fish have evolved from early observations of mortality in hard water and high doses of Al, to more sophisticated analyses of blood of fish exposed to low, environmentally realistic concentrations of Al in acidic soft water. Freeman and Everhart (1971) exposed fingerlings of rainbow trout (Salmo gairdneri) to 5.2, 0.52, or 0.05 $mg.L^{-1}$ Al in neutral to alkaline tapwater (pH 7.0-9.0). These workers recommended that Al concentrations not exceed 100 $\mu g.L^{-1}$ in circumneutral or alkaline water to ensure trout survival and normal growth. Decker and Menedez (1974) reported 50% mortality of adult brook trout (Salvelinus fontinalis) in 96 h when exposed to 3-5 $mg.L^{-1}$ Al in hard water of pH 5.5-6.5. These and other, earlier studies of Al toxicity, mostly run in hard water, were reviewed by Burrows (1977).

Dickson (1978) and Cronan and Schofield (1979) first documented the connection between the partial neutralisation of acidic precipitation by soils and the release of Al into lakes. Dickson (1978) presented Swedish data which showed that acidic lakes had higher concentrations of Al than did neutral lakes, as predicted by Al solubility in water (Fig. 1). Concentrations of Al were 100-200 $\mu g.L^{-1}$ in lakes near pH 5 (Dickson 1978). Mortality of brook trout exposed to Adirondack water of >200 $\mu g.L^{-1}$ Al was associated with necrosis of gill epithelia (Cronan and Schofield 1979).

The realisation that all forms of Al are not equally toxic was made by Driscoll et al. (1980), who found that the addition of citrate or fluoride increased survival time of brook trout exposed to soft water containing 400-500 $\mu g.L^{-1}$ Al. In addition, Al caused greater fish mortality at pH 5.2 than at pH 4.4. From these results, it became clear that measurements of total Al could overestimate potential Al toxicity, and that total Al needed to be separated into biologically-active and complexed, less-reactive Al.

Since these early studies, acute toxicity of Al in soft water has been examined in a large number of studies using various fish species (summarised in Havas and Jaworski 1986). Toxic effects occurred at 75 to 450 $\mu g.L^{-1}$ Al, pH 4.2-6.1, and ranged from mild stress to mortality. In general, Al is most toxic to fish near pH 5, is least toxic when complexed by organic material, and its

toxicity is reduced by higher concentrations of Ca^{2+}. Susceptibility to Al may change during fish development (eg. Baker and Schofield 1982; McCormick et al. 1989; Palmer et al. 1989), but these changes are species dependent and cannot easily be generalised.

Physiological basis of low pH and Al toxicity to fish

Acidic water by itself affects ion balance in fish. Fish normally take up Na^+ and Cl^- ions through the gills by Na^+/H^+(or NH_4^+) and Cl^-/HCO_3^-(or OH^-) exchanges, to replace diffusive losses of these ions (reviewed by McDonald 1983a; Wood 1989). Hydrogen ions reduce active uptake of Na^+ and Cl^- (Na^+ to a greater extent), and increase passive ion effluxes. The latter effect may be due to displacement of Ca^{2+} from paracellular channels, making the gills more "leaky" (McDonald 1983a). Fish blood may become acidic, because of net acidic equivalent entry into the fish associated with differential Na^+ and Cl^- losses, although this effect is generally small with Ca^{2+} concentrations typical of soft water (Wood 1989). Only very acidic conditions (pH~4) cause respiratory effects in fish, a result of gill damage and excess mucus production impairing gas transfer (reviewed by McDonald 1983a).

Toxic effects of Al, like those of acidity alone, were assumed to take place at the gills (eg. Freeman and Everhart 1971), but it was not until 1980 that the first physiological

studies of Al toxicity indicated the mechanisms of its action. Ionoregulatory disturbances of Al were shown by Cl^- losses from blood of brown trout (Salmo trutta) exposed to ~1 $mg.L^{-1}$ Al, losses which were greater at pH 5.1 and 5.5 than at pH 4.3 or 4.5 (Muniz and Leivestad 1980). Water hardness affected Cl^- ion losses: less plasma Cl^- was lost when trout were exposed to Al in water containing ~200 $\mu equiv.L^{-1}$ Ca^{2+} than in water containing 40 $\mu equiv.L^{-1}$ Ca^{2+} (Muniz and Leivestad 1980). Paradoxically, at pH 4.0, 380 $\mu g.L^{-1}$ Al reduced the decrease in plasma Cl^- owing to acidity alone. Stressful concentrations of Al were often accompanied by mucus clogging of the gills and lowered oxygen tension of venous blood (Muniz and Leivestad 1980); ventilation frequency increased in brook trout exposed to Al (Rosseland 1980). These studies were the first to establish the ionoregulatory and respiratory effects of Al, plus the curious reduction of the ionoregulatory effects of extreme acidity by Al. However, in these early physiological studies, fish were usually exposed to unrealistically high concentrations of Al.

In more recent studies, Neville (1985) exposed cannulated rainbow trout to 75 $\mu g.L^{-1}$ Al in soft water. A mixture of ionoregulatory and gas exchange problems was seen after 10 d: fish lost blood electrolytes at pH 4.0 and 4.5, developed hypoxemia at pH 6.1, and showed a combination of the two effects at pH 5.0 and 5.5. Aluminum was found on the gills, but did not enter the trout (Neville 1985), which supports the assumption of a

gill surface mechanism for Al toxicity. Malte and Weber (1988) found that rainbow trout exposed to 860 $\mu g.L^{-1}$ Al in soft water at pH 5.0 died primarily from impeded gas exchange, not ion losses. Witters (1986) exposed rainbow trout to pH 4.1, 350 $\mu g.L^{-1}$ Al for 3.5 h in water containing either 40 or 190 $\mu equiv.L^{-1}$ Ca^{2+}, and found no reduction in whole body Na^+ and Cl^- losses with the higher Ca concentration. Ion loss was twice as high in the presence of Al than during exposure to 4.1 alone, unlike the results of Muniz and Leivestad (1980), where Al reduced ion losses at very low pH.

Malte (1986) and Jensen and Weber (1987) observed hypoxemia in rainbow trout and tench (Tinca tinca) exposed to very high Al concentrations (2 $mg.L^{-1}$) in hard water (Ca^{2+} ~7000 $\mu equiv.L^{-1}$) at pH 5.0. However, the environmental relevance of their observations is doubtful because waters of such high Ca concentrations rarely become acidified. Impaired gas diffusion at the gills could have been caused by Al precipitation at the gills, mucus accumulation on the gills, or gill damage.

Blood acidosis has also been seen in fish exposed to Al. Depending on conditions, decreases in blood pH associated with Al exposure have been attributed to respiratory acidosis due to CO_2 accumulation, or to metabolic acidosis due to lactic acid accumulation as a result of anaerobic respiration (Neville 1985; Malte 1986; Jensen and Weber 1987). However, in some conditions neither mechanism appeared to be responsible.

A recent series of studies of the effects of Al on brook trout has shed more light on the physiological effects of Al, as modified by pH, Ca, and pre-exposure to Al. Brook trout lost Na^+ and Cl^- across the gills, and showed decreases in plasma Na^+ and Cl^- (terminal samples) when exposed to pH 4.8 or 4.4 soft water in the absence of Al (Booth et al. 1988). Ion losses increased in the presence of 333 $\mu g.L^{-1}$ Al at pH 4.8 and 5.2, and mortality was greatest at pH 5.2. Increased Ca^{2+} (400 vs 25 $\mu equiv.L^{-1}$) generally reduced ion losses and fish mortality due to acidity or Al (Booth et al. 1988).

Aluminum generally exacerbated the decreased Na^+ influx and increased Na^+ efflux at the gills associated with acid exposure alone (Booth et al. 1988). Over the first 12-24 h, efflux was the larger component of the problem, but in the longer term, the persistence of influx inhibition was more serious. Ion loss correlated well with fish death: if a fish lost more than 4% of its total body Na^+ in the first 24 h of Al exposure, its chances of survival for 10 days were <10%. Much larger amounts (10-35%) were generally lost by the time death actually occurred (1-4 d). Aluminum accumulated on the gills, but was not found in fish plasma or livers (Booth et al. 1988).

Using cannulated fish, Wood et al. (1988a) demonstrated ionic disturbances in brook trout exposed to pH 4.8, 333 $\mu g.L^{-1}$ Al, 25 $\mu equiv.L^{-1}$ Ca^{2+}, and respiratory disturbances (eg. decreased arterial O_2 tension) in fish exposed to the same pH and Al

concentration but with 400 μequiv.L^{-1} Ca^{2+}. Here, higher Ca concentrations did not reduce Al toxicity, but changed its form from ionic to respiratory distress. At pH 4.4, 25 μequiv.L^{-1} Ca^{2+}, in the absence of Al, some fish died due to ion losses, but the addition of 333 μg.L^{-1} Al caused more fish deaths, less ionic disturbance, and more extreme blood gas problems (Wood et al. 1988a). Walker et al. (1988b) further documented the respiratory problems, in particular a marked hyperventilation.

Physiological acclimation to Al was demonstrated in brook trout exposed to 75-150 μg.L^{-1} Al at pH 5.2 for 10 weeks before an Al challenge (Wood et al. 1988b, c). Relative to trout previously unexposed to Al, these fish were able to reduce their ion losses when exposed to 333 μg.L^{-1} Al at pH 4.8, and showed much lower mortality over 2 d. Acclimation was partly a result of increased Na^+ transport activity (McDonald and Milligan 1988), but pre-exposure to Al also alleviated the respiratory distress associated with the Al challenge (Wood et al. 1988c). These results agree with toxicological studies on rainbow trout: pre-exposure to 90 μg.L^{-1} Al for 1 or 2 weeks nearly doubled their threshold lethal concentraton of Al (Orr et al. 1986).

Current ideas on mechanisms of Al toxicity

Current information about Al toxicity to fish suggests that ionoregulatory disturbances are due to both reduced active uptake of ions and increased diffusive loss of ions. Reduced ion uptake

across the gills could be due to reductions in carbonic anhydrase and Na-K-ATPase activity, needed for osmotic and acid-base regulation (Staurnes et al. 1984). Increased ion efflux may be a result of disruption of paracellular channels where ion losses - especially those due to H^+ ions - may normally occur (McDonald 1983a). Calcium could reduce ionoregulatory effects of Al by competing for binding sites of Al at the gills, as it probably does against H^+ ions.

Respiratory effects of Al have been attributed to mucus accumulation on gills. Mucus produced in response to Al can accumulate between gill lamellae (eg. Muniz and Leivestad 1980), clogging the gills and impeding gas diffusion. Arterial O_2 tension would decrease, and CO_2 tension increase (eg. Wood et al. 1988a). Cough rate may increase (eg. Neville 1985) as fish try to remove excess mucus and Al from the gills, and hyperventilation (Walker et al. 1988b) may develop in an attempt by the fish to restore normal blood gas levels. Blood acidosis may occur because of CO_2 accumulation (respiratory acidosis), or because of lactic acid accumulation during anaerobic respiration (metabolic acidosis; Neville 1985; Malte 1986; Jensen and Weber 1987).

Ionoregulatory, respiratory, and acid-base disturbances require that Al accumulate on the branchial surfaces. If ion losses are caused by displacement of Ca^{2+} from paracellular channels, positively charged Al species such as Al^{3+} could be responsible. Very high concentrations of Al (eg. Malte 1986;

Jensen and Weber 1987) may be oversaturated, and flocs of Al caught on gill lamellae could well be the explanation for respiratory distress and acid-base disturbances observed in exposures to high Al concentrations. However, respiratory toxicity and blood acidosis seen with more realistic exposures (eg. Neville 1985; Wood et al. 1988a), which are undersaturated with Al, are more difficult to explain. An idea that has developed to explain this anomaly is that acidic, inspired water is rendered more alkaline in the gill micro-environment (Neville 1985; Wood et al. 1988a). A rise in pH would decrease Al solubility (Fig. 1), so that Al would precipitate from solution onto the gills. Alternatively, the pH rise could change Al species to forms that more readily attach to gills. Both these possibilities have been presented by Neville (1985) and Booth et al. (1988).

The idea that water chemistry and pH next to the gills is different than in the bulk water inhaled by the fish is not new. Lloyd and Herbert (1960) deduced from ammonia toxicity data that CO_2 released by fish probably makes water near the gills more acidic. They proposed that production of H^+ ions, as CO_2 dissociated to HCO_3^- and H^+, detoxified ammonia (NH_3) by converting it to NH_4^+. Wright et al. (1986) were able to measure this acidification of the gill micro-environment by CO_2 using opercular catheters and fish ventilation masks. Besides CO_2, ammonia is also released at fish gills (eg. Wright and Wood 1985),

and would be expected to render acidic water more alkaline as it passes over the gills (Randall and Wright 1989). Carbon dioxide released at fish gills may aid ammonia excretion, because H^+ formed from CO_2 dissociation would combine with excreted NH_3 in the gill micro-environment to form NH_4^+, creating a sink for NH_3 (Wright et al. 1989). In acidic water (pH<5), CO_2 will not acidify water because it no longer dissociates to HCO_3^- and H^+ (Stumm and Morgan 1981). Ammonia released at the gills into acidic water containing Al may be the alkalinizing agent which results in deposition of Al onto fish gills, although there is not yet any experimental evidence in favour of this idea.

Thesis objectives

The overall objective of this research was to obtain more complete knowledge of the physiological and toxicological effects of Al in acidic soft water on a reference fish species, the rainbow trout, and to elucidate the mechanisms of Al deposition at the gills. The aim of the first set of experiments (Chapter 2) was to separate, in environmentally relevant exposures, the interactive effects of pH, Al, and Ca on ionoregulatory, respiratory, and acid-base status in rainbow trout. Cannulated trout were used, to allow repetitive blood sampling to monitor the development of these responses with time. Results of these experiments have now been published (Playle et al. 1989).

From these experiments, it became clear that real understanding of Al toxicity must take into account water chemistry changes near fish gills. The goal of Chapter 3 was to measure pH changes at trout gills, quantify the CO_2, ammonia, and

base transfers producing those changes, then model the system using classical aquatic chemistry and the measured acid and base transfers. Rainbow trout fitted with ventilation masks and opercular catheters were used; opercular catheters allowed collection of water samples from within the gill micro-environment. Results of this study are now in press (Playle and Wood 1989a).

The objective of Chapter 4 was to determine the effects of Al on pH changes in the gill micro-environment, and the influence of the pH changes on Al solubility and speciation at the gills. Short (2-3 h) and longer term (44 h) exposures to Al were run using fish fitted with ventilation masks and opercular catheters, to assess changes in fish ventilation and gill water chemistry with time. In addition, calculated deposition of Al on the gills was compared to measured accumulation, as an indication of Al removed from the gills. Results of Chapter 4 are also in press (Playle and Wood 1989b).

The goal of Chapter 5 was to establish the relative importance of Al precipitation and speciation changes at the gills in determining Al toxicity, through frequent, simultaneous measurements of Al extraction and pH of the gill micro-environment. Correlation analysis of Al extraction and deposition at the gills with solubility, oversaturation, and species of Al, calculated from measured gill expired pHs, was used to establish which mechanisms of Al deposition were most probable. Short (6 h) experiments were carried out on fish fitted with

ventilation masks and opercular catheters. Accurate calculations of Al deposition on the gills were compared with measured Al accumulation, to better determine the extent of Al sloughing from the gills. Experiments were also run to determine how fast Al can precipitate from solution under conditions similar to those at the gills. Results from the Al precipitation experiments have been submitted for publication (Playle and Wood, submitted).

Finally, a model to explain Al interactions at fish gills is presented in Chapter 6. The model integrates proposed mechanisms of Al deposition and accumulation at fish gills with the observed respiratory, ionoregulatory, and acid-base disturbances of Al.

Chapter 2

Physiological disturbances in rainbow trout during acid and aluminum exposures in soft water

Introduction

In Chapter 1, current ideas on the physiological basis of Al toxicity to fish were discussed. Aluminum may cause ion losses in fish (eg. Muniz and Leivestad 1980; Neville 1985; Witters 1986), losses which Ca may (Muniz and Leivestad 1980) or may not (Witters 1986) reduce. Aluminum itself can reduce ion losses caused by H^+ in very acidic water (eg. Muniz and Leivestad 1980), may reduce ion losses only temporarily (Neville 1985), or may have no protective effect against H^+ (Witters 1986).

A second aspect of Al toxicity at low pH is respiratory disturbance (eg. Rosseland 1980; Wood et al. 1988a). Respiratory effects of Al occur at moderate acidity (pH~5-6), and are distinct from respiratory effects of acidity alone, which occur in very acidic water (pH~4). Blood acidosis may develop during respiratory distress caused by Al, as CO_2 in the blood increases or as lactic acid accumulates because of anaerobic respiration (eg. Neville 1985; Malte and Weber 1988). However, blood acidosis in fish may not always be explained by these two mechanisms.

To date, there has been no systematic study of the interactive effects of water pH, Al, and Ca in causing

ionoregulatory, respiratory, and acid-base disturbances in fish. The objective of the present investigation was to carry out such a study under environmentally relevant conditions. The specific goals were to separate the effects of acidity from those of Al, to examine the influence of acidity on Al toxicity (or vice versa), and to assess the protective effects of Ca.

Rainbow trout were cannulated to allow repetitive blood sampling with minimal disturbance, and were exposed to conditions designed to simulate those occurring during acidic pulses such as snow melt (eg. Gunn and Keller 1984; Abrahams et al. 1989) or rainstorm runoff (Harvey 1980). The acidities used (pH 5.2, 4.8, 4.4) represent moderate to highly acidic conditions. An Al exposure of 105 $\mu g.L^{-1}$ was chosen because at pH 5.2 this concentration is close to the solubility limit of Al, yet is still a representative concentration of Al in moderately acidic water (Dickson 1978; Dillon et al. 1984). The Ca concentrations used represent very soft water (45 $\mu equiv.L^{-1}$ Ca^{2+}) and moderately soft water (410 $\mu equiv.L^{-1}$ Ca^{2+}). A flow-through system was used to minimize the complexation and precipitation of Al that can occur in static exposures.

Materials and methods

Experimental animals and water

Adult rainbow trout (*Salmo gairdneri* = *Oncorhynchus mykiss*) of both sexes, weight=420 ±10 g (mean ±1 SEM, n=101), were purchased from Spring Valley Trout Farm, New Dundee, Ont. They were held in dechlorinated Hamilton city tapwater (hard water; Ca^{2+} ~2 mequiv.L^{-1}; Na^{+} ~0.6 mequiv.L^{-1}; Cl^{-} ~0.8 mequiv.L^{-1}, titratable alkalinity ~1.9 mequiv.L^{-1}, pH ~8.0) at 15-20°C and were fed floating trout pellets (Martin Feed Mills, Elmira, Ontario) twice weekly.

At least two weeks before an experiment the fish were placed in a flowing soft water acclimation tank and feeding was suspended. Soft water was produced from dechlorinated tapwater passed through a reverse osmosis unit (Culligan MP1000) or through deionising resin cannisters (J.W. Anderson Co. Ltd.). Appropriate amounts of analytical grade NaCl and $CaCl_2$ (BDH) were added by peristaltic pump. Acclimation conditions were approximately pH 6.5, 15°C, Ca^{2+} 45 or 410 μequiv.L^{-1}, Na^{+} 55 μequiv.L^{-1}, Cl^{-} 95 μequiv.L^{-1}, titratable alkalinity 130 μequiv.L^{-1}, and background Al concentrations of 5 μg.L^{-1}. This water composition is typical of natural, poorly buffered soft waters from the Pacific coast of Canada, in which rainbow trout are endemic, much of northeastern North America, and Scandinavia.

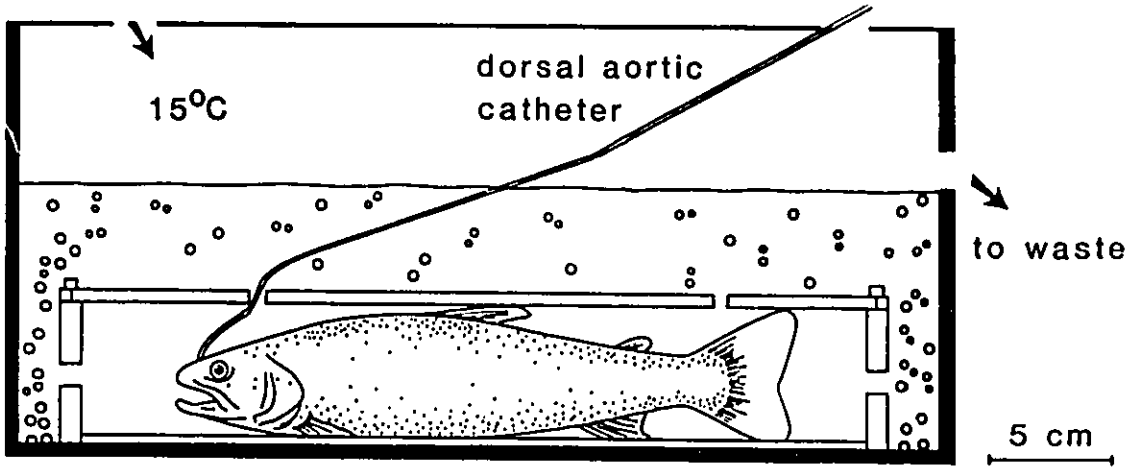
Water pH was measured daily (Radiometer PHM82 pH meter and a Radiometer GK2401C electrode), and cation concentrations every few days (atomic absorption spectrophotometry (AAS); Varian 1275). Total aqueous Al concentrations were determined using the pyrocatechol violet method (Dougan and Wilson 1974), Cl^- by the mercuric-thiocyanate method (Zall et al. 1956), and titratable alkalinity (to pH 4.0) by the method described in detail in Chapter 3.

Fish were anaesthetised with 0.5 $mg.L^{-1}$ MS222 (Sigma) in soft water buffered to pH ~6.5 with KOH, and cannulated with Clay-Adams PE-50 polyethylene tubing via the dorsal aorta (Soivio et al. 1972). The catheters were filled with heparinized Cortland saline (Wolf 1963; Sigma sodium heparin, 45 $i.u..mL^{-1}$). Cannulated fish were placed individually in one of 13 darkened, aerated, plexiglass boxes (vol. ~3 L; after McDonald and Rogano 1986), with a flow of acclimation water of about 100 $mL.min^{-1}$ to each fish (Fig. 3). Water passed through the boxes into a surrounding bath which kept box temperatures at 14-16°C, then went to waste.

Experimental protocols

After about 44 h recovery from cannulation, initial blood samples were taken. Flow to all fish boxes was then changed to acidified water by acidifying the head tank supplying the boxes. A Radiometer PHM82 pH meter with a Radiometer GK2401C combination electrode, connected to a magnetic valve, controlled delivery of 1

Fig. 3. Summary of experimental conditions used. Cannulated rainbow trout were placed in 3 L, aerated boxes. Flow rates to each box were about 100 $mL.min^{-1}$. Water O_2 and CO_2 tensions were ~140 and <1 torr, respectively.



420 g rainbow trout

pH: 5.2, 4.8, 4.4

Al: 5, 105 µg/L

Ca: 45, 410 µeq/L

Na: 55 µeq/L

Fish acclimated for 2^{+} wk in softwater at pH~6.5

Fish sampled over 66 h for blood gasses and blood ions.

N reagent grade H_2SO_4 to the strongly aerated head tank. Flow from the head tank was split, and a concentrated stock Al solution ($AlCl_3.6H_2O$ (Sigma); 0.39 $g.L^{-1}$; pH ~4.0) was delivered by peristaltic pump into one-half the flow. Fish exposed to low pH plus Al were run simultaneously with those exposed to the same low pH alone; fish boxes were interspersed to avoid position effects.

Each experiment was at one pH (pH 5.2, 4.8, or 4.4) and one Ca concentration (45 or 410 $\mu equiv.L^{-1}$), with (105 $\mu g.L^{-1}$) and without added Al. Background Al was about 5 $\mu g.L^{-1}$. Water pH in the head tank was set below the desired pH to counteract the neutralizing influence of the fish on the water (see Chapter 3) as it passed through the fish boxes. Water pH was monitored in the boxes near the head of the fish, using a second Radiometer electrode and meter, and was adjusted by changing water flows to individual boxes; pH was kept within $\pm$0.1 units of the desired pH. Oxygen and carbon dioxide tensions in the fish boxes were about 140 torr and <1 torr, respectively.

Blood samples (1000 μL) were drawn anaerobically into gas-tight, ice-cold Hamilton syringes before the start of the acid and Al exposure (initial values), and at 4, 18, 28, 42, and 66 h thereafter, if fish death did not occur earlier. Blood removed was replaced with Cortland saline. Blood was analyzed for pH, total CO_2 (whole blood and true plasma), O_2 tension, hematocrit, hemoglobin, lactate, and plasma concentrations of Cl^-, Na^+, K^+, Ca^{2+}, protein, and glucose.

Analytical methods

Whole blood arterial pH (pHa) and O_2 tension (Pa_{O_2}) were measured at experimental temperature using Radiometer micro-electrode units (E5021, E5046) connected to a Radiometer PHM72 acid-base analyzer. Total CO_2 in whole blood and true plasma was measured using either a Cameron chamber equipped with a Radiometer E5036 P_{CO_2} electrode (Cameron 1971a), or a Corning 965 CO_2 analyzer.

Hematocrit was measured by centrifugation at ~5000 G for 5 min; plasma samples were then aspirated from the hematocrit tubes for plasma CO_2 analysis. Hemoglobin was measured colorimetrically as cyanmethemoglobin (Blaxhall and Daisley 1973) using Drabkin's reagent (Sigma). Lactate was measured enzymatically (L-lactate dehydrogenase/NADH method; Loomis 1961; Sigma reagents) on whole blood which had been immediately deproteinized in two volumes of ice-cold 8% perchloric acid. Remaining blood was spun at ~9000 G for 2 min, and the plasma stored at -70°C for later analyses. A drop of plasma was used to determine plasma protein concentration using a hand-held refractometer (American Optical; Alexander and Ingram 1980).

Frozen plasma was later thawed, and glucose was measured using the hexokinase method of Bondar and Mead (1974; Sigma reagents). Plasma Cl^- was measured either using a Radiometer CMT10 chloridometer or a mercuric-thiocyanate spectrophotometric

method (Zall et al. 1956); results by the two methods differed negligibly. Plasma Na^+, K^+, and Ca^{2+} were measured by atomic absorption spectrophometer (Varian AA-1275) after suitable dilution; 0.2% $LaCl_2$, to reduce Na^+ interference, was used for Ca^{2+} measurements.

After 66 h, surviving fish in some treatments were stunned with a blow to the head and a section of their third right gill arch was removed for Al determinations. Each gill sample was placed for 1 min in 15 mL distilled water (to remove excess, loosely bound Al), then frozen. Filaments were later cut from the frozen gill portions, weighed, and then digested in 5x their weight of 0.1 N reagent-grade H_2SO_4 for 8 h at 80°C. The supernatent was analyzed for Al using the pyrocatechol violet method (Dougan and Wilson 1974). Gill supernatent was added to the Al standards to account for tissue interferences.

Calculations

Arterial CO_2 tension (P_{aCO_2}) was calculated using the following form of the Henderson-Hasselbalch equation:

$$P_{aCO_2} = \frac{\text{total plasma } CO_2}{\alpha CO_2 \cdot (1 + \text{antilog } (pHa - pK'))}.$$

Values of αCO_2 and pK' at experimental temperatures were taken from values for trout plasma determined by Boutilier et al.

(1984). Whole blood and plasma bicarbonate concentrations were calculated by:

$[HCO_3^-]$ = total plasma CO_2 - ($\propto CO_2$ · P_{aCO_2}).

Metabolic acid load of whole blood (ΔH^+m) was calculated cumulatively (McDonald et al. 1980) using the following equation:

$[\Delta H^+m] = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pHa_1 - pHa_2)$.

Total ΔH^+m is the sum of ΔH^+m for each interval from the initial sample onwards. In this equation both $[HCO_3^-]$ and β (the non-bicarbonate buffer value) are for whole blood. Beta is largely a function of hemoglobin concentration (Wood et al. 1982), so β was calculated from hemoglobin using the following empirical relationship determined by Wood et al. (1982):

$\beta = -1.073\ [Hb] - 2.48$.

Values of ΔH^+m were calculated for whole blood rather than plasma for the sake of direct comparison to Δlactate values, which were also measured in whole blood. There is likely a small error (<5%) associated with the calculation because $\propto CO_2$ values from plasma (Boutilier et al. 1984) were used; values for $\propto CO_2$ in whole blood are not available. Mean cell hemoglobin concentration (in $g.mL^{-1}$) was calculated as the ratio of hemoglobin ($g.dL^{-1}$) to hematocrit ($mL.dL^{-1}$).

Treatment of data

The presentation of physiological data from toxicological experiments is complicated by the fact that different fish die at different times. Simple averaging of all data from all fish at each time can be misleading because the most sensitive fish showing the greatest physiological disturbances generally die first. Loss of their values from the mean at subsequent sample times can produce an artifical trend of group recovery. To overcome this problem, physiological data are presented in two ways in the present study.

For representative parameters, data from only those fish which survived at least 42 h of acid or Al exposure have been averaged at each time up to 42 h. This illustrates changes in parameters over time in the most resistant individuals in each experiment. For brevity, only data for the two extreme acidities (pH 5.2 and 4.4) are shown in these figures; trends at pH 4.8 were generally intermediate. Results are also presented as final minus initial values ("terminal changes") for all fish in all treatments. Here, final values represent either the 66 h sample or the last sample taken before a fish died. Presentation of "terminal changes" emphasises, but does not change, trends in the data, and allows comparisons amongst all twelve treatments.

Data are generally represented as means ± 1 standard error of the mean (SEM). The "n" number represents the number of different

fish contributing to each mean. Differences in mortality were assessed by a X^2 test with Yate's correction. Paired Student's t-tests were used to determine if a parameter in a treatment changed with time, and unpaired t-tests to compare, within a treatment, fish exposed to Al with fish not exposed to Al. Analysis of variance followed by Duncan's Multiple Range test was used to compare terminal changes among treatments. Gill Al data were log(x+1) transformed before statistical analysis. Unless otherwise stated the level of significance used was $P \leq 0.05$ (two-tailed tests).

Results

Mortality

Mortality associated with exposure to acidity alone in cannulated rainbow trout was 0-35%, and not significantly different amongst the three acidities and two Ca concentrations (Fig. 4). Aluminum was most toxic to cannulated rainbow trout at pH 5.2 and least toxic at pH 4.4. Higher water Ca concentrations reduced mortality due to Al at pH 5.2 and 4.8, but had no significant effect at pH 4.4, where mortality was 0-35% in the presence or absence of Al. Most fish deaths occurred between 42 and 66 h, with the exception of the Al exposure at pH 5.2, low Ca, where 4 of 10 fish died at about 30 h. In general, mortality due to Al was greater at higher pH, and Ca ameliorated mortality caused by Al at higher pH but not at pH 4.4.

Ionoregulatory responses

In trout surviving to 42 h, decreases in plasma Cl^- concentrations caused by acidity alone were seen only in the pH 4.4, low Ca treatment; decreases were approximately linear over time, and were significant by 4 h (Fig. 5C). Decreases in plasma Cl^- concentrations owing to the presence of Al were seen at both pH 5.2 and 4.8 (not shown), but there was no additional effect of Al at pH 4.4 (Fig. 5A, B, C, D). Higher Ca concentrations appeared to reduce but not eliminate Cl^- losses caused by acidity or Al (Fig. 5B, D). It is not known why the initial plasma Cl^-

Fig. 4. Mortality in cannulated rainbow trout in the presence (105 $\mu g.L^{-1}$) or absence of Al, in low (45 $\mu equiv.L^{-1}$) or higher (410 $\mu equiv.L^{-1}$) Ca, in water of three acidities (pH 5.2, 4.8, and 4.4; 66-h tests). Number of fish exposed in each treatment, from left to right: 10, 7, 8, 5, 11, 8, 6, 5, 8, 12, 10, and 11. Significant differences in mortalities between an Al treatment and the same pH and Ca concentration without Al are indicated by: + ($P \leq 0.05$) and +++ ($P \leq 0.001$). Significant differences in mortalities between treatments are indicated below. Numbers refer to the twelve treatments, as given in the Figure. Single lines underscore mortalities which are not significantly different from one another ($P > 0.05$).

1 5 2 6 12 10 4 9 8 3 7 11

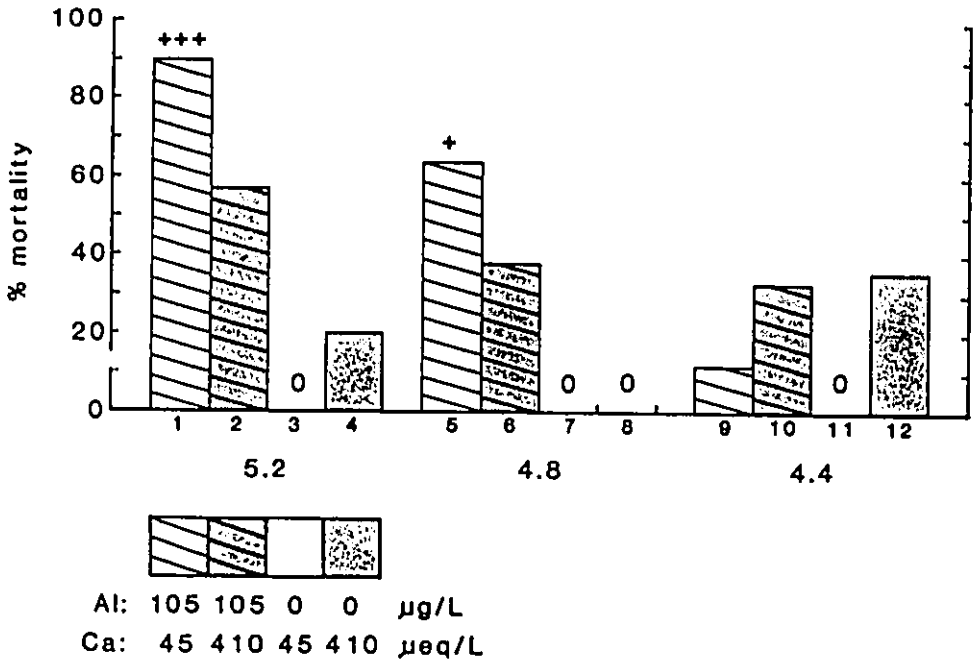

100
80
60
40
20
0
% mortality
+++
+
0
0
0
0
1 2 3 4 5 6 7 8 9 10 11 12
5.2
4.8
4.4
Al: 105 105 0 0 µg/L
Ca: 45 410 45 410 µeq/L

Fig. 5. Plasma Cl^- concentrations of cannulated rainbow trout during 42 h exposure to pH 5.2 or 4.4, 45 or 410 $\mu equiv.L^{-1}$ Ca, in the presence (105 $\mu g.L^{-1}$) or absence of Al. Means ± 1 SEM. The number of 42-h survivors for each treatment was: (A), no Al, n=8; with Al, n=4; (B), no Al, n=5; with Al, n=7; (C), no Al, n=10; with Al, n=8; (D), no Al, n=9; with Al, n=12. Asterisks (*, **, ***) denote significant differences ($P \leq 0.05$, ≤ 0.01, ≤ 0.001) in mean plasma Cl^- concentration compared to the same fish at time 0. Crosses (+) indicate significant differences between fish exposed to Al and fish not exposed to Al. "I" = initial value, taken before acid and Al exposures started at time 0.

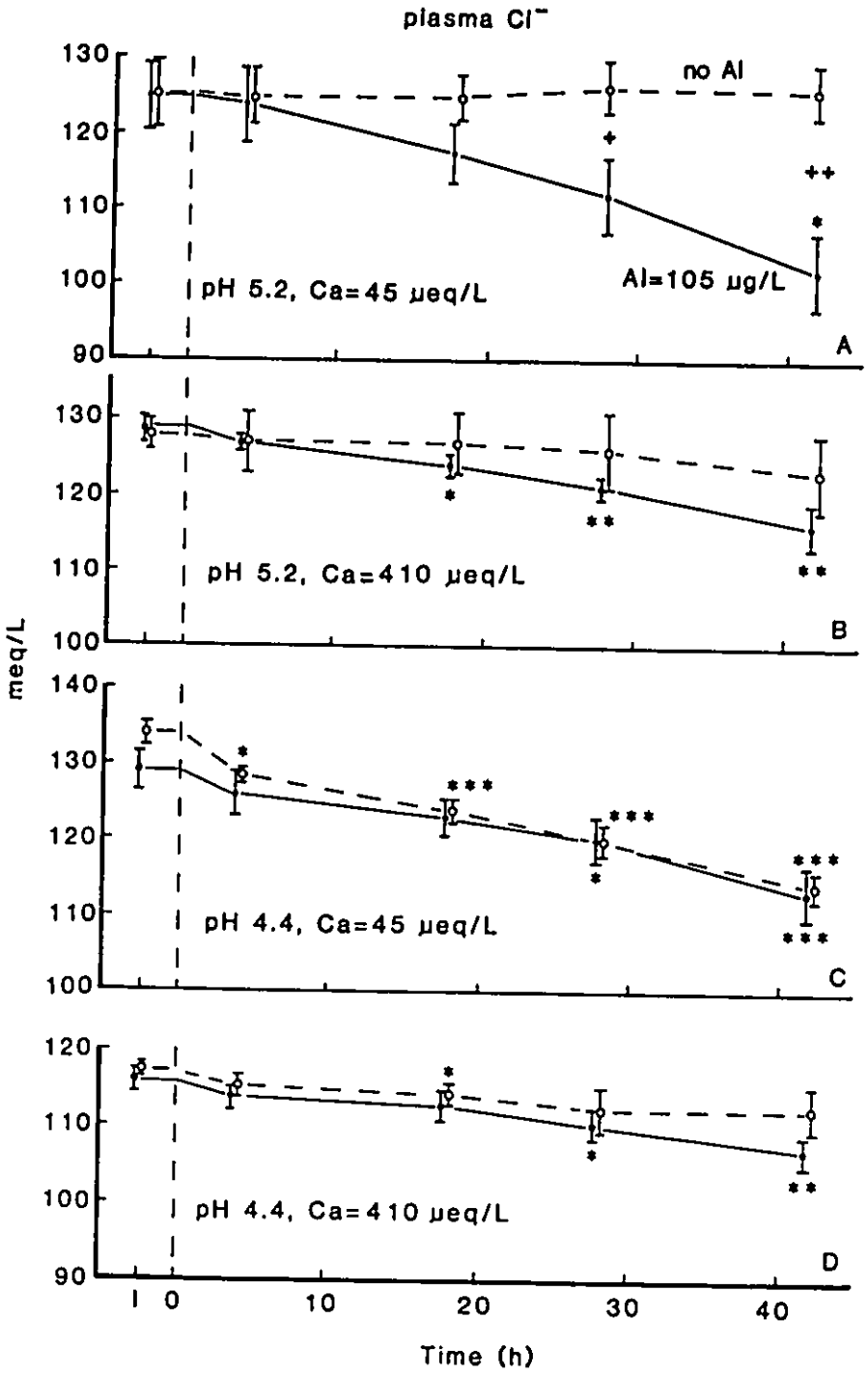

plasma Cl⁻
no Al
Al=105 µg/L
pH 5.2, Ca=45 µeq/L
pH 5.2, Ca=410 µeq/L
pH 4.4, Ca=45 µeq/L
pH 4.4, Ca=410 µeq/L
A
B
C
D
meq/L
Time (h)
130
120
110
100
90
140
0
10
20
30
40

Fig. 6. Plasma Na^+ concentrations of cannulated rainbow trout during 42 h exposure to pH 5.2 or 4.4, 45 or 410 $\mu equiv.L^{-1}$, in the presence (105 $\mu g.L^{-1}$) or absence of Al. See legend of Fig. 5 for other details.

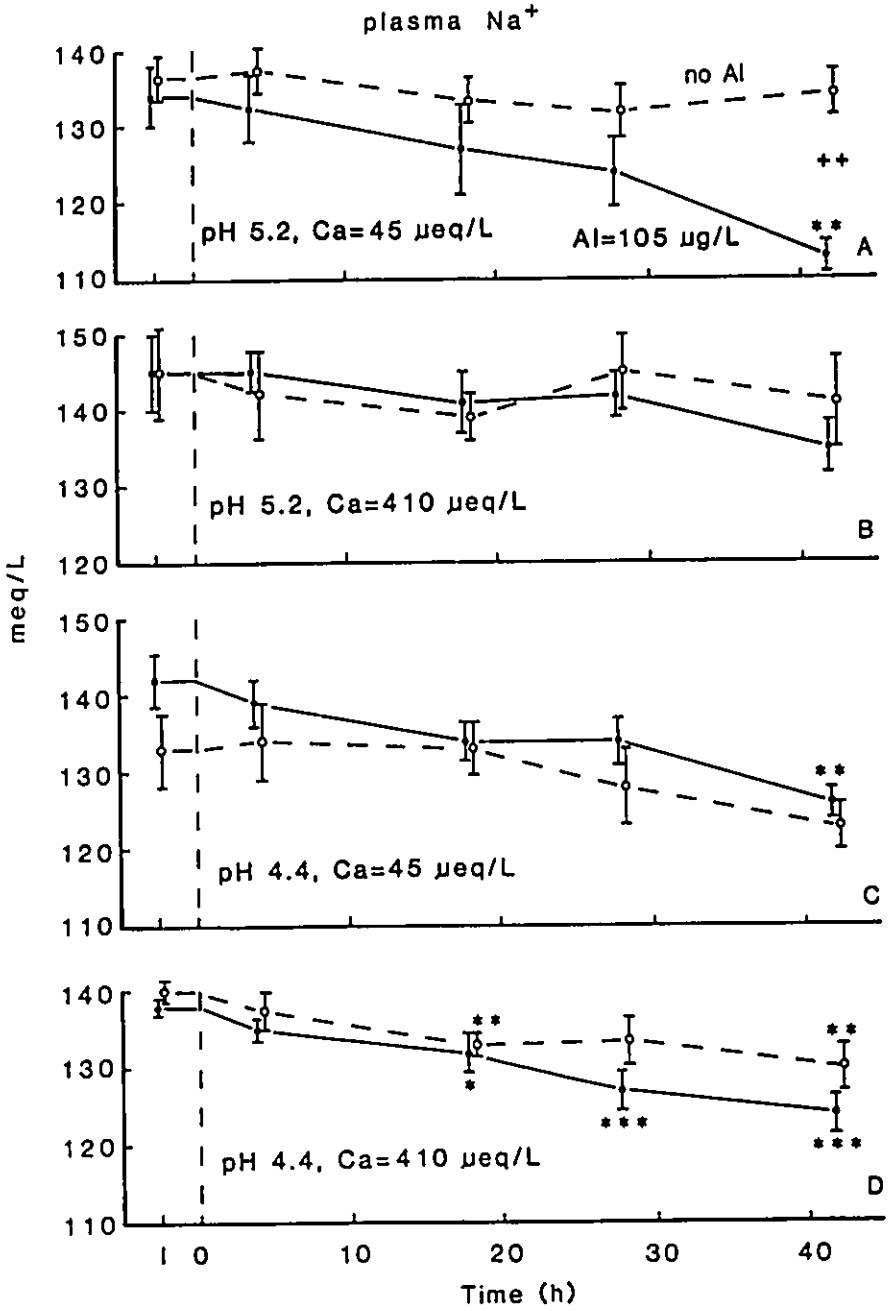

plasma Na+
no Al
pH 5.2, Ca=45 µeq/L
Al=105 µg/L
A
pH 5.2, Ca=410 µeq/L
B
pH 4.4, Ca=45 µeq/L
C
pH 4.4, Ca=410 µeq/L
D
meq/L
Time (h)

Fig. 7. Terminal changes in plasma concentrations of Cl^- and Na^+ for cannulated rainbow trout exposed to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 $\mu g.L^{-1}$) or absence of Al. Means $\pm$1 SEM, n values as in Fig. 4. Asterisks (*, **, ***) denote significant differences ($P \leq 0.05$, ≤ 0.01, ≤ 0.001) between terminal and initial values for the same treatment. The mean initial concentrations for all 12 treatments are given in each panel. Significant differences in terminal changes between treatments are indicated below. Numbers refer to the twelve treatments, as given in the Figure. Single lines underscore terminal changes which are not significantly different from one another ($P > 0.05$).

Cl^-: 11 9 1 6 2 5 10 12 8 4 7 3

Na^+: 9 10 2 6 12 1 11 5 3 8 7 4

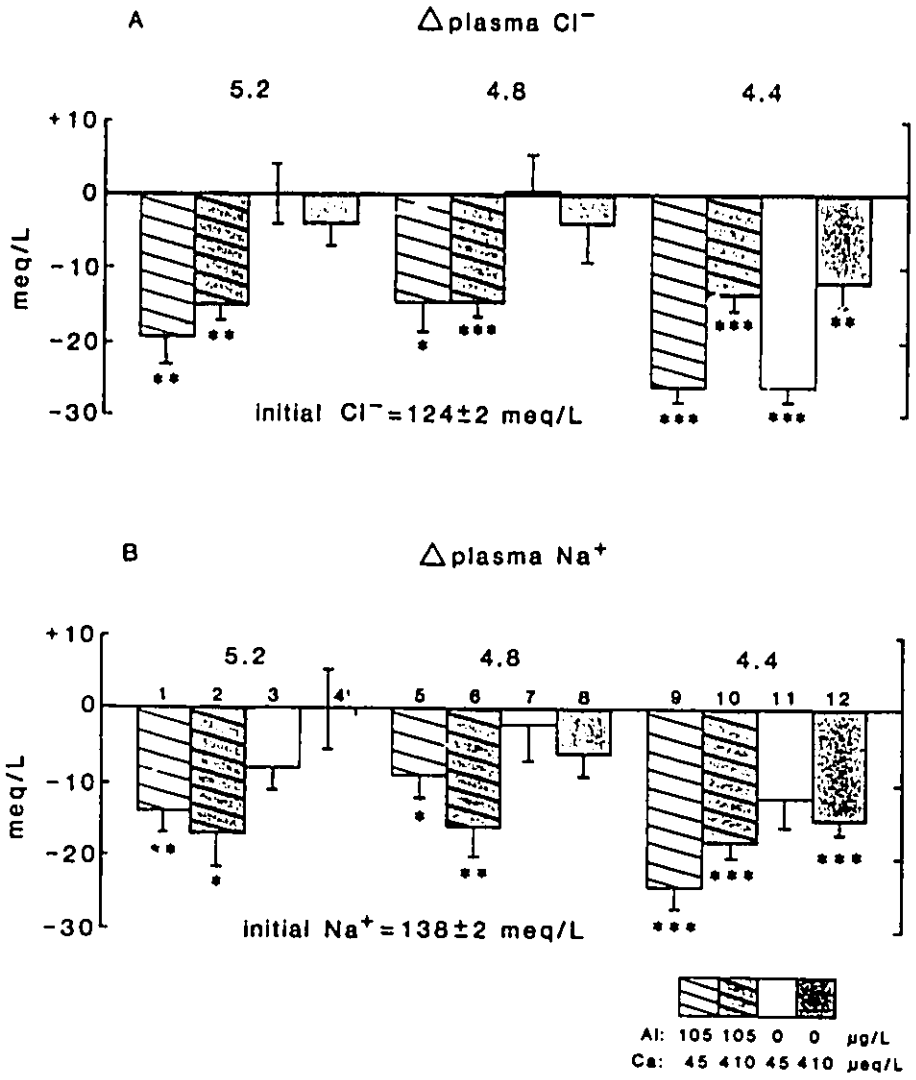

A
Δplasma Cl⁻
5.2
4.8
4.4
+10
0
-10
-20
-30
meq/L
initial Cl⁻=124±2 meq/L
B
Δplasma Na⁺
1 2 3 4 5 6 7 8 9 10 11 12
initial Na⁺=138±2 meq/L
Al: 105 105 0 0 μg/L
Ca: 45 410 45 410 μeq/L

Fig. 8. Terminal changes in plasma concentrations of K^+, Ca^{2+}, protein, and glucose, and mean cell hemoglobin concentration (MCHC) for cannulated rainbow trout exposed to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence or absence of Al. Significant differences in terminal changes amongst treatments are indicated below. See legend of Fig. 7 for other details.

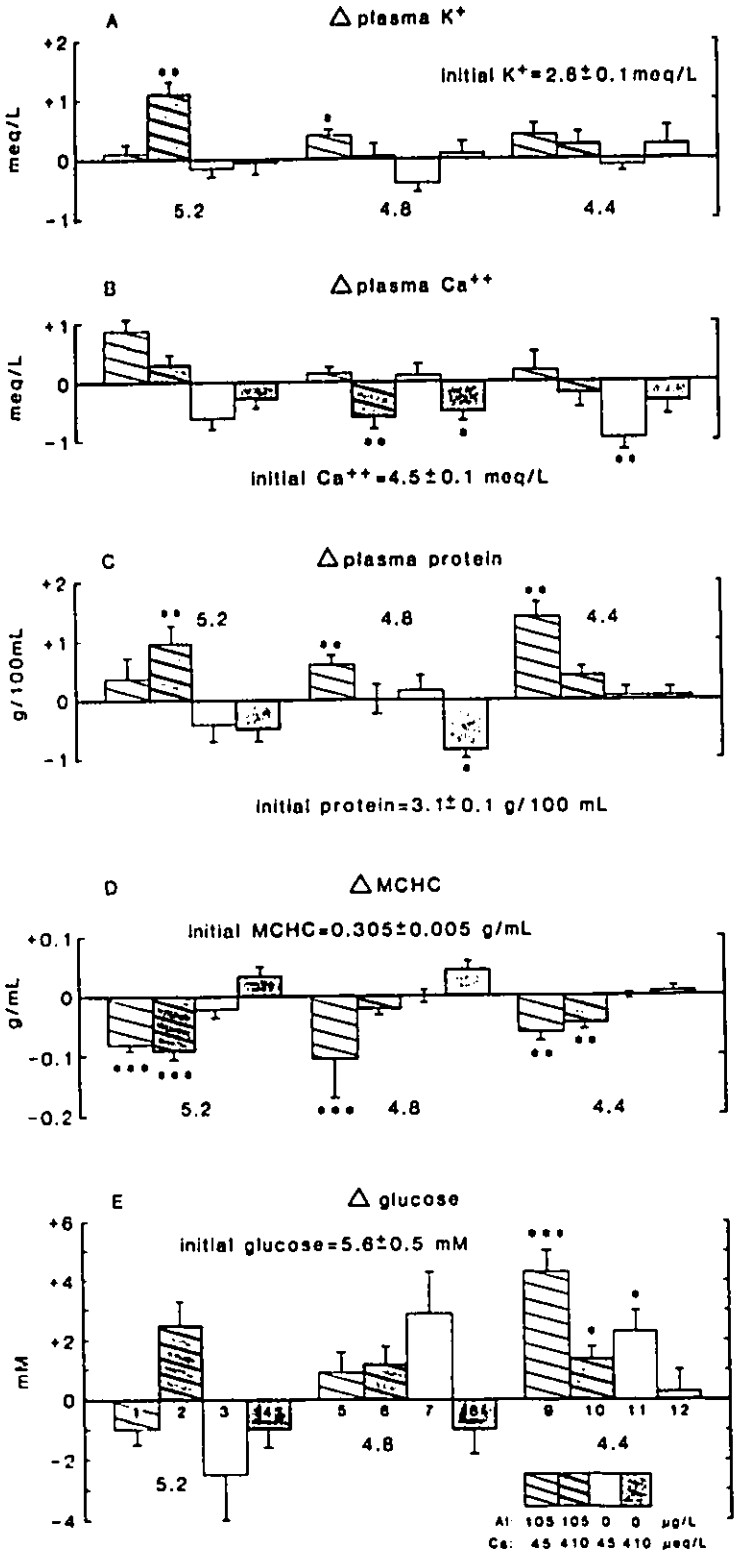
K^+: 2 5 9 10 12 1 6 8 4 11 3 7

Ca^{2+}: 1 2 5 9 7 10 12 8 4 3 6 11

protein: 9 2 5 10 1 7 11 12 6 3 4 8

MCHC: 5 2 1 9 10 6 3 7 4 12 11 8

glucose: 9 7 11 2 10 6 5 12 1 8 4 3


A
Δ plasma K+
initial K+=2.8±0.1 meq/L
meq/L
+2
+1
0
-1
5.2
4.8
4.4
B
Δ plasma Ca++
meq/L
+1
0
-1
initial Ca++=4.5±0.1 meq/L
C
Δ plasma protein
g/100mL
+2
+1
0
-1
5.2
4.8
4.4
initial protein=3.1±0.1 g/100 mL
D
Δ MCHC
initial MCHC=0.305±0.005 g/mL
g/mL
+0.1
0
-0.1
-0.2
5.2
4.8
4.4
E
Δ glucose
initial glucose=5.6±0.5 mM
mM
+6
+4
+2
0
-2
-4
1
2
3
4
5
6
7
8
9
10
11
12
5.2
4.8
4.4
Al: 105 105 0 0 μg/L
Ca: 45 410 45 410 μeq/L

concentrations in the pH 4.4, high Ca exposures were lower than in the other treatments; initial Na^+ concentrations were normal.

The summary of terminal changes emphasizes that, at pH 4.4, acidity alone caused large decreases in plasma Cl^- whereas there was little change in plasma Cl^- at pH 5.2 and 4.8 in the absence of Al (Fig. 7A). Plasma Cl^- losses caused by Al were high at pH 5.2 and 4.8, but Al neither added to nor reduced the Cl^- losses already due to acidity alone at pH 4.4. Calcium had little effect on Cl^- losses caused by Al at pH 5.2 or 4.8, but reduced by about half the plasma Cl^- losses caused by acidity in the pH 4.4 treatments (Fig. 7A).

In general, trout surviving to 42 h showed decreases in Na^+ ions (Fig. 6) that were similar to the plasma Cl^- losses, but the terminal change summary revealed some subtle differences in the overall patterns. Acidity alone caused large reductions in plasma Na^+ concentrations in the pH 4.4 treatments (Fig. 7B), which agrees with the results for plasma Cl^- (Fig. 7A). However, exposure to Al appeared to worsen plasma Na^+ losses at all three acidities, whereas Al had no effect on Cl^- losses in the pH 4.4 treatments. Furthermore, higher water Ca did not reduce Na^+ losses at pH 4.4, and tended if anything to worsen Na^+ losses due to Al at pH 5.2 and 4.8 (Fig. 7B). Overall, Al caused decreases in plasma Cl^- and Na^+ ion concentrations in the pH 5.2 and 4.8 treatments, where Cl^- and Na^+ losses were low in the absence of

Al. Decreases in plasma Cl^- and Na^+ ions at pH 4.4 were caused mainly by acidity alone.

Plasma K^+ concentrations generally increased when Na^+ and Cl^- ions were lost from the plasma (Fig. 8A), although there were only two statistically significant changes. There was an overall trend towards decreasing plasma Ca^{2+} concentrations over time in most exposures, likely a result of repetitive blood sampling (as shown by McDonald et al. 1980), but the effect was reduced or even reversed at pH 5.2 in the presence of Al (Fig. 8B). Plasma protein concentration (Fig. 8C) tended to increase, and mean cell hemoglobin concentration (Fig. 8D) tended to decrease, as plasma Cl^- and Na^+ ions decreased (Fig. 7A, B). At all three acidities these effects were more pronounced in the presence of Al, and were not systematically affected by Ca (Fig. 8C, D). Glucose data were rather variable, but a significant increase in plasma glucose concentration associated with acidity alone was seen in the pH 4.4, low Ca treatment (Fig. 8E). Plasma glucose also increased significantly in the presence of Al in the pH 4.4, low and high Ca treatments (Fig. 8E).

Respiratory responses

Acidity alone had little effect on arterial oxygen tension (Pa_{O_2}) in 42-h survivors (Fig. 9), but Al caused large and rapid decreases in Pa_{O_2} (significant by 4-18 h) in the pH 5.2 treatments (Fig. 9A, B). Similarly, in the pH 4.8, low Ca treatment, Al

caused a drop in P_{aO_2} from 100 to 40 torr in 42 h (not shown). Calcium did not reduce the effect of Al on P_{aO_2} at pH 5.2 (Fig. 9A, B), but the decrease in P_{aO_2} caused by Al at pH 4.8 was eliminated by higher Ca (not shown); a similar protective effect of Ca occurred at pH 4.4 (Fig. 9C, D).

Responses in arterial carbon dioxide tension (P_{aCO_2}) in 42-h survivors generally mirrored those in P_{aO_2}. The P_{aCO_2} was little affected by acidity alone: in the pH 4.4, no Al treatments, P_{aCO_2} increased by only about 0.5 torr (Fig. 10C, D). Aluminum caused large, rapid increases in P_{aCO_2} at pH 5.2 (Fig. 10A, B) and pH 4.8, low Ca (not shown), and increased P_{aCO_2} to a lesser degree in the pH 4.4, low Ca treatment (Fig. 10C). Calcium did not alter the effect of Al on P_{aCO_2} at pH 5.2 (Fig. 10A, B) but reduced it by half at pH 4.8 (not shown), and eliminated the effect at pH 4.4 (Fig. 10C, D). The reciprocal nature of the P_{aO_2} and P_{aCO_2} responses is well illustrated by the summary of terminal changes in all treatments (Fig. 11A, B).

In broad overview, acidity alone had little effect on blood gasses, but Al caused severe respiratory toxicity in rainbow trout at pH 5.2 (shown by the large decreases in P_{aO_2} and increases in P_{aCO_2}; Figs. 11A, B), caused less severe respiratory toxicity at pH 4.8, and induced only moderate respiratory distress at pH 4.4. Calcium reduced the respiratory effects of Al at pH 4.4 and especially at pH 4.8, but had no effect at pH 5.2. Increases in

Fig. 9. Arterial oxygen tension of cannulated rainbow trout during 42 h exposure to pH 5.2 or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 μg.L^{-1}) or absence of Al. One torr = 0.133 kPascal. See legend of Fig. 5 for other details.

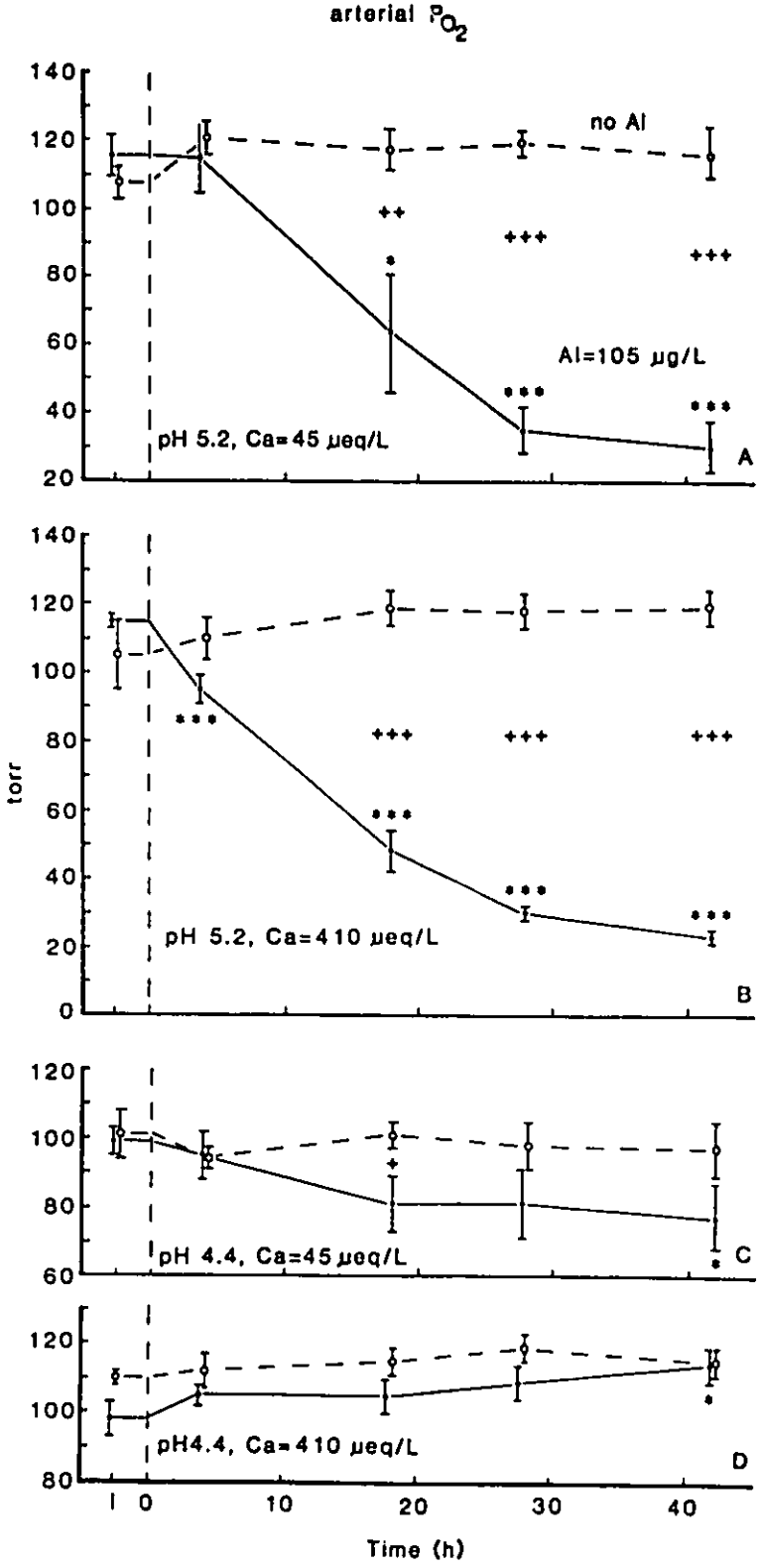

arterial P_{O_2}
no Al
Al=105 µg/L
pH 5.2, Ca=45 µeq/L
A
pH 5.2, Ca=410 µeq/L
B
pH 4.4, Ca=45 µeq/L
C
pH4.4, Ca=410 µeq/L
D
torr
Time (h)

Fig. 10. Arterial carbon dioxide tension of cannulated rainbow trout during 42 h exposure to pH 5.2 or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 μg.L^{-1}) or absence of Al. One torr = 0.133 kPascal. See legend of Fig. 5 for other details.

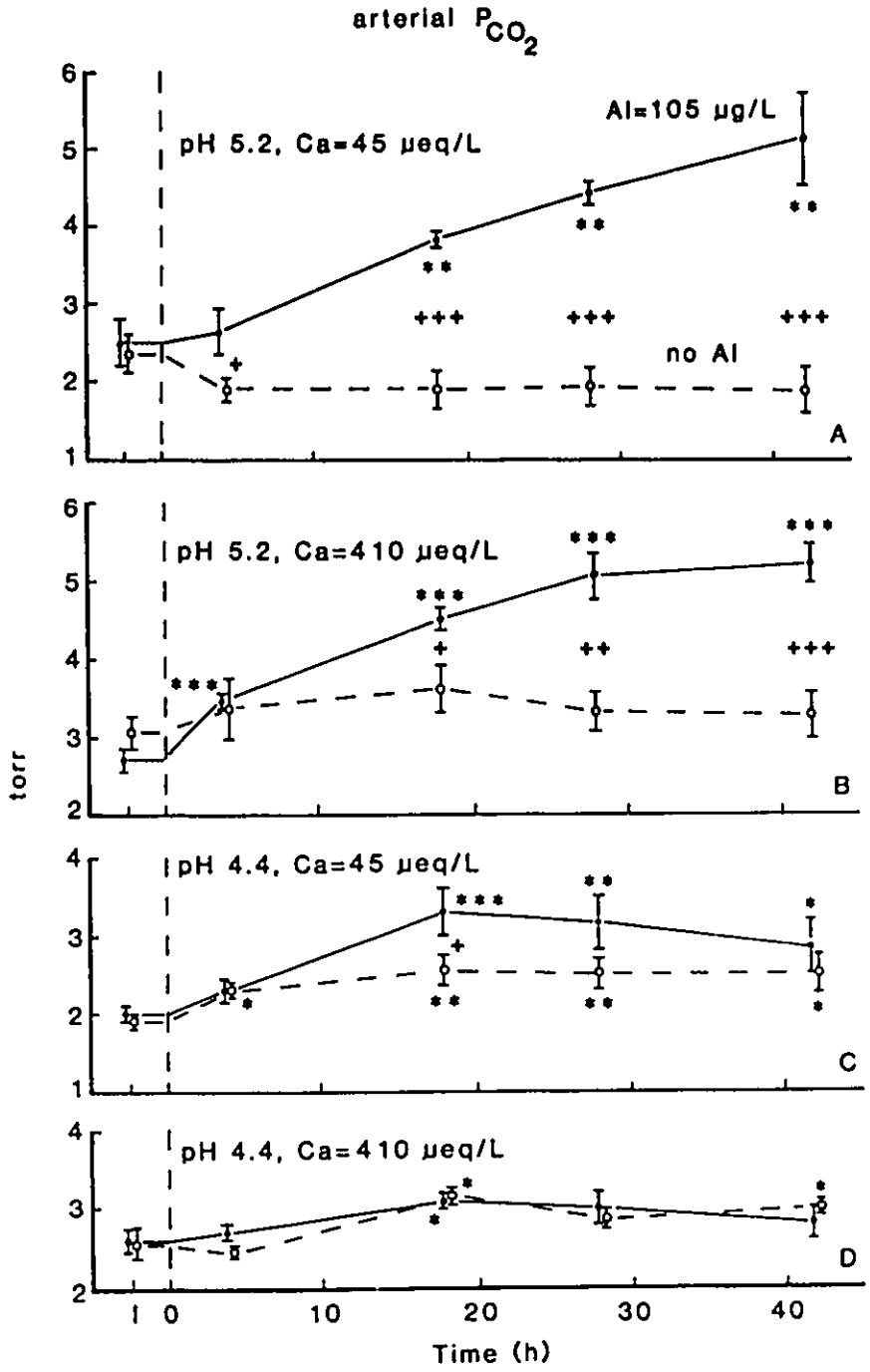
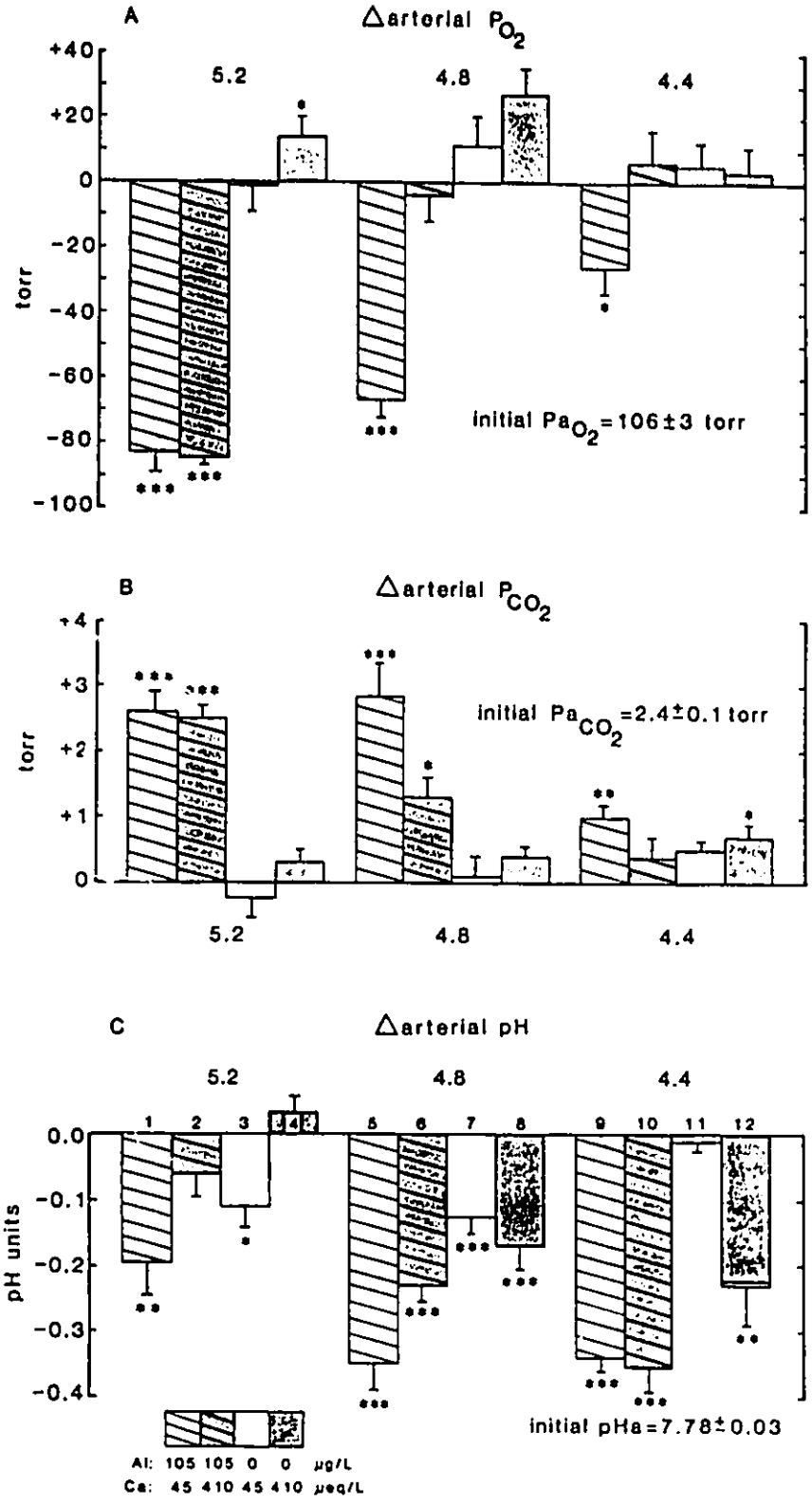

arterial P_{CO_2}
Al=105 µg/L
pH 5.2, Ca=45 µeq/L
no Al
A
pH 5.2, Ca=410 µeq/L
B
pH 4.4, Ca=45 µeq/L
C
pH 4.4, Ca=410 µeq/L
D
torr
Time (h)

Fig. 11. Terminal changes in arterial oxygen tension, carbon dioxide tension, and pH in cannulated rainbow trout exposed to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 μg.L^{-1}) or absence of Al. One torr = 0.133 kPascal. Significant differences in terminal changes amongst treatments are indicated below. See legend of Fig. 7 for other details.

P_{aO_2}: 2 1 5 9 6 3 10 12 7 11 4 8

P_{aCO_2}: 5 1 2 6 9 12 8 10 11 7 4 3

pHa: 5 9 10 6 12 1 8 7 3 2 11 4


A
Δarterial P_{O_2}
+40
+20
0
-20
-40
-60
-80
-100
torr
5.2
4.8
4.4
initial Pa_{O_2}=106±3 torr
B
Δarterial P_{CO_2}
+4
+3
+2
+1
0
torr
initial Pa_{CO_2}=2.4±0.1 torr
5.2
4.8
4.4
C
Δarterial pH
5.2
4.8
4.4
1 2 3 4 5 6 7 8 9 10 11 12
0.0
-0.1
-0.2
-0.3
-0.4
pH units
initial pHa=7.78±0.03
Al: 105 105 0 0 μg/L
Ca: 45 410 45 410 μeq/L

blood lactate (Fig. 16B), indicating anaerobic metabolism, correlated well with the extent of respiratory toxicity.

Acid-base responses

In 42-h survivors, arterial pH (pHa) decreased quickly due to acidity alone in the pH 4.4, high Ca treatment (significant by 18 h; Fig. 12D), but not in the pH 4.4, low Ca treatment (Fig. 12C). Acidity alone had no effect on pHa at pH 5.2 (Fig. 12A, B). Aluminum had little effect on pHa in the pH 5.2 treatments (Fig. 12A, B), but caused large, linear decreases in pHa in the pH 4.4, low Ca treatment (Fig. 12C), and worsened the blood acidification already present in the pH 4.4, high Ca treatment without added Al (Fig. 12D). Arterial pH decreased from about pH 7.8 to pH 7.6-7.7 in 42-h survivors in all four pH 4.8 treatments (not shown).

Accumulation of CO_2 in the blood due to respiratory toxicity should shift the carbon dioxide-bicarbonate equilibrium, resulting in increases in arterial concentrations of HCO_3^- and H^+ ions, thereby decreasing blood pH (respiratory acidosis; Davenport 1974). Although pHa fell in almost every treatment where P_{aCO_2} increased (Fig. 11C, 12), and HCO_3^- increased in some of the exposures (Fig. 13), the relationships were not proportional. Fish showed greater acidosis at pH 4.4 in the presence of Al, where P_{aCO_2} elevations were smallest and HCO_3^- actually declined, than at pH 5.2 where Al caused large accumulations of both CO_2 and

HCO_3^- in the blood. Blood acidification at pH 4.8 was worse than expected if due solely to respiratory acidosis.

Metabolic acid load (ΔH^+m) was calculated to separate the metabolic component of acidosis from the respiratory component. This calculation indicated that at pH 4.4, in the presence or absence of Al, blood acidification was caused mainly by entry of "metabolic" H^+ ions into the blood (Fig. 14, 16A), and that this factor also made a substantial contribution to acidosis at pH 4.8 (Fig. 16A). This "entry" was probably from the external, acidic water. ("Entry" is used here in its broadest sense; acid entry cannot be distinguished from base efflux with current technology.) At pH=5.2, ΔH^+m was significantly negative at low Ca in the presence of Al, and at high Ca in the presence or absence of Al (Fig. 16A). This explained the stability or alkalinization of pHa in these treatments in the face of substantial P_{aCO_2} elevations (Fig. 11B, C).

Changes in blood lactate and ΔH^+m showed very different trends. In general, lactate rose considerably in the treatments where P_{aO_2} decreased and P_{aCO_2} increased (Al at higher pHs; Fig. 15), not in those where blood gasses showed little change (acidity alone, and Al at lower pHs). ΔH^+m exceeded the elevations in blood lactate in most treatments (Fig. 16). Thus H^+ ions from lactic acid production were generally not responsible for blood metabolic acidosis. For example, blood lactate increased by about 4 mM overall in the pH 5.2 plus Al exposures (Fig. 15, 16B), yet

Fig. 12. Arterial pH of cannulated rainbow trout during 42 h exposure to pH 5.2 or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 μg.L^{-1}) or absence of Al. See legend of Fig. 5 for other details.

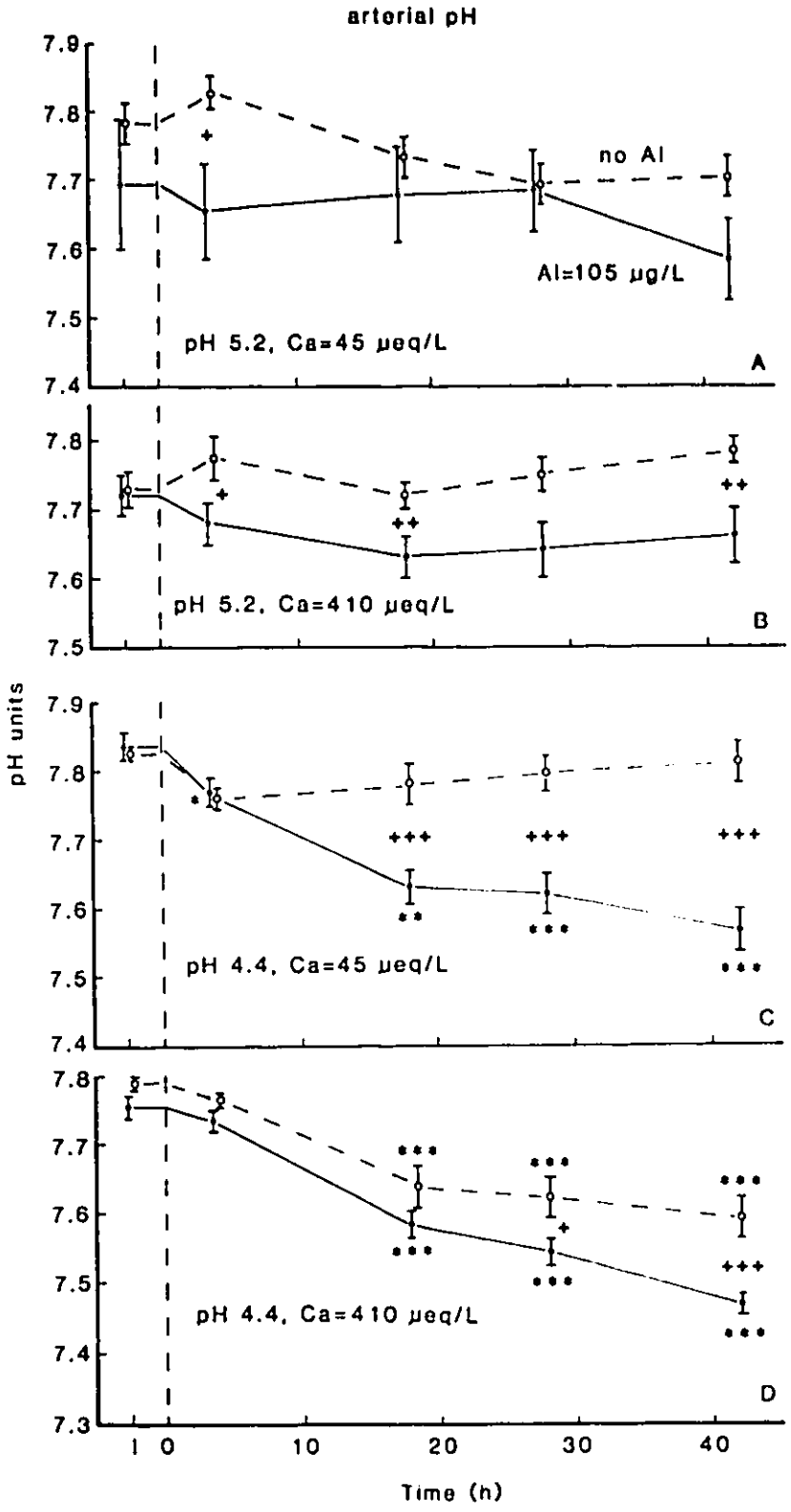

arterial pH
no Al
Al=105 µg/L
pH 5.2, Ca=45 µeq/L
A
pH 5.2, Ca=410 µeq/L
B
pH 4.4, Ca=45 µeq/L
C
pH 4.4, Ca=410 µeq/L
D
pH units
Time (h)

Fig. 13. Arterial plasma HCO_3^- concentrations of cannulated rainbow trout during 42 h exposure to pH 5.2 or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 μg.L^{-1}) or absence of Al. One torr = 0.133 kPascal. See legend of Fig. 5 for other details.

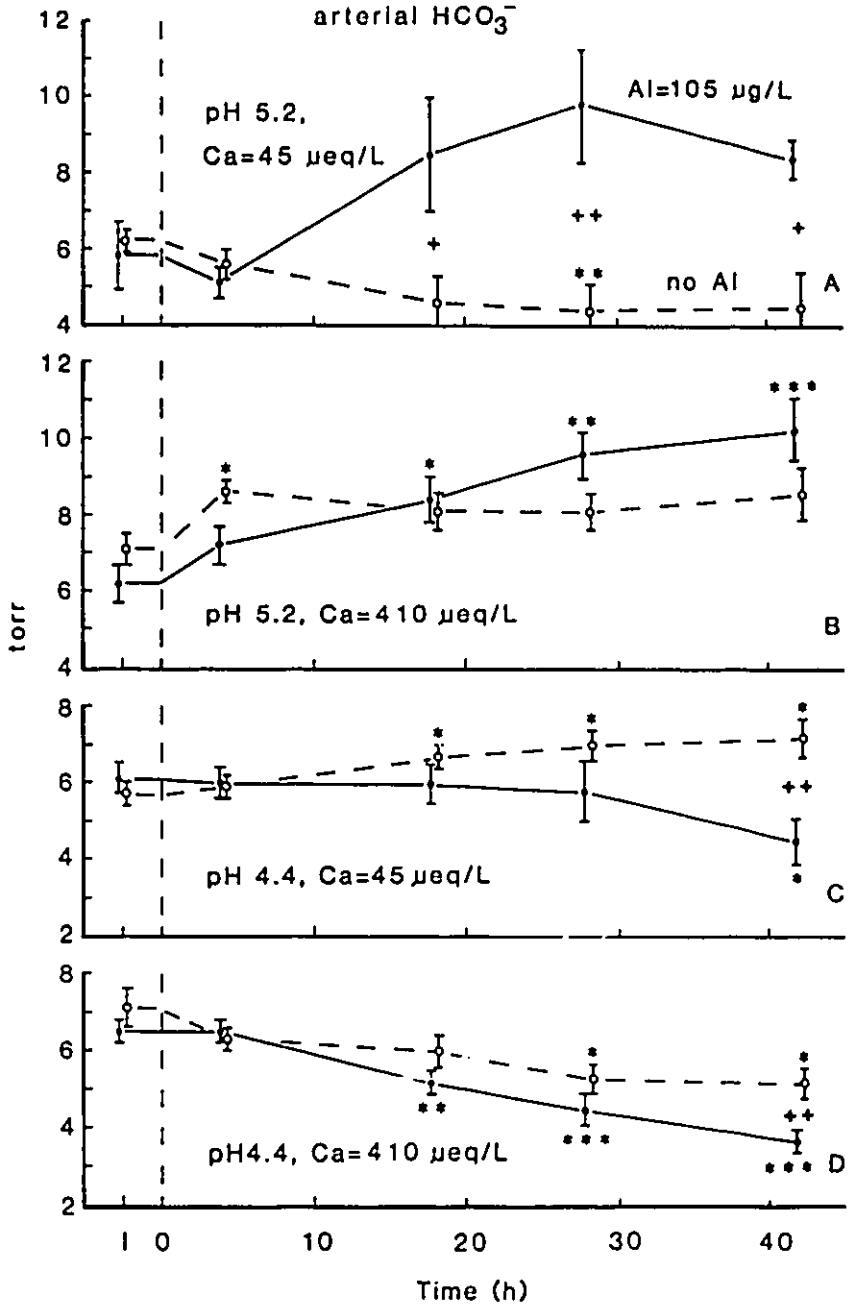
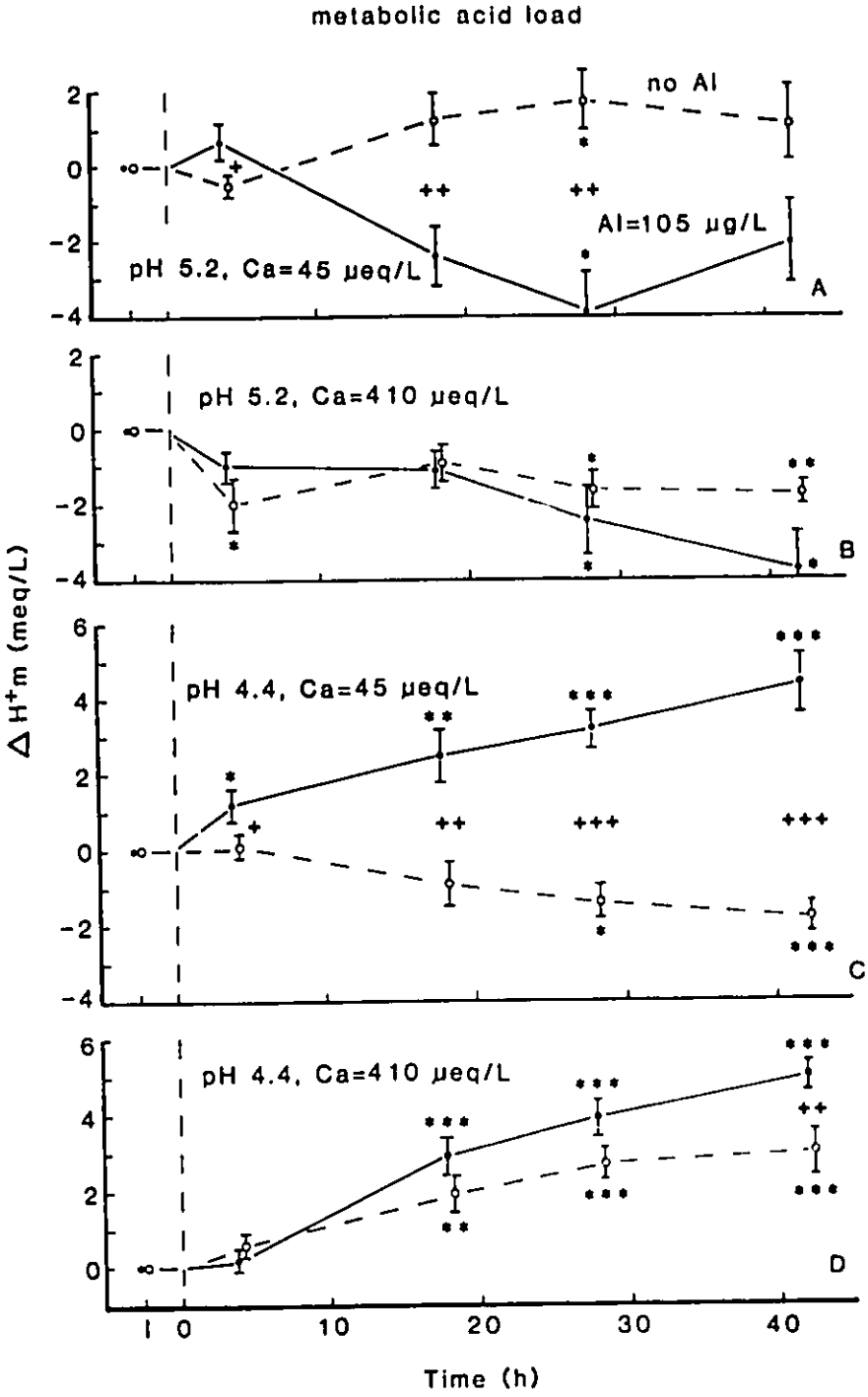

arterial HCO_3^-
pH 5.2,
Ca=45 µeq/L
Al=105 µg/L
no Al
A
pH 5.2, Ca=410 µeq/L
B
pH 4.4, Ca=45 µeq/L
C
pH4.4, Ca=410 µeq/L
D
torr
Time (h)

Fig. 14. Blood metabolic acid load of cannulated rainbow trout during 42 h exposure to pH 5.2 or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 μg.L^{-1}) or absence of Al. See legend of Fig. 5 for other details.


metabolic acid load
no Al
Al=105 µg/L
pH 5.2, Ca=45 µeq/L
A
pH 5.2, Ca=410 µeq/L
B
pH 4.4, Ca=45 µeq/L
C
pH 4.4, Ca=410 µeq/L
D
Δ H+m (meq/L)
Time (h)

(9)

Fig. 15. Blood lactate concentrations of cannulated rainbow trout during 42 h exposure to pH 5.2 or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 μg.L^{-1}) or absence of Al. See legend of Fig. 5 for other details.

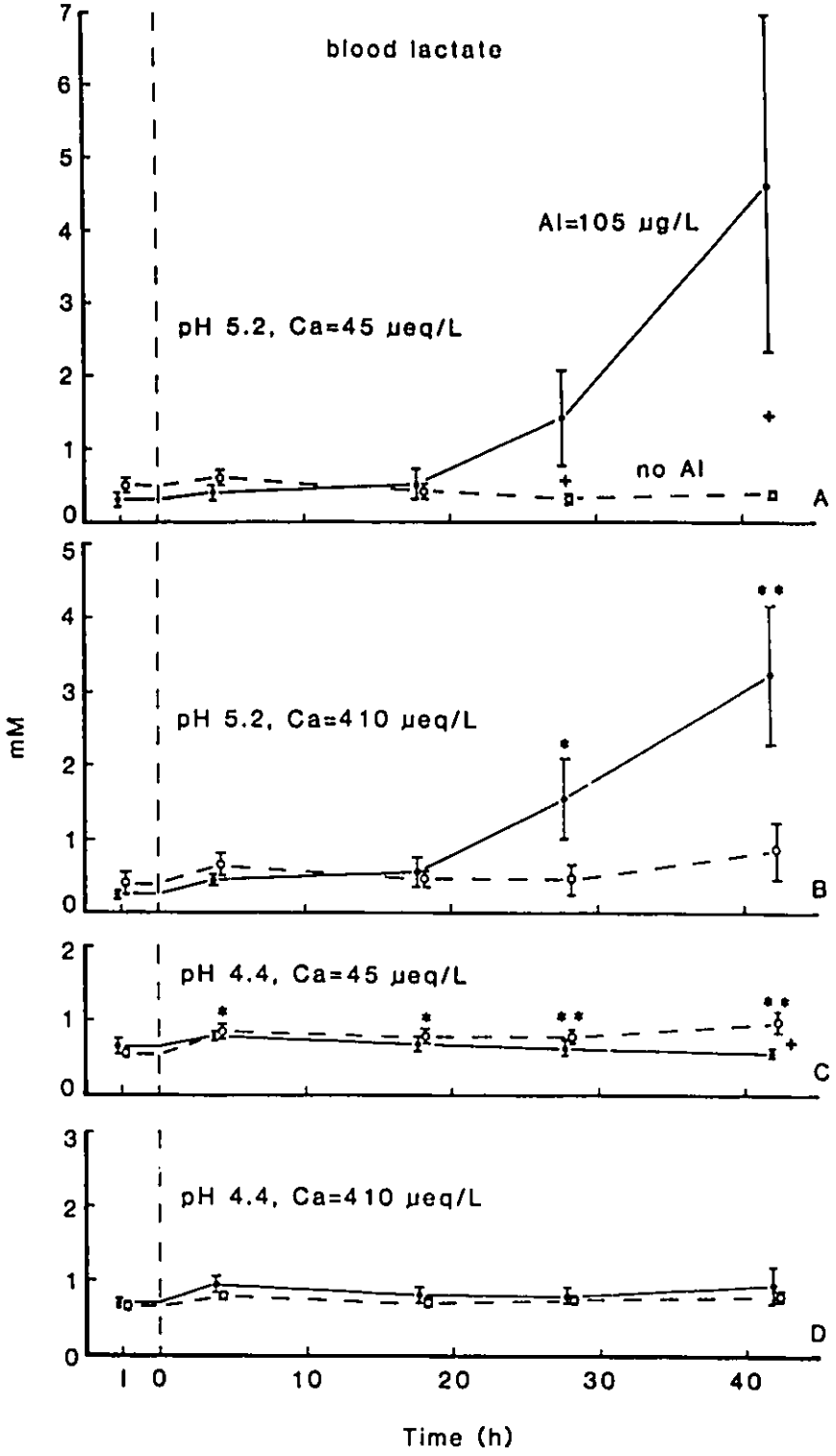
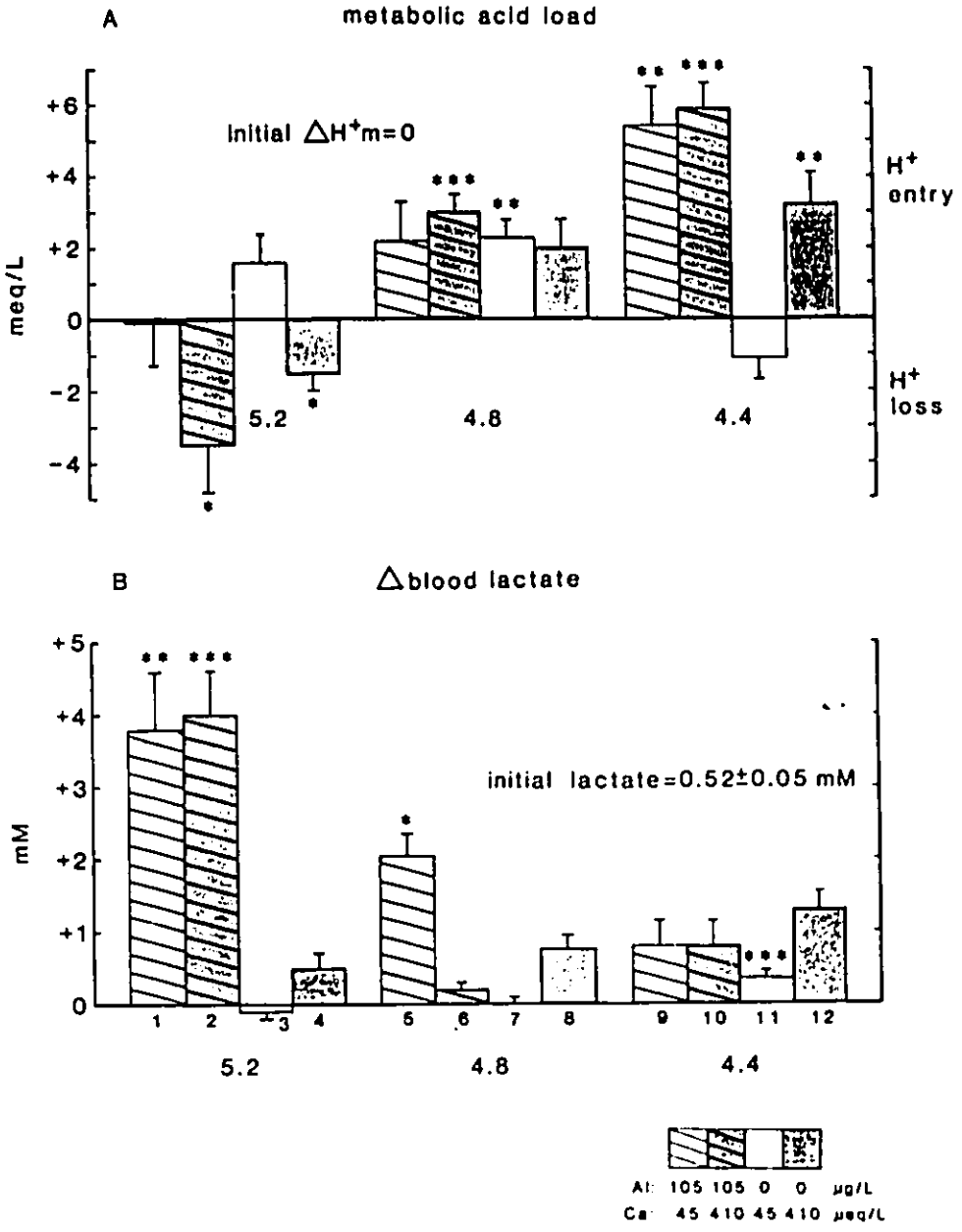

blood lactate
Al=105 µg/L
pH 5.2, Ca=45 µeq/L
no Al
A
pH 5.2, Ca=410 µeq/L
B
pH 4.4, Ca=45 µeq/L
C
pH 4.4, Ca=410 µeq/L
D
mM
Time (h)

Fig. 16. Terminal blood metabolic acid load and terminal changes in blood lactate for cannulated rainbow trout exposed to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv.L^{-1} Ca, in the presence (105 μg.L^{-1}) or absence of Al. Significant differences between treatments are indicated below. See legend of Fig. 7 for other details.

ΔH^+m: 10 9 12 6 7 5 8 3 1 11 4 2

lactate: 2 1 5 12 9 10 8 11 4 6 7 3


A
metabolic acid load
Initial ΔH+m=0
meq/L
+6
+4
+2
0
-2
-4
H+ entry
H+ loss
5.2
4.8
4.4
B
Δblood lactate
mM
+5
+4
+3
+2
+1
0
initial lactate=0.52±0.05 mM
1 2 3 4 5 6 7 8 9 10 11 12
5.2
4.8
4.4
Al: 105 105 0 0 µg/L
Ca: 45 410 45 410 µeq/L

blood acidification was low (Fig. 11C), and ΔH^+m was zero or negative. Here, lactate accumulation in the blood reflected anaerobic metabolism due to low Pa_{O_2}, but did not result in blood acidification.

Gill aluminum accumulation

In order to assess whether gill Al accumulation was correlated with physiological disturbances, gills were sampled from surviving fish at the end of some experiments. The number of samples was low, and some treatments were not sampled (i.e. pH 5.2 and 4.8 low Ca treatments, with or without Al). Nevertheless, at all three acidities gill Al concentrations were elevated in the presence of Al (Table 1). Higher water Ca reduced gill Al accumulation in the one available comparison, at pH 4.4.

Table 1. Gill Al concentrations (μg Al.g^{-1} wet tissue) in cannulated rainbow trout surviving 66 h exposure to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv.L^{-1} Ca^{2+}, in the presence (105 μg.l^{-1}) or absence of Al. Means ±1 SEM (n). Significant differences among exposures are indicated below the table. The numbers refer to the eight treatments, numbered from left to right; see legend of Fig. 7 for other details.

	pH 5.2		pH 4.8		pH 4.4			
Al (μg.L^{-1})	105	0	105	0	105		0	
Ca (μequiv.L^{-1})	410	410	410	410	45	410	45	410
Gill Al	20	5	18*	1	17**	4+	2	3
(μg.g^{-1})	±18	±2	±3	±1	±4	±4	±1	±2
	(2)	(4)	(4)	(3)	(5)	(6)	(5)	(7)

*, ** = significantly different ($P \leq 0.05$, $P \leq 0.01$; t-test, log(x+1) transformed data) from the comparable mean in the absence of Al.

\+ = significantly different ($P \leq 0.05$; t-test, log(x+1) transformed data) from the comparable mean at lower Ca (45 μequiv.L^{-1}).

3 5 1 2 6 8 7 4

Discussion

Mortality

Mortality of cannulated rainbow trout exposed to Al and acidity in combination was greatest at pH 5.2 and least at pH 4.4. Mortality was caused by a combination of respiratory and ionoregulatory toxicity. Respiratory toxicity was caused solely by Al and was greatest at higher pH, in contrast with ionoregulatory toxicity which was due to Al at pH 5.2 and 4.8 but caused mainly by acidity at pH 4.4. In general, Ca reduced both ionoregulatory and respiratory toxicity at lower pH, but not at pH 5.2.

Ionoregulatory responses

Ionoregulatory effects of acidity and Al on rainbow trout can be described using a simple model proposed by Wood and McDonald (1987). The presence of H^+ in the external environment inhibits active uptake of Na^+ and Cl^- at the gills, and stimulates passive effluxes through paracellular channels, perhaps by displacement of Ca^{2+} from the tight junctions (McDonald 1983a). These changes lead to net plasma Na^+ and Cl^- losses as seen in the present study in the pH 4.4 treatments. Acid-induced decreases in plasma ions have been reported in adult rainbow trout exposed to pH 4.0-4.8 in both soft and hard water (McDonald et al. 1980; McDonald and Wood 1981; McDonald, 1983b; Lee et al. 1983; Holeton et al. 1983a; Giles et al. 1984; Neville 1985), but not at more neutral pH.

In this model, Al also reduces active Na^+ and Cl^- uptake and increases Na^+ and Cl^- efflux, resulting in net ion losses at moderate pH where acidity alone causes little or no decrease in these ions (i.e. pH 5.2 and 4.8 treatments, Fig. 7). Previous reports of Na^+ and Cl^- losses in adult salmonids exposed to Al in soft water at physiologically "safe" pHs include Muniz and Leivestad (1980), Neville (1985), Booth et al. (1988), Goss and Wood (1988), Wood et al. (1988a), and McDonald et al. (submitted). At moderate acidities (pH-5), Al accumulation at the gills may lead to ion losses because of inflammation, cell swelling, and distortion of the branchial epithelium, resulting in increased paracellular permeability. Gill damage of this nature attributed to Al in the pH range 5-6 has been observed through both light and electron microscopy in several fish species (Chevalier et al. 1985; Malte 1986; Karlsson-Norrgren et al. 1986a,b; Youson and Neville 1987; Goossenaerts et al. 1988; Mueller et al. submitted).

The present study showed little if any effect of Al on plasma Na^+ and Cl^- losses due to acidity alone (pH 4.4 treatments, Fig. 7). The transition between harmful effects of Al on ionoregulation at higher pH to benign effects at lower pH may be due to greater Al precipitation at the gills at pH 5-6 where Al solubility is lowest (Roberson and Hem 1969), or to a change in toxicity as the Al species shift from Al-hydroxides (higher pH) to the Al^{3+} cation (lower pH). These possibilities are explored further in Chapters 4 and 5. At very low pH, Al^{3+} may be

protective (eg. Muniz and Leivestad 1980; Neville 1985; Chapter 5; pH~4.0), perhaps through its ability to mimic the effects of Ca^{2+} on limiting membrane permeability (Baker and Schofield 1982). However, in the present experiments, as in those of Neville (1985) and Witters (1986) at pH 4.0-4.1 using rainbow trout, the presence of Al never reduced ionoregulatory disturbances caused by acidity alone. Besides depending on pH, the protective effects of Al in acidic water may vary by species or be related to stage of fish development (eg. Baker and Schofield 1982).

Calcium reduced Cl^- losses due to acidity alone (pH 4.4), but did not reduce Cl^- losses caused by Al at higher pH (Fig. 7A). Calcium did not reduce Na^+ losses caused by acidity alone, and may even have worsened Na^+ losses caused by Al (Fig. 7B). The differential effect of Ca in reducing acid-induced Cl^- loss more than acid-induced Na^+ loss has been reported before (summarised by McDonald 1983a; Wood 1989), and is attributed largely to differential effects on the passive efflux components rather than the active uptake components of branchial Na^+ and Cl^- exchange. The inability of Ca to reduce the effects of Al on ion losses is likely related to the specific toxic action of Al at the gill membranes: if gill inflammation and damage caused by Al accumulation at the gills are responsible for Al-induced ion losses, it is probable that these effects of Al are not ameliorated by Ca.

Sodium and Cl^- ion losses are accompanied by fluid volume shifts out of the plasma into muscle because extracellular osmolarity decreases faster than intracellular osmolarity (Milligan and Wood 1982). Observed increases in plasma concentrations of K^+, Ca^{2+}, and protein (Fig. 8A, B, C) were likely a result of decreased plasma volume, which would translate into higher concentrations of these parameters. Increased plasma K^+ may also be related to acidosis, because intracellular K^+ is released from muscle as H^+ enters (Ladé and Brown 1963).

Mean cell hemoglobin concentration (MCHC) usually decreased as plasma Na^+ and Cl^- decreased (Fig. 8D). The decrease in MCHC was likely associated with entry of fluid into the red blood cells in response to osmotic disequilibrium caused by the decrease in plasma ions. In addition, mobilization of catecholamines into the blood may also have promoted red cell swelling (Vermette and Perry 1988). Preliminary work has shown large but transitory increases in plasma epinephrine and norepinephrine in rainbow trout exposed to 105 $\mu g.L^{-1}$ Al at pH 4.8 (G.G. Goss, R.C. Playle, and C.M. Wood, unpubl. results). Such increases in plasma catecholamines were probably responsible for the observed elevations of plasma glucose concentrations (Fig. 8E; Perry et al. 1988). Glucose mobilization is a commonly observed response to general stress in fish, and during acid and Al exposures glucose may be particularly useful as a method for supplementing plasma osmolarity in the face of ion loss (McDonald 1983b; Goss and Wood 1988).

Respiratory responses

Respiratory toxicity in rainbow trout was caused by Al but not by acidity alone, and was worse at higher pH (Fig. 11A, B). In the simple model (Wood and McDonald 1987), the branchial epithelium becomes inflamed, swollen, and coated with mucus as Al precipitates on the gills. Inflammation and cell swelling would decrease gas transfer because of increased diffusion distance across the gills. Mucus accumulation would also decrease gas transfer because of lower diffusion through mucus and an increased boundary layer (Ultsch and Gros 1979).

Accumulation of Al on gills has been reported by Neville (1985), Chevalier et al. (1985), Harvey and McArdle (1986), Lee and Harvey (1986), Karlsson-Norrgren et al. (1986a,b), Jensen and Weber (1987), McCahon et al. (1987), Youson and Neville (1987), Goossenaerts et al. (1988), Handy and Eddy (1989), and the present study (Table 1). Accumulation of mucus on gills, or increased mucus production at the gills during exposures to Al have been reported by Muniz and Leivestad (1980), Rosseland (1980), Harvey and McArdle (1986), Lee and Harvey (1986), Karlsson-Norrgren et al. (1986a), Jensen and Weber (1987), McCahon et al. (1987), and by Handy and Eddy (1989). Overall, these studies suggest that gill mucification, Al accumulation, and damage are worst between about pH 5 to 6, where Al solubility is low (Fig. 1) and Al precipitation on the gills would be expected to be high. Furthermore, ammonia excretion at the gills probably raises the pH

of the branchial micro-environment (Wright and Wood 1985), intensifying Al precipitation as the solubility of Al is exceeded. Alternatively, alkalinization of the gill micro-environment could alter Al speciation to species of Al which better bind to the gills. These topics are examined experimentally in Chapters 3, 4, and 5.

Calcium reduced respiratory disturbances in the pH 4.8 and 4.4 treatments but not at pH 5.2 (Fig. 11A, B). As discussed earlier, Ca did not ameliorate ionoregulatory disturbances caused by Al at higher pH (Fig. 7). Gill Al data for the pH 4.4 treatments (Table 1) suggest that Ca may have reduced respiratory disturbances caused by Al at lower pHs by reducing Al accumulation on the gills. Unpublished rainbow and brook trout fingerling data also suggest that Ca can reduce gill Al accumulation (D.G. McDonald and C.M. Wood, pers. comm). In addition, brook trout yolk-sac fry and swim-up fry at pH 4.8 and 5.2 accumulated less Al in water of higher Ca than lower Ca (Wood et al. 1989a, b).

How Ca reduces Al accumulation at the gills is not known, but perhaps Ca competes with Al for its binding sites. Curiously, this postulated effect of Ca on Al binding at the gills did not reduce the respiratory effects of Al in the pH 5.2 treatments, possibly because Al precipitation at that pH may simply be too great to be ameliorated by Ca (see Chapters 4 and 5). Equally curious is why the respiratory toxicity of Al in the pH 4.8 treatments was reduced by Ca but the ionoregulatory toxicity was

not. Perhaps only a small amount of bound Al is needed to cause gill inflammation and thereby ion losses, and larger amounts of precipitated Al are necessary to impair gas transfer through cell swelling and mucus accumulation.

A further complication is that 400 μequiv.L^{-1} Ca^{2+} worsened the respiratory effects of 330 $\mu g.L^{-1}$ Al at pH 4.8 in brook trout (Wood et al. 1988a) but reduced the respiratory effects of 105 $\mu g.L^{-1}$ Al at pH 4.8 in rainbow trout (this study). Whether there is some basic difference between gills of the two species (brook trout are more resistant to acidity and Al; Grande et al. 1978) or whether this contrast is solely a result of the different Al concentrations used remains to be seen.

Acid-base responses

Calculations of metabolic acid load (Fig. 14, 16A) indicated that blood acidification in the pH 4.4 and 4.8 treatments was caused mostly by metabolic acid entry, probably from the acidic water. In agreement with theory (Wood 1989) and previous experimental results on rainbow trout (McDonald et al. 1980; McDonald and Wood 1981; McDonald 1983b), apparent H^+ entry at pH 4.4 (no Al) was associated with higher water Ca concentration. This effect is explained by a differential action of Ca^{2+} on Na^+ and Cl^- losses at low pH, which constrains net H^+ entry through the "strong ion difference" relationship (Stewart 1978). In simple terms, any excess of Na^+ over Cl^- loss to the water is made

up by H^+ entry, resulting in blood acidification. In the high Ca, pH 4.4 treatment, Al worsened metabolic acid load, and therefore blood acidification (Fig. 11C, 16A), possibly because Al enhanced the Ca-induced effect of greater Na^+ over Cl^- losses (Fig. 7). Aluminum also worsened metabolic acid load and blood acidification in the low Ca, pH 4.4 treatment, but Na^+ and Cl^- losses from the blood were of similar magnitude.

Impaired gas transfer in Al-exposed rainbow trout caused CO_2 accumulation in the blood, especially at higher pH (Fig. 11B), which by itself would decrease arterial pH (respiratory acidosis; Davenport 1974). Blood acidification in the Al treatments at pH 5.2 was due solely to respiratory acidosis, and respiratory acidosis added to the metabolic acidosis already present in the pH 4.8 plus Al treatments, and in the pH 4.4, low Ca, Al treatment (Fig. 11B, C). Neville (1985), Malte (1986), Jensen and Weber (1987), Malte and Weber (1988), and Wood et al. (1988a) have also demonstrated respiratory acidosis caused by Al. The pH 5.2, 105 $\mu g.L^{-1}$ Al, high Ca treatment is interesting because a metabolic alkalosis (Fig. 16A) counteracted the respiratory acidosis due to arterial CO_2 build-up (Fig. 11B), resulting in only minor blood acidification (Fig. 11C). Metabolic alkalosis was also seen in this treatment in the absence of Al. The causes of these alkaloses remain unknown.

Small increases in P_{aCO_2} in fish not exposed to Al (Fig. 10C, D; Fig. 11B) were not due to excess CO_2 in acidified water because

the head tank and fish boxes were well aerated, and measured water P_{CO_2} stayed below 1 torr. Increased P_{aCO_2} in these fish may have been a result of catecholamine mobilization (Milligan and Wood 1982), which would raise both P_{aO_2} and P_{aCO_2}, the latter possibly by inhibiting HCO_3^- dehydration through the red blood cells (Wood and Perry 1985; Vermette and Perry 1988).

Lactate production reflected anaerobic metabolism caused by hypoxemia during Al exposures. For example, in the pH 5.2 plus Al treatments blood lactate concentrations increased sharply when P_{aO_2} fell below 40 torr and P_{aCO_2} increased above 4 torr, conditions where the blood was probably only 40-70% saturated with oxygen (Cameron 1971b). However, metabolic acid load was lowest when blood lactate was highest (Fig. 16). Lactate production did not cause blood metabolic acidosis, probably because H^+ ions from lactic acid were retained in muscle and not released into the bloodstream (Wood and Perry 1985). Neville (1985) also reported high (5 mM) blood lactate concentrations, probably a result of anaerobic metabolism (arterial blood O_2 saturation was about 40%) in her pH 6.1 plus Al treatments.

In summary, the results of this Chapter have demonstrated that rainbow trout exposed to an environmentally realistic concentration of Al in soft water develop Na^+ and Cl^- losses at pH 5.2 and 4.8, pHs where ion losses due to acidity alone are negligible. However, at pH 4.4 the presence of Al does not add to ion losses caused by acidity alone. Aluminum causes respiratory

disturbances that are not seen with acidity alone; these disturbances are greatest at higher pH. At higher pH, Al deposition at the gills likely causes gill inflammation and damage, leading to ion losses and gas transfer impairment. These effects of Al at the gill surface (inflammation and damage) are distinct from the effects of H^+ alone, which probably increases gill permeability by displacing Ca^{2+} from the tight junctions.

Acid-base disturbances are a combination of metabolic acidosis caused by entry of acidic equivalents from the water and, at higher pH, respiratory acidosis caused by P_{CO_2} build-up. Calcium reduces Cl^- losses caused by acidity alone, but worsens blood acidosis at pH 4.4 through unequal effects on net Na^+ and Cl^- fluxes. Calcium does not ameliorate ion losses caused by Al at higher pH. Calcium also reduces the respiratory effects of Al at lower pH but not at higher pH, where Al precipitation may be too high to be ameliorated by Ca. The next step to better understanding of the effects of acidity and Al at the gill is to study the gill micro-environment itself, concentrating on how the pH at the gill affects Al precipitation and speciation. These topics are explored in Chapters 3, 4, and 5.

Chapter 3

Water chemistry changes in the gill micro-environment of rainbow trout: experimental observations and theory

Introduction

Fresh water fish modify the water they breathe by extracting oxygen and ions, and releasing carbon dioxide, ammonia, and other metabolic endproducts at the gills. Transfers of carbon dioxide and ammonia may acidify or alkalinize expired water, respectively, depending on water pH (Lloyd and Herbert 1960; Randall and Wright 1989). In well buffered water the changes in pH at the gills may be small, but in poorly buffered soft water the pH changes at the gills could be large.

Accordingly, any environmental contaminant whose toxicity varies with pH could be more or less toxic at the gills than would be predicted from bulk water pH, because the different pH at the gills could change toxicant species or solubility. This situation was originally proposed to explain the toxicity of ammonia (Lloyd and Herbert 1960; Szumski et al. 1982), and later aluminum (Neville 1985; Booth et al. 1988; Chapter 2) at fish gills, although the relevant experimental measurements have not yet been made.

Before exploring the effects of pH changes at the gills on the toxicity of Al, through alterations of Al solubility and

speciation near the gills (Chapters 4 and 5), it was important to study the general mechanisms causing pH changes in the gill micro-environment. Recently, Wright et al. (1986) developed a method to measure water pH near the gills of rainbow trout during normal ventilation. The method consisted of fish fitted with latex ventilation masks, to separate inspired water from expired, and catheters placed within the opercular cavity to draw water from the gill micro-environment. Once collected, water samples from near the gills could be analysed for virtually any parameter of interest.

The objectives of the present study were (i) to use the ventilation mask and opercular catheter technique to quantify changes in pH occurring at rainbow trout gills over a wide range of inspired water pH (4.0-10.1) in a defined soft water of low buffer capacity, (ii) to determine the accompanying carbon dioxide, ammonia, and titratable base transfers at the gills, and (iii) to model the system using classical aquatic chemistry and the measured acid and base transfers.

Materials and methods

Experimental animals and water

Adult rainbow trout of both sexes, weight = 281±6 g (mean ±1 SEM, n=52), were purchased from Spring Valley Trout Farm, New Dundee, Ont. They were held in dechlorinated Hamilton city tapwater at 15-20°C and were fed floating trout pellets twice weekly. At least two weeks before an experiment the fish were placed in a flowing soft water acclimation tank and feeding was suspended. Production of soft water and routine water chemistry monitoring is described in Chapter 2. Acclimation conditions in the standard soft water were Ca^{2+} ~47 μequiv.L^{-1}, Na^{+} ~68 μequiv.L^{-1}, Cl^{-} ~95 μequiv.L^{-1}, titratable alkalinity ~130 μequiv.L^{-1}, pH~6.7, at 15°C.

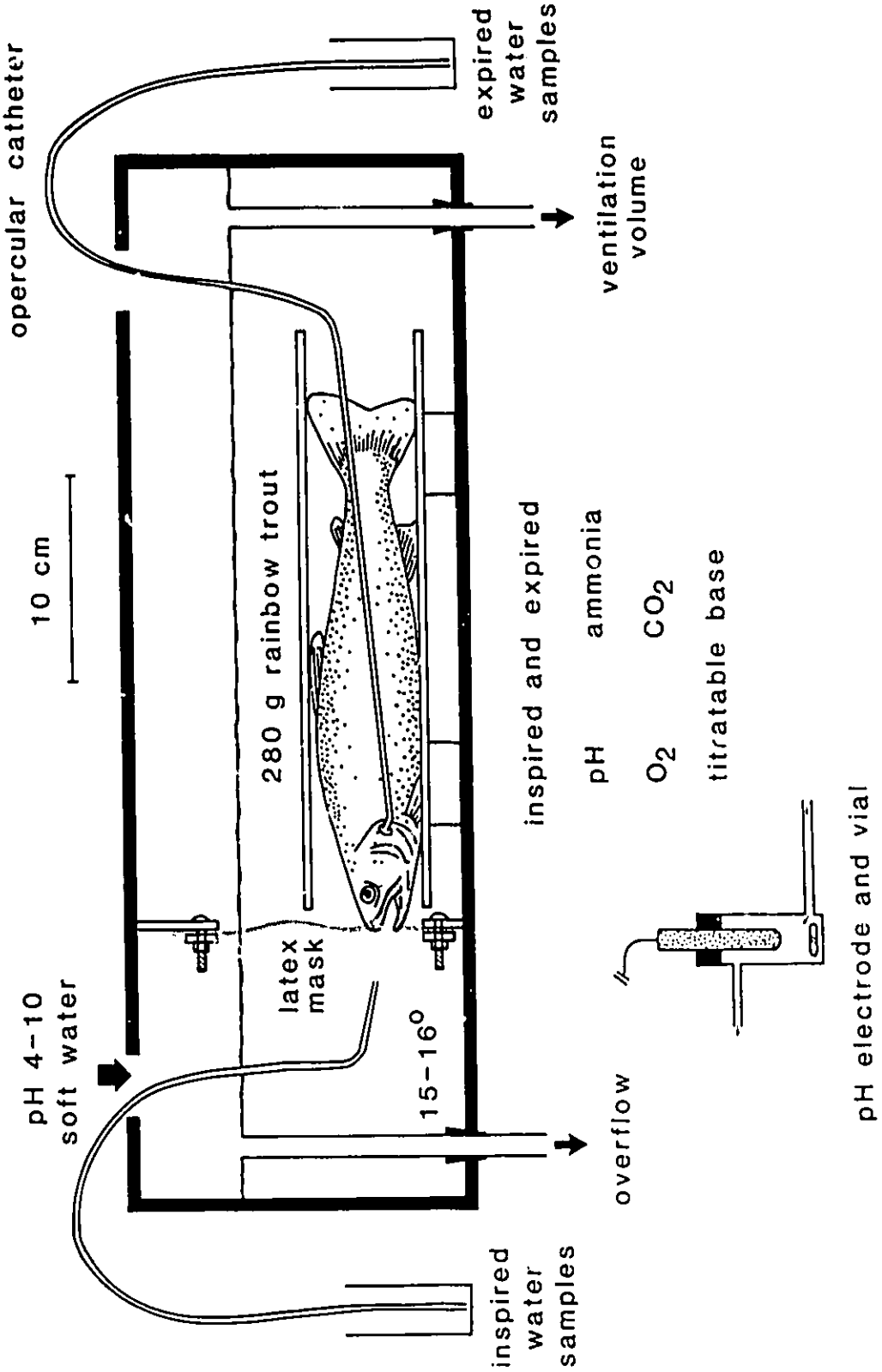
For the operations, fish were initially anaesthetised with 0.5 mg.L^{-1} MS222 (Sigma, Saint Louis, Missouri) buffered to pH ~6.5 with KOH, then three-quarter strength anaesthetic was used during the rest of the operation. A latex surgical glove with thumb and fingers removed was sewn around each fish's mouth to serve as a ventilation mask; the thumb hole fitted over the fish's head (Fig. 17; see Cameron and Davis 1970, and Wright et al. 1986 for more details). After this operation was completed, a hole was punched with a 2 mm O.D. trochar about 1 cm from the posterior margin of an operculum, and an 85 cm piece of Clay-Adams PE-190 polyethylene tubing was threaded through the hole. The tubing was

heat-flared, and the flange rested against the inside of the operculum. A 0.5 cm flanged piece of PE-240 tubing was placed against the outside of the operculum, and was held tightly in place by a right angle bend in the catheter near the operculum and by a knot of surgical silk. The opercular catheters stayed in place well and did not appear to hinder normal opercular movements. Affixing a mask and catheter took a total of about 45 min.

Fish fitted with latex masks and opercular catheters were placed individually in one of 5 darkened and compartmentalised Plexiglas boxes (Fig. 17). These ventilation collection boxes were identical in design to those described by Cameron and Davis (1970). The latex masks were fitted over pegs on a retaining ring which formed a seal between anterior and posterior chambers of the boxes. Water breathed by the fish passed from the anterior chamber, over the gills, into the posterior chamber. Standpipes in each chamber were set so that there was no pressure differential between the two chambers. Vigorously-aerated water flowed from a headtank into the anterior chamber of each fish box at a rate greater than the fish's ventilatory demand, then overflowed to waste.

After 24 h the opercular catheters were tested to ensure they would provide an adequate flow (2-4 $mL.min^{-1}$) by siphon, and to ensure they were sampling from a site which provided a representative oxygen extraction (cf. Davis and Watters 1970). If

Fig. 17. Illustration of a rainbow trout fitted with a latex ventilation mask and opercular catheter. The ventilation mask divides the box into an anterior and posterior chamber. Water breathed by the fish passes into the posterior chamber of the box; overflow from this chamber is fish ventilation volume. The opercular catheter siphons water from within the gill micro-environment. Parameters measured in inspired and expired water are indicated.


opercular catheter
expired water samples
ventilation volume
10 cm
280 g rainbow trout
inspired and expired
pH
ammonia
O_2
CO_2
titratable base
latex mask
15-16°
pH 4-10 soft water
overflow
inspired water samples
pH electrode and vial

the difference between inspired and expired O_2 concentrations was below about 20 μM the catheter was repositioned on the operculum (a 15 min operation); this was done to avoid using a catheter that was drawing water from an anatomical dead space. The fish were allowed to recover for ~48 h after the initial operations, and ~24 h after any catheter reimplantations, before an experiment was begun.

Experimental protocols

Inspired pH of the standard soft water was varied using either 0.5 M H_2SO_4 or 1 M KOH, delivered to the head tank by a magnetic valve controlled by a Radiometer PHM82 pH meter and Radiometer GK2401C combination electrode. Water was vigorously aerated to ensure that inspired P_{O_2} (>140 torr) and P_{CO_2} (<1 torr) remained constant.

Five fish at a time were exposed to basic or acidic soft water for 2-3 h, measurements taken, then another pH for another 2-3 h. The order of exposures was random, apart from extreme pHs. In general, fish recovered from moderate acid or base exposures quickly (pH 5.0-9.0); as a precaution exposures to extreme pHs (<5.0, >9.0) were done at the end of the day, followed by a return to circumneutral pH overnight, as a recovery period. Soft water Ca^{2+} and Na^{+} concentrations averaged 54±1 and 63±1 μequiv.L^{-1} (±1 SEM; n=97), respectively, over the course of the experiments; experimental temperatures were 15-16°C.

Analytical methods

Opercular catheters siphoned water from near the gills at a rate of 2-4 $mL.min^{-1}$; inspired water was similarly siphoned from the anterior chamber of each fish box using PE-190 tubing. Siphoned water flowed continuously through a closed 7.5 mL polyethylene vial into which was sealed a Radiometer GK2401C pH electrode; the actual volume of water surrounding the pH electrode was about 4.5 mL (see Fig. 17). A Radiometer PHM82 pH meter was used. Siphoned water was stirred continuously with a magnetic flea and flowed through the vial and out another port. Alternate inspired and expired water pH measurements were made using the same pH electrode. After drainage of the vial for a new sample, and the vial had refilled, three minutes were allowed to elapse for thorough flushing. Usually the pH reading stabilized by one min, which corresponded to total delay in the system, including flow from gills to vial. Mean pH in a continually flowing system was measured, so electrode response time was not a complication.

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 ($\dot{V}_w$) for each fish was measured as the water volume overflowing from the posterior chamber of the ventilation collection boxes in 1 min (see Fig. 17). Oxygen tension of inspired and expired water was measured immediately on samples drawn anaerobically from the pH vial and injected into a Radiometer E5046 micro-electrode unit kept at 15°C and connected to a Radiometer PHM72 acid-base analyzer. Water P_{O_2} values (torr) were converted to O_2 concentrations (μM) using the solubility of O_2 at 0% salinity (Boutilier et al. 1984). Inspired water was always near saturation at 15°C (~300 μM). Ammonia samples were collected by filling 7.5 mL polyethylene vials from the inspired and expired siphons. Samples were frozen and later thawed and analyzed using the salicylate-hypochlorite method (Verdouw et al. 1978).

Samples for CO_2 analysis were collected via the siphons into 2 mL glass vials, capped tightly, and analyzed within 4 h. One mL aliquots of sample were mixed in a 5 mL glass syringe with 0.5 mL HCl (0.1 M) and 4.5 mL helium, to liberate all CO_2 as gas. The gas was injected into a Shimadzu GC-8A gas chromatograph with Shimadzu C-R3A integrator. 0.0, 0.1, 0.2, and 0.5 mM $NaHCO_3$ standards were used. There was no indication of CO_2 production or consumption during sample storage (<4 h) in the glass vials.

Titratable base (=acid neutralizing capacity) of inspired and expired water samples was measured by titrating 10 mL samples to pH 4.0 with 0.02 M HCl using Gilmont microburettes. The samples

were at room temperature and were bubbled with air during the titrations. Total titration time was 14 min, to allow adequate time for the conversion of HCO_3^- to CO_2, and its subsequent diffusive loss. Titratable base measures strong bases in water and excludes alkalinity due to dissolved CO_2.

Data have generally been expressed as the difference between expired and inspired values for a given parameter, and are referred to as "Δ" or "transfer", eg. ΔpH, ammonia transfer. Oxygen transfer, ammonia transfer, and ΔpH were determined in all experiments. Measurements of CO_2 and titratable base transfer are more difficult and time consuming, so were done only in a subset of experiments representative of the inspired pH range as a whole.

Titration curves in vitro of the acidic or basic water at 15°C were determined on 10 mL, stirred samples using 0.02 M HCl or NH_4OH, delivered by Gilmont microburettes. An electrode equilibration time of 3 min between each addition of titrant was used. Samples at all pH_{in} starting values were titrated down by about 2 pH units with HCl. Samples at pH_{in} values below ~6.5 were also titrated up with NH_4OH by about 2 pH units, to determine the effects of both base and acid additions at these pHs. Stirring, instead of aerating samples was done in order to assess all the buffering in the water, including HCO_3^-.

Inspired and expired water samples from three fish were also titrated, to assess buffering effects of substances released at

the gills. In this instance inspired (circumneutral) water and expired water were brought to pH 4.0 quickly with HCl, then the titration curves determined up to pH~8.5 with NH_4OH additions, as above. Expired and inspired water samples from these three fish were also analysed for anions released at the gills. High pressure liquid chromatography (HPLC; Waters 510 pump, Waters 430 conductivity detector, and Waters IC-Pak anion exchange column) was used to assay for phosphate (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$), Cl^-, NO_2^-, NO_3^-, and SO_4^{2-} in 100 µL filtered samples of inspired or expired water.

In order to determine the effect of CO_2 additions on the pH of the standard soft water in vitro (in relation to model predictions), samples at various pH_{in} values were bubbled for 1-3 min with 0.3% CO_2 (in air) at 15°C. A Wösthoff 301-AF gas mixing pump was used. The aim was to achieve a ΔCO_2 approximately equivalent to that observed in vivo (~100 µM; see Results), and of course this was reached more quickly at the higher starting pHs. In practice, a variety of gassing durations were tried, and those producing the ΔCO_2 closest to 100 µM were used. The CO_2 samples and the pH readings were taken simultaneously. Carbon dioxide in the water was measured in the usual manner by gas chromatography; ΔpH was measured using a Radiometer GK2401C pH electrode and Radiometer PHM82 pH meter.

Experimentally observed data are presented in the Figures as means ±1 SEM (n). When experimental data were recalculated for

the modelling exercise, or when model predicted curves were compared with experimentally observed values, 95% confidence limits around the means of the observed data have been used. For these data, 95% confidence limits are 2.1-2.8x larger than 1 SEM. The least squares method was used for linear regression calculation.

Results

Gill water chemistry and ventilation

Rainbow trout were exposed to acidic and basic soft water to study the influence of inspired water pH on ventilation volume, oxygen consumption, ammonia excretion, and pH changes in water passing over the gills. Sixteen different soft water pHs in the pH range 4.0 to 10.1 were examined. Data from all 52 trout are included in these analyses (Fig. 18, 19).

Expired water drawn from near the gills of rainbow trout was more basic than inspired soft water if the inspired water was acidic (pH_{in}=4-6; Fig. 18). For example, expired water was about 0.7 pH units higher (i.e. pH-5.7) than inspired water of pH-5. Expired water was more acidic than inspired water if the inspired water was circumneutral or basic (pH_{in}=6-10; Fig. 18). For example, if inspired water was about pH 9 then expired water was about pH 7.3 (ΔpH--1.7). The ΔpH was low (+0.1) at pH_{in}-4, most positive at pH_{in}-5 (+0.7), zero near pH_{in}=6, most negative at pH_{in}-9 (-1.7), and was only about -0.7 at pH_{in}-10 (Fig. 18).

Ventilation volume ($\dot{V}_w$) of the fish was approximately constant (-0.37 $L.kg^{-1}.min^{-1}$) over the inspired soft water pH range 4.6 to 10.1 (Fig. 19A). However, $\dot{V}_w$ increased 1.6-fold at pH_{in}=4.4 and 2.4-fold at pH_{in}=4.0. Oxygen consumption by the fish ($\dot{M}_{O_2}$), measured as oxygen transfer at the gills (inspired

[O_2]-expired [O_2]) multiplied by $\dot{V}_w$, was about 1.7 mmol.kg^{-1}.h^{-1} over the same pH_{in} range 4.6 to 10.1 (Fig. 19B). Oxygen transfer at the gills was also approximately constant at about 85 μM. In accord with the increase in $\dot{V}_w$ at very low pH, $\dot{M}_{O_2}$ at pH_{in}<4.6 was ~1.6x higher than over the rest of the pH_{in} range. Oxygen transfer at the gills decreased only to 50 μM at pH_{in}=4.0 (Fig. 20), in spite of the 2.4-fold increase in $\dot{V}_w$, which resulted in the net increase in $\dot{M}_{O_2}$ at this inspired pH.

Ammonia excretion ($\dot{M}_{amm}$) was also approximately constant (~300 μmol.kg^{-1}.h^{-1}) over the pH_{in} range 4.6 to 10.1 (Fig. 19C; ammonia transfer ~15 μM), and increased ~1.7x at pH_{in}<4.6. As for oxygen transfer, mean ammonia transfer at the gills decreased only to 8 μM at pH_{in}=4.0 (Fig. 20) in spite of the 2.4x increase in $\dot{V}_w$, so a net increase in $\dot{M}_{amm}$ at this pH_{in} was seen. In summary, $\dot{V}_w$, $\dot{M}_{O_2}$, and $\dot{M}_{amm}$ were remarkably constant over a wide range of inspired pH during these relatively short (2-3 h) exposures, and increased only under very acidic conditions (pH_{in}<4.6).

Changes in water chemistry at the gills were examined in greater detail in 10 trout exposed to soft water of seven different inspired pHs. In these fish CO_2 and titratable base transfers to the water were measured, as well as the usual O_2 and ammonia measurements. Mean CO_2 and O_2 transfers at the gills of these fish were about 100 and 85 μM, respectively, excluding values from pH_{in}=4.0 (Fig. 20). Oxygen and CO_2 transfers at the gills were lower at pH_{in}=4.0, associated with the higher

Fig. 18. The difference between pH of expired (pH_{ex}) and inspired soft water (pH_{in}) is plotted against inspired water pH for rainbow trout fitted with opercular catheters and latex masks. Positive ΔpH: expired water is more basic than inspired water; negative ΔpH: expired water is more acidic than inspired water. Means ±1 SEM are indicated. A total of 52 trout were exposed in fish boxes in a flow-through system to water of different acidities for 2-3 h. Mean number of fish represented at each point is 12; minimum number is 5 (pH_{in}=5.2, 5.8, 7.0, 8.2); and the maximum number represented is 26 (pH_{in}=6.5).

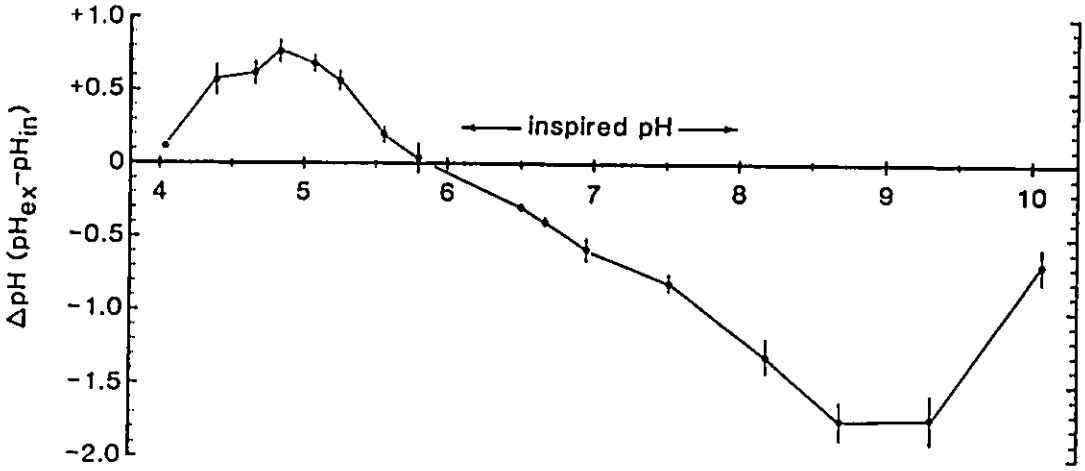

+1.0
+0.5
0
-0.5
-1.0
-1.5
-2.0
ΔpH (pHex-pHin)
inspired pH
4
5
6
7
8
9
10

Fig. 19. A) Ventilation volume ($\dot{V}_w$) of rainbow trout fitted with latex masks, in acidic and basic soft water. $\dot{V}_w$ was approximately constant in the pH_{in} 4.6-10.1 range, but was about 1.6x and 2.4x higher at pH_{in}=4.4 and 4.0, respectively. Means ±1 SEM; number of fish at each point as given in Fig. 18.

B) Oxygen consumption ($\dot{M}_{O_2}$) of rainbow trout in acidic and basic soft water. $\dot{M}_{O_2}$ was approximately constant at pH_{in} 4.6-10.1, but increased by about 1.6x at pH_{in}<4.6. Means ±1 SEM; number of fish at each point as given in Fig. 18.

C) Ammonia excretion ($\dot{M}_{amm}$) of rainbow trout in acidic and basic soft water. $\dot{M}_{amm}$ was approximately constant at pH_{in} 4.6-10.1, but was about 1.7x higher at pH_{in}<4.6. Means ±1 SEM; number of fish as given in Fig. 18.

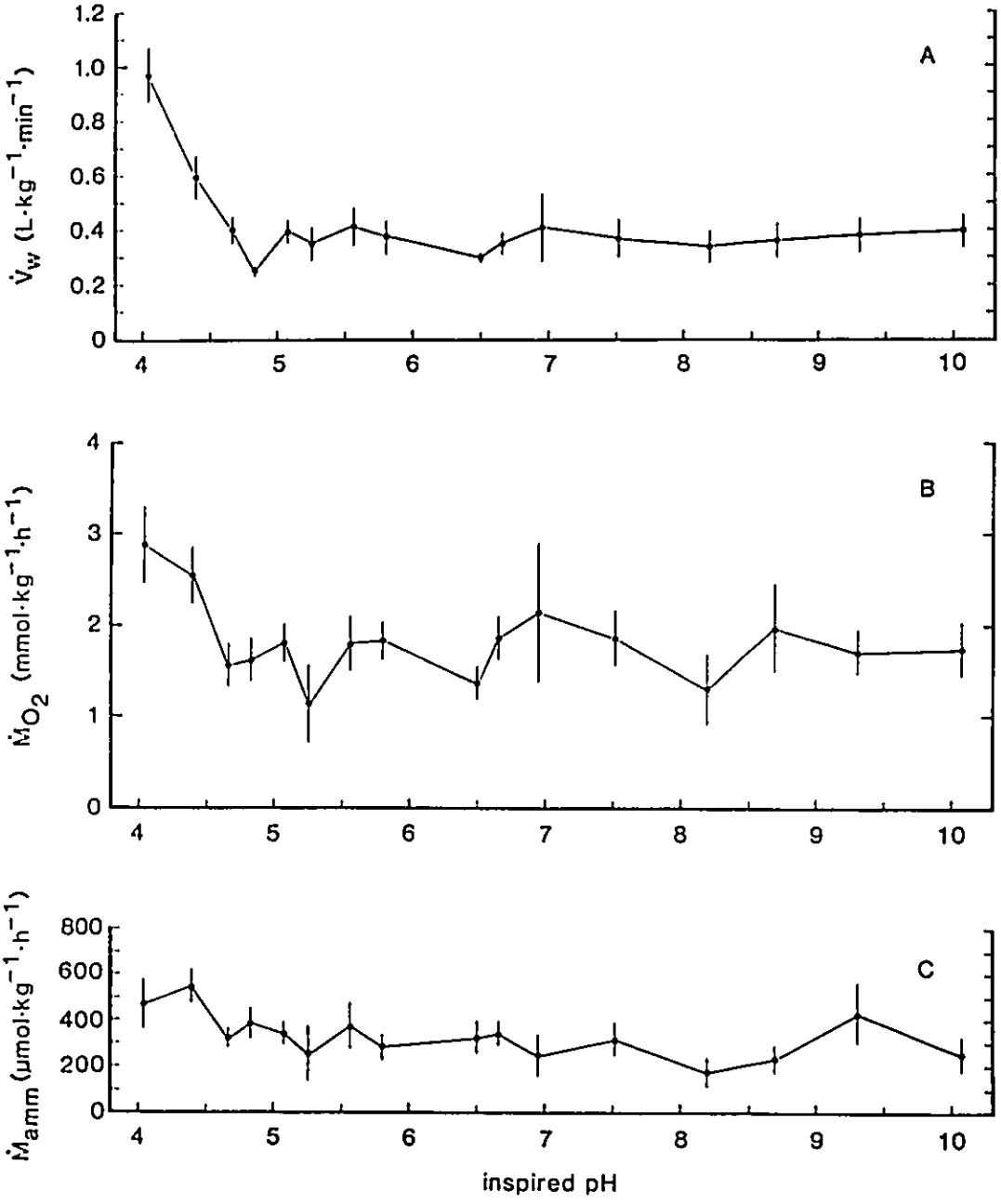

A
B
C
$\dot{V}_w$ (L·kg^{-1}·min^{-1})
$\dot{M}_{O_2}$ (mmol·kg^{-1}·h^{-1})
$\dot{M}_{amm}$ (µmol·kg^{-1}·h^{-1})
inspired pH
0
0.2
0.4
0.6
0.8
1.0
1.2
1
2
3
4
200
400
600
800
5
6
7
8
9
10

Fig. 20. Carbon dioxide, oxygen, titratable base, and ammonia transfers at the gills of rainbow trout held in soft water of various acidities. Transfers were approximately constant at pH_{in} 5.1-10.1, but were reduced at pH_{in}=4.0, in accord with increased $\dot{V}_w$ at that pH. Means $\pm$1 SEM; n=10 except at pH_{in}=5.6 and pH_{in}=7.5, where n=5 and 6, respectively.

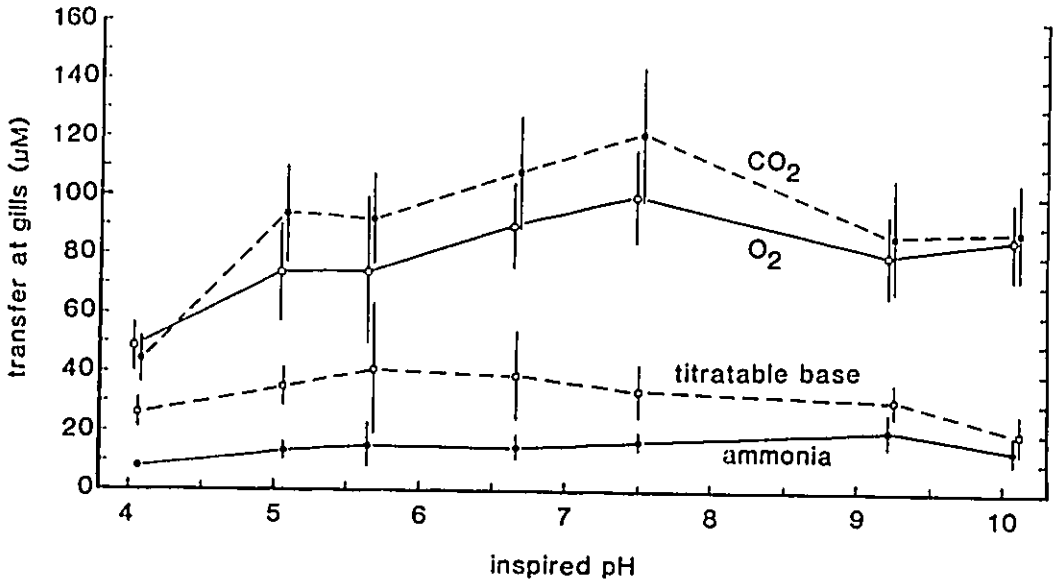
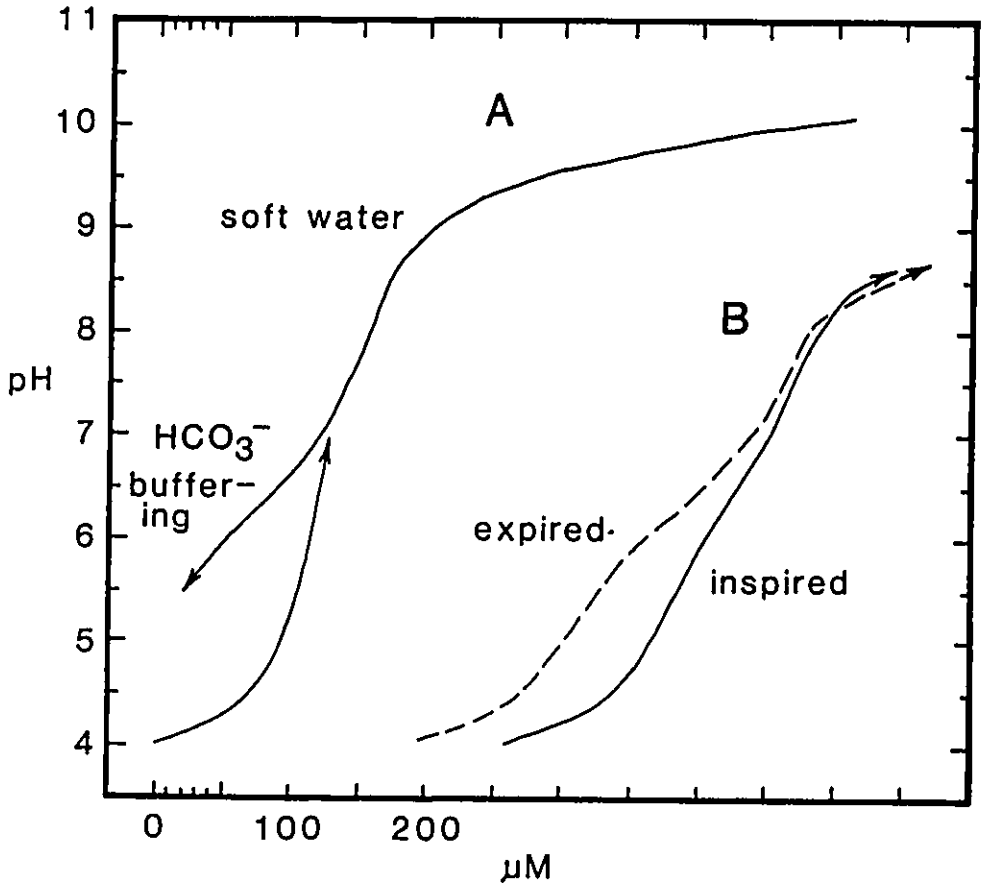

160
140
120
100
80
60
40
20
0
transfer at gills (μM)
4
5
6
7
8
9
10
inspired pH
CO_2
O_2
titratable base
ammonia

Fig. 21. A) Composite titration curves for the soft water used. Buffer capacity of alkalinized water was higher in the pH range 5.5-7.0 than that of acidified or un-modified water, because of HCO_3^- buffering. Arrows indicate the direction to which the titrations apply. Horizontal axis: amount of acid or base, in μM (μequiv.L^{-1}). See text for further details.

B) Titration curve of expired water of a single fish, ccmpared with the titration curve of the inspired water it was breathing. Bicarbonate buffering was added to the expired water because of CO_2, ammonia, and titratable base transfers at the gills. Inspired water pH was 6.6. Two other fish which were examined in the same fashion showed similar titration curves and added buffering in the expired water. Titration curves in B are displaced to the right of those in A for clarity only; the same vertical and horizontal scales apply to all curves.


11
10
9
8
7
6
5
4
pH
A
soft water
B
HCO_3^-
buffer-
ing
expired
inspired
0
100
200
µM

ventilation volume at that pH. The relative decrease in CO_2 transfer (~60%) was greater than that in O_2 transfer (~40%) at pH_{in}=4.0. Mean titratable base added at the gills was about 30 μM, and ammonia transfer at the gills was about 15 μM (Fig. 20). Again, the notable feature of all these transfers at the gills, except at the lowest inspired pH (4.0), was their constancy over a wide range of inspired pH (4.6-10.1).

Composite titration curves of the soft water used are given in Fig. 21A. Two curves are presented, because the buffer capacity of the un-modified or acidified inspired water was very low (i.e. negligible bicarbonate alkalinity), whereas the buffer capacity of alkalinized inspired water was higher in the pH range 5.5-7.0. This difference was due to the fact that addition of base (KOH) for alkalinization resulted in the fixation of atmospheric CO_2 as HCO_3^-; when the alkalinized soft water was titrated downwards, the buffering effect of the extra HCO_3^- became apparent in the pH range 7.0-5.5. Similarly, bicarbonate buffering was added to water breathed by the fish as CO_2, ammonia, and titratable base were released at the gills (cf. expired, inspired curves of Fig. 21B).

Model of pH changes at the gills

Using the titration curves appropriate to the inspired pH, as illustrated in Fig. 21A, calculations were made of the amount of base or acid needed to change the inspired water pH to the

experimentally observed expired water pH, given in Fig. 18. That is, the quantity of base or acid released at the gills. The results of this analysis are presented in Fig. 22. For example, at pH_{in}=5.0, about 15 μM of "base" were apparently needed to raise the expired water pH to about pH 5.7 (Fig. 22); at pH_{in}=9.0, about 80 μM of "acid" were needed to lower the expired pH to pH 7.3. Near pH_{in}=6 no net base or acid addition was apparent at the gills (i.e. observed ΔpH=0).

Next, I tried to explain the apparent base and acid additions by the fish to the water near the gills using the measured CO_2, ammonia, and titratable base transfers, and aquatic CO_2 and ammonia equilibria. In water, CO_2 dissociates to HCO_3^- and H^+ (pK~6.3), then from HCO_3^- to CO_3^{2-} and H^+ (pK~10.3; Stumm and Morgan 1981). Ammonia dissociation ($NH_4^+ \rightleftharpoons NH_3 + H^+$) has a pK of about 9.5 (Cameron and Heisler 1983). Using these equilibria the theoretical base and acid additions at trout gills were calculated. Five assumptions were made: (i) CO_2 released at the gills was 100 μM (Fig. 20), (ii) titratable base released at the gills was 30 μM, of which ammonia (assumed to all be released as NH_3) contributed 15 μM (Fig. 20), (iii) instantaneous reactions at the gills, (iv) constant fish ventilation volumes, and (v) a situation in which water pH did not change as base or acid were added (i.e. a perfectly buffered system). The last assumption is clearly untrue, but simplified initial stages of the modelling process.

Theoretical base and acid additions at rainbow trout gills, using the above five assumptions, are portrayed in Fig. 23. Thirty μM of base are added between pH 4 and 10, of which 15 μM are added as NH_3. Above pH 8.5, the base addition owing to NH_3 decreases to 7.5 μM at pH 9.5 (the pK of ammonia) and decreases to zero by about pH 10.2. Acid addition by 100 μM CO_2 is negligible below about pH 5, is 50 μM at pH 6.3 (the first pK of CO_2), and increases to its full 100 μM by pH 8. At pH 10.3 (the second pK of CO_2), 50% of the CO_2 released at the gills is further converted to CO_3^{2-}, so 150 μM of H^+ are produced. The theoretical sum of titratable base and CO_2-acid added at the gills is about 28 μM base at pH 5, no net addition at pH 6.1, about 70 μM acid added near pH 8, and 100 μM acid added at pH 10.1 (Fig. 23).

The next step was to convert the theoretical acid and base added at the trout gills to predicted ΔpH at the gills by means of the soft water titration curves (Fig. 21A). To do this conversion an iterative calculation was used to account for changes in pH at the gills in the poorly buffered water as acid (CO_2) and base were released into it; changes in pH would affect further CO_2 dissociation. The iterative calculation overcame the problems with assumption (v) above. The calculation was the equivalent of adding 100 μM CO_2 and 30 μM base in 10 equal parts to soft water at each inspired pH. The final pH_{ox} was the cumulative effect of each small addition of acid and base on pH and therefore on dissociation of the CO_2 portion of the next small acid and base

Fig. 22. Amount of base or acid needed to change inspired pH to the experimentally observed expired pH (from Fig. 18), calculated from the appropriate titration curves given in Fig. 21A. See text for further details. Above the horizontal axis base is added; below, acid is added, in μM ($\mu equiv.L^{-1}$). 95% confidence limits are indicated about the mean values.

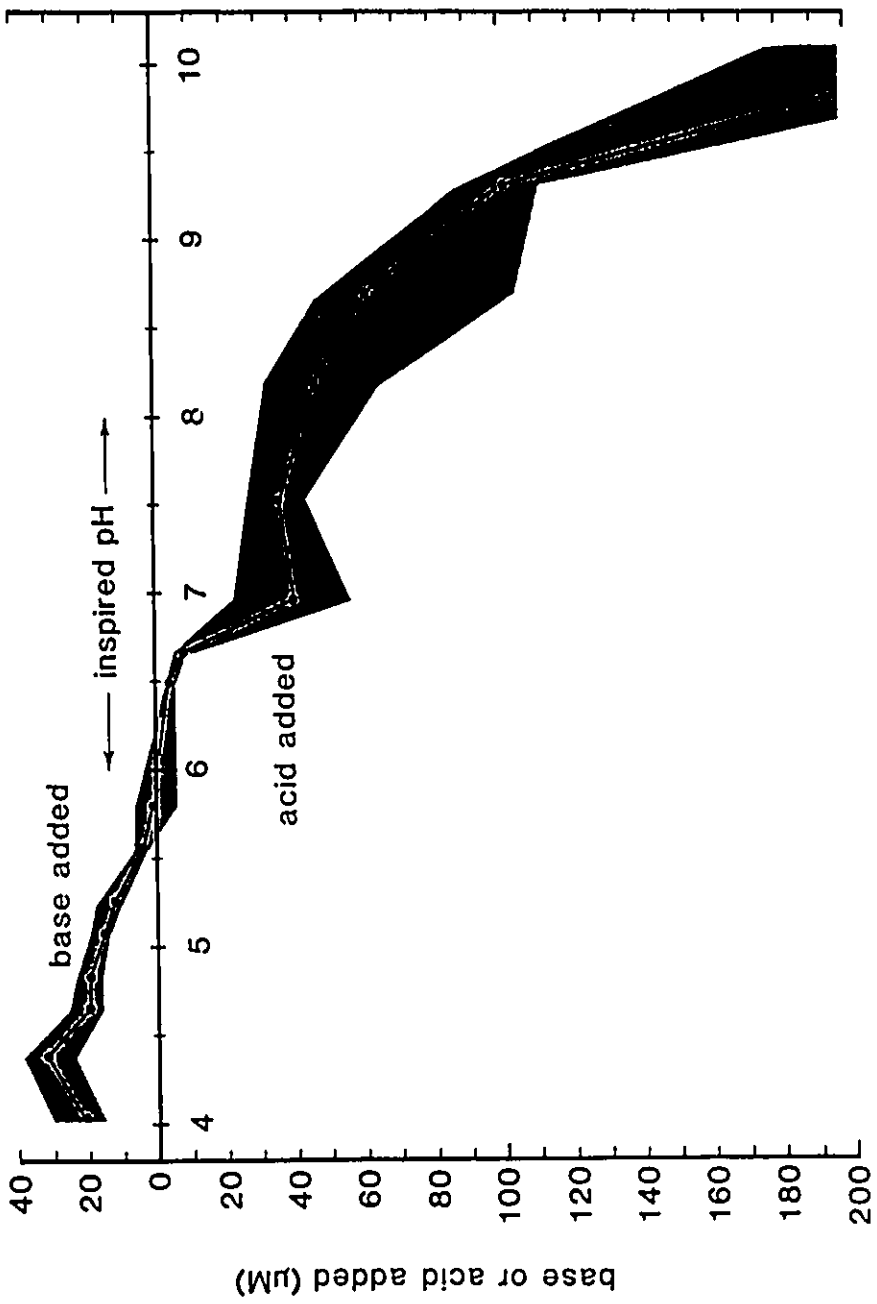

base added
inspired pH
acid added
4
5
6
7
8
9
10
40
20
0
20
40
60
80
100
120
140
160
180
200
base or acid added (μM)

Fig. 23. Theoretical acid or base contributions of 100 μM CO_2 and 30 μM base (including 15 μM as NH_3) at equilibrium in water of various acidities. See text for further details. Above the horizontal axis base is added; below, acid is added.

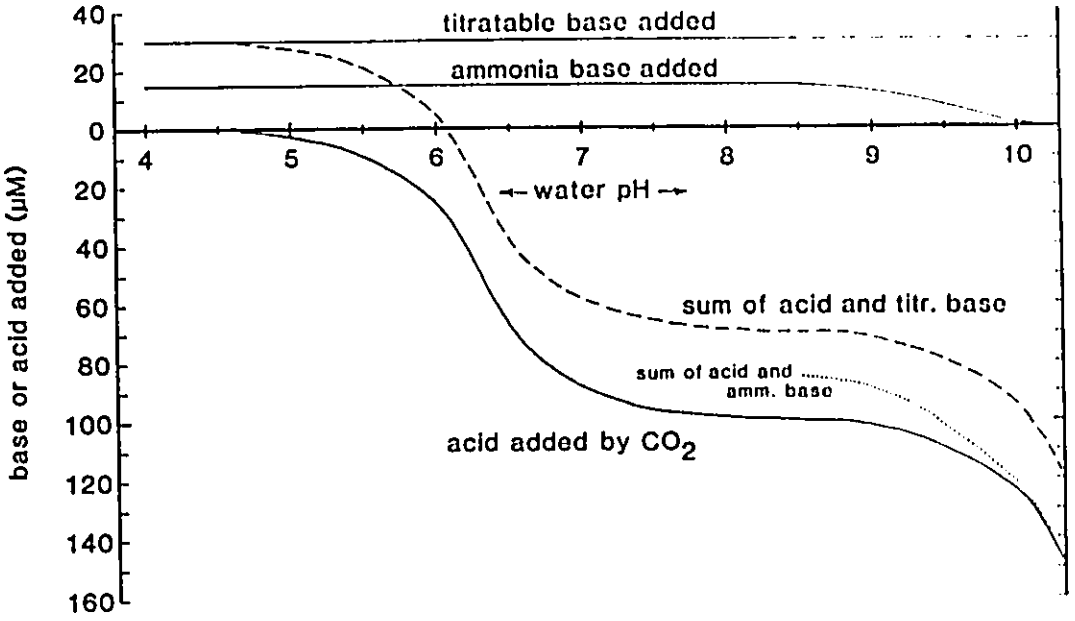

titratable base added
ammonia base added
water pH
sum of acid and titr. base
sum of acid and amm. base
acid added by CO_2
base or acid added (μM)
40
20
0
20
40
60
80
100
120
140
160
4
5
6
7
8
9
10

Fig. 24. Model predicted ΔpH vs pH_{in} contrasted with experimentally observed ΔpH vs pH_{in} (from Fig. 18). 95% confidence limits of the means are indicated for the observed ΔpH vs pH_{in} curve. The predicted curve was calculated from the theoretical acid and base contributions at rainbow trout gills (Fig. 23) and the appropriate titration curves of the water used in the experiments (Fig. 21A). An iterative calculation was used to account for the changes in water pH as acid or base were released into the poorly buffered water. Predicted ΔpHs assuming that no base was released at the gills are also presented for $pH_{in}>8.6$. See text for further details.

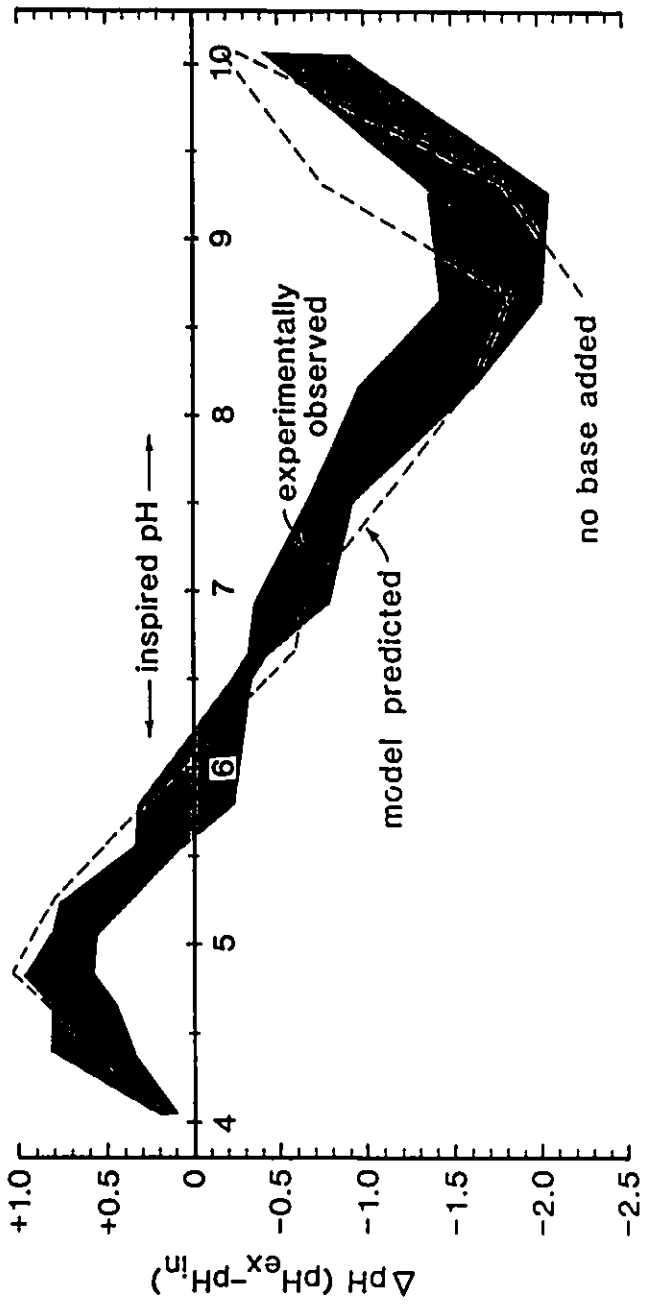
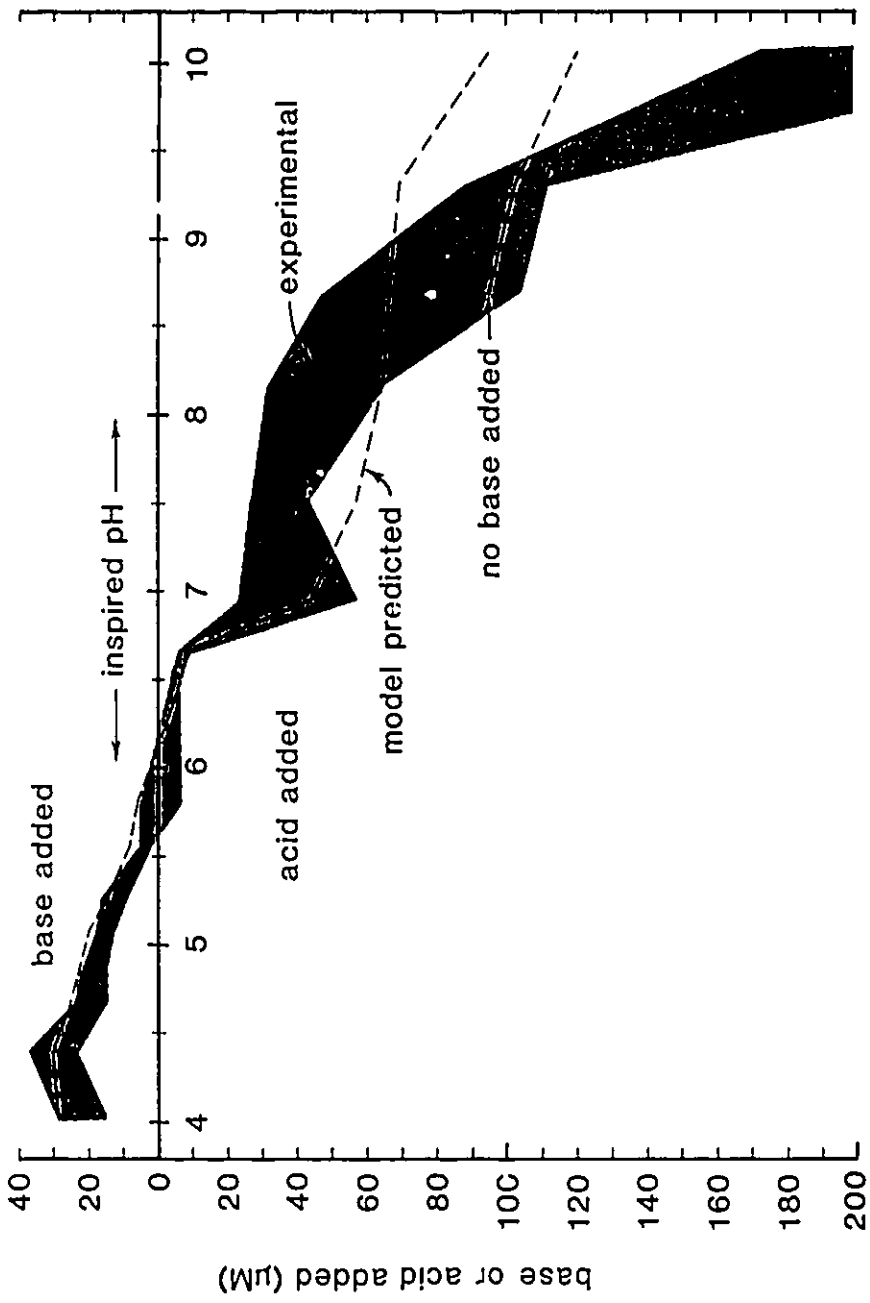

+1.0
+0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
ΔpH (pH_ex−pH_in)
4
5
6
7
8
9
10
inspired pH
experimentally observed
model predicted
no base added

Table 2. Comparison of observed ΔpH and predicted ΔpH when 0.3% CO_2 was gassed into poorly buffered soft water of various starting pHs (see text for details). Correlation coefficient of observed and predicted ΔpH is 0.98. Equation of the line is:
observed ΔpH = 0.70·(predicted ΔpH)-0.15.

initial pH	measured ΔCO_2 (μM)	ΔpH (observed)	ΔpH (predicted)
4.74	85	-0.06	-0.02
5.42	114	-0.41	-0.22
6.15	103	-0.74	-0.75
6.64	99	-0.84	-0.64
7.26	102	-0.80	-1.23
8.30	148	-1.72	-2.50
9.07	156	-2.39	-2.97
10.03	174	-0.41	-0.53

Fig. 25. Base or acid needed to change inspired pH to the model predicted expired pH, contrasted with the base or acid needed to change inspired pH to the experimentally observed expired pH (from Fig. 22). 95% confidence limits of the means are given for values derived from observed expired pHs. Above the horizontal axis base is added; below, acid is added. For $pH_{in}>8.6$ the predicted acid added is also indicated assuming no base is released at the gills. See text for further details.


base added
inspired pH
acid added
experimental
model predicted
no base added
base or acid added (μM)
40
20
0
20
40
60
80
100
120
140
160
180
200
4
5
6
7
8
9
10

addition. A good fit was obtained between theoretically predicted and experimentally observed ΔpH at the gills (Fig. 24), except at inspired pH>8.6. I also calculated the theoretical ΔpH assuming that no base was released at the gills at pH_{in}>8.6 (Fig. 24). This relationship fits the observed data better at high inspired pH, where errors in assumed pKs of CO_2, or in titratable base measurements, may have affected the model (see Discussion).

To check that CO_2 did lower water pH as predicted, 0.3% CO_2 was used to add, in vitro, about 100 μM CO_2 to soft water of different starting pH. The actual ΔCO_2 added was measured, and the ΔpH was predicted using the equilibria of Fig. 23, the iterative calculation, and the appropriate soft water titration curve (Fig. 21A). Predicted ΔpHs were compared to observed ΔpHs (Table 2). Observed and predicted ΔpH correlated well (correlation coefficient =0.98, $P<0.001$); the slope of the line was 0.70, not significantly different from a slope of 1.0 ($P>0.10$).

Finally, using the titration curves of the soft water I calculated (Fig. 25) how much base or acid would have to be added to the poorly buffered water to produce the predicted ΔpH versus pH_{in} curve shown in Fig. 24. In essence Fig. 25 is a re-drawing of Fig. 23, with the 100 μM CO_2 and 30 μM base added into a poorly buffered solution instead of into a perfectly buffered system (again overcoming the problems with model assumption v). The acid or base needed to produce the theoretically predicted ΔpH curve

agrees well with the amount of acid or base needed to change the inspired water pH to the experimentally observed expired water pH (Fig. 25). For $pH_{in}>8.6$, the acid needed to produce the theoretical ΔpH curve assuming no base was added at the gills was also calculated (see Fig. 25 and Discussion).

A potential, small source of error in this analysis could be the addition of buffer substances (besides CO_2/HCO_3^- and NH_3/NH_4^+) to the soft water as it passes over the gills. For example, it is possible that some of the change in slope and position of the titration curves of expired water relative to inspired water (e.g. Fig. 21B), attributed here to net CO_2, ammonia, and titratable base transfers, could reflect an addition of some other buffer at the gills. Inorganic phosphate, with a pK ~6.8, was an obvious candidate.

Using HPLC, I surveyed the inorganic anion content of inspired and expired water samples from three fish. Neither phosphate, NO_2^-, NO_3^-, nor SO_4^{2-} were released at the gills in greater than 1 μM concentrations. Up to 10 μM Cl^- were released at the gills, but this ion is fully dissociated in water so would be detected in the titratable base measurement (if not matched by equimolar cation loss). Therefore, the release of inorganic buffers at the gills does not appear to be a complicating factor in this analysis, although the possibility of organic buffer release at the gills (e.g. mucus glycoproteins) cannot be eliminated at present.

Discussion

Gill water chemistry

These results clearly show that water chemistry in the gill micro-environment is different than the chemistry of the bulk water in which a fish lives and which a fish breathes. Poorly buffered soft water near the gills has a higher pH than does acidic inspired water, and water near the gills has a lower pH than circumneutral or basic inspired water, with a crossover point near pH_{in}=6.0. In similar soft water, Wright et al. (1986) reported a gill ΔpH of about -0.6 to -0.8 at pH_{in}-7.2, and Holeton and Randall (1967) reported a ΔpH of about -0.2 to -0.3 for pH_{in} 6.9-7.4, close to the present values at these inspired pHs (Fig. 18).

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 close to inspired pH at the leading edges of gill lamellae, and changes 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, Univ. of British Columbia, Canada; pers. comm.). Expired pH may well approximate the overall, mean pH near the gill surface.

Model of pH changes at the gills

Experimentally determined CO_2, titratable base, and ammonia transfers at the gills (about 100, 30, and 15 μM, respectively), and the *in vitro* titration characteristics of the defined soft water medium, were used in a model to explain the pH changes seen at the gills. A good fit between theoretical and observed values was obtained (Fig. 24). This good fit suggests that CO_2, base, and ammonia transfers at fish gills adequately account for pH changes at the gills, and that the assumptions of the model are reasonable. The model assumptions are discussed below.

It was assumed, based on experimental measurements, that CO_2 transfer at rainbow trout gills was about 100 μM (Fig. 20). Expressed as CO_2 excretion ($\dot{M}_{CO_2}$), 100 μM CO_2 for these fish is 2.2 $mmol.kg^{-1}.h^{-1}$ (using $\dot{V}_w$ of 0.37 $L.kg^{-1}.min^{-1}$; Fig. 19A). This number agrees well with previously-reported $\dot{M}_{CO_2}$ values for rainbow trout (fitted with masks) of 2.4-3.9 $mmol.kg^{-1}.h^{-1}$ (Wright et al. 1986), and about 1.1-2.7 $mmol.kg^{-1}.h^{-1}$ (Iwama et al. 1987). Conversely, using average reported fish weights and ventilation volumes, these published $\dot{M}_{CO_2}$ values can be converted to CO_2 transfers: 95-109 μM (Wright et al. 1986) and 52-127 μM (Iwama et al. 1987). It appears then, both from my own data and published values, that 100 μM CO_2 transfer at the gills is a reasonable estimate to use in the present model.

Although it will have little effect on the model, a puzzling aspect of my results is the higher than expected ratio between CO_2 and O_2 transfers at the gills. From standard metabolic theory the predicted CO_2:O_2 ratio would be 0.7-1.0, depending on the aerobic substrate used, but the respiratory exchange ratio here was approximately 1.2 (i.e. 100/85; Fig. 20). Iwama et al. (1987) reported a respiratory quotient (R.Q.) of 0.87 for rainbow trout, in agreement with theory, as did Kutty (1968; R.Q.=0.96). However, R.Q. values of >1 have been reported for hypoxic rainbow trout (R.Q.=1.4) and trout at the beginning of exercise (R.Q.=1.2; Kutty 1968), and in resting coho salmon and starry flounder (R.Q.~1.1 for both; Milligan and McDonald 1988).

Causes of the discrepancies between observations and theory in resting fish in well oxygenated water are unknown, but could be related to nutritional status of the fish (Kutty 1972) or to stress-induced anaerobic respiration (Kutty 1968). I cannot eliminate the possibility that the restraints involved in the experiments caused increases in plasma cortisol and catecholamine concentrations, resulting in CO_2, ammonia, and acid-base fluxes across the gills different from a true resting situation. However, in a separate set of experiments to study ventilation effects of infused catecholamines in rainbow trout, similar CO_2 but higher O_2 transfers were measured (Playle et al., submitted), so that the CO_2:O_2 ratio was ~0.9. This indicates that the ΔCO_2

values from the present study are reasonable: it is the ΔO_2 values that are more likely in error.

For the predictive model, titratable base released at the gills was assumed to be about 30 μM, of which ammonia contributed 15 μM, based on the experimental observations (Fig. 20). Net base release (titratable base minus ammonia transfer, = acid entry), the value normally reported for the acid-base status of fish, would therefore be about 330 $\mu mol.kg^{-1}.h^{-1}$ (using $\dot{V}_w$=0.37 $L.kg^{-1}.min^{-1}$). Fish in the present study were acclimated to soft water (Ca^{2+}~50 $\mu equiv.L^{-1}$) for about 2 weeks, and were not fed. McDonald (1983b) and Audet et al. (1988) reported net base releases of 70-80 $\mu mol.kg^{-1}.h^{-1}$ for rainbow trout acclimated to Ca^{2+}=50-60 $\mu equiv.L^{-1}$. Holeton et al. (1983b) reported net base release of 330 $\mu mol.kg^{-1}.h^{-1}$ in rainbow trout in hard water, as opposed to the usual small net base uptake (acid excretion) in non feeding animals in hard water (e.g. Wood 1988). They attributed the net base release to lack of recovery after arterial cannulation of their fish. The results of McDonald (1983b) with starved trout at a range of Ca concentrations suggest, however, that low Ca concentrations in water may also be responsible for the observed base releases at fish gills. The long acclimation period (2-4.5 mo) of the fed fish studied by Audet et al. (1988) suggests that base release to the water is a steady state condition of rainbow trout in very soft water.

Fifteen μM of the 30 μM titrable base were added as ammonia (from experimental observations), assuming that all ammonia was released as NH_3 at the gills. Ammonia excretion in soft water acclimated rainbow trout has been reported at about 200 $\mu mol.kg^{-1}.h^{-1}$ (McDonald 1983b; Audet et al. 1988), which is similar to the 15 μM (330 $\mu mol.kg^{-1}.h^{-1}$) value used in the model. Ammonia transfer in trout is anywhere between 45 and 100% NH_3, the remainder occurring as NH_4^+ (Cameron and Heisler 1983; Wright and Wood 1985). Since ammmonia transfer is included within the 30 μM titratable base transfer, the ratio of NH_3:NH_4^+ is immaterial, except at pH_{in}>8.6, where the assumption of 100% ammonia transfer as NH_3 becomes important (see below).

Instantaneous reactions at the gills were assumed in the model. The NH_3 to NH_4^+ and HCO_3^- to CO_3^{2-} reactions are essentially instantaneous (i.e. mseconds; Stumm and Morgan 1981). The CO_2 to HCO_3^- reaction is slow in the absence of carbonic anhydrase (i.e. minutes). This fact probably explains why acidification of water near the gills by CO_2 transfer, as originally proposed by Lloyd and Herbert (1960), was not generally accepted within the scientific community; it was unclear how an instantaneous reaction could occur, unless carbonic anhydrase was present at the external gill surface (see Wright and Wood 1985; Wright et al. 1986). Szumski et al. (1982) speculated that carbonic anhydrase was indeed present at the gills, speeding CO_2 dissociation, but gave no evidence to support this idea. Now,

however, carbonic anhydrase has been localized in fish mucus (Wright et al. 1986) and in gill epithelia (Conley and Mallatt 1988; Rahim et al. 1988). Wright et al. (1986) concluded that CO_2 disequilibrium was negligible in water near the gills. With regards to bases released at the gills, it is not known whether or not their reactions are instantaneous, because the precise bases involved have not been identified.

Constant fish ventilation volumes were assumed for the model, a valid assumption at inspired pHs greater than about 4.6 (Fig. 19A). Ventilation increased greatly below pH_{in}=4.6, presumably due to irritation of the gills by H^+ ions and accumulation of mucus on the gills (McDonald 1983a). In spite of increased $\dot{V}_w$ at low inspired pH, the ΔpHs predicted by the model are very close to the observed values (Fig. 24), because the buffer capacity of the very acidic water is so high in this region (cf. Fig. 21A) that increased $\dot{V}_w$ is inconsequential to ΔpH. The assumption of constant $\dot{V}_w$ is most important between pH 5 and 9, where buffering in the soft water is low (Fig. 21A). The experimental results showed approximately constant ventilation in that pH range (Fig. 19A), so using constant $\dot{V}_w$ in the model is reasonable.

The last assumption of the model, that water pH did not change as base or acid were added at the gills, is clearly not valid for the poorly buffered water used. This invalid assumption was used solely as a necessary first step in preparing the model as presented in Fig. 23. When applying the model to the poorly

buffered system I used the titration curves for the system (Fig. 21A) and an iterative calculation that accounted for the pH changes in the soft water as base and CO_2-acid were added.

In general the fit between model predicted and experimentally observed ΔpH at rainbow trout gills is good (Fig. 24). The major discrepancy is at pH_{in} 9.3 and 10.1, where predicted ΔpH is less negative than observed. (Likewise, theoretical acid added at pH_{in} 9.3 and 10.1 is less than the observed acid added; Fig. 25). A possible modification of the model to better reflect reality would be to assume that no effective base is released at the gills if inspired pH is greater than ~8.5 (Fig. 24, 25).

The rationale for this modification is that the titratable base transfer (30 μM) was determined by comparing the titratable base of inspired and expired water titrated to pH 4.0; the unknown products released (or taken up) at the gills to add base to the water would have no alkalinizing effect at $pH_{in}>8.5$ if their (unknown) pKs were below pH 8, but would show up as titratable base using the present methodology. Above pH_{in}~8.5 the postulated 15 μM ammonia addition at the gills might also have less alkalinizing effect at the gills than predicted (Fig. 23) because rainbow trout may decrease the proportion of ammonia excreted as NH_3 at high pH (i.e. more excreted as NH_4^+; Wright and Wood 1985). Without better knowledge of the nature of the bases released at trout gills in soft water it is difficult to decide whether 0 μM,

30 μM, or an intermediate value for base transfer should be used in the model for pH_{in}>8.5.

An alternative explanation of the discrepancy between predicted and observed ΔpH at high inspired pH is that the second pK of CO_2 is less than 10.3 in the gill micro-environment, resulting in more H^+ released near pH_{in}>9 than was modelled. The pKs of CO_2 vary with ionic strength, decreasing as ionic strength increases (Stumm and Morgan 1981). I assumed CO_2 pKs of 6.3 and 10.3 as reasonable for low ionic strength fresh water; release of electrolytes and ammonia at the gills might decrease these pKs in the gill micro-environment as ionic strength is increased. If, for example, the second pK of CO_2 was 10.0 instead of 10.3 in the gill micro-environment then 25-30 μM more acid would be released near pH_{in}=10.1 than is indicated in Fig. 25.

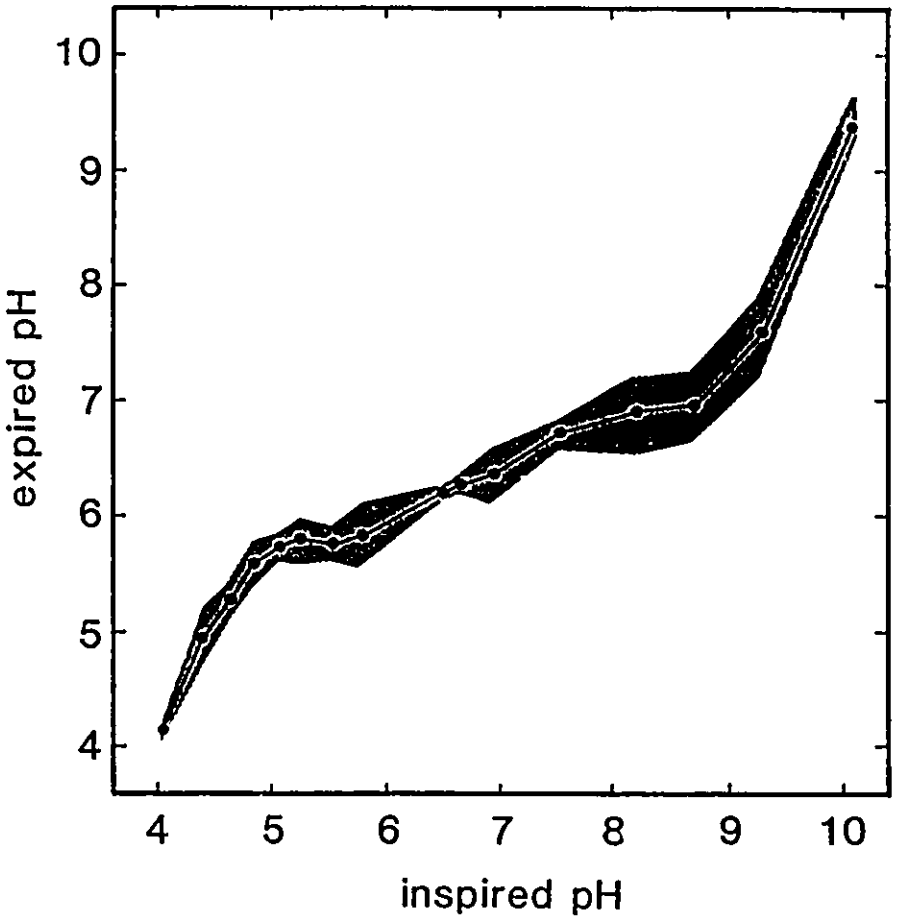
Which of the two modifications to the model at high inspired pH is more valid is difficult to resolve at present; in any case the model quantitatively mimics experimentally observed results well in the pH_{in} 4.0-8.5 range, and qualitatively follows the observed trends above pH_{in}=8.5. The assumption that no base is released at the gills at high inspired pH is a reasonable method of fine-tuning the model fit.

In the poorly buffered soft water the release of CO_2, base, and ammonia at the gills kept expired pH between 5.6 and 7.6 over the inspired water pH range 4.8-9.3 (Fig. 26). Below pH_{in}=4.8 and

above pH_{in}=9.3 the "buffering" of the gill micro-environment breaks down, and eventually the pH near the gills approaches inspired water pH (i.e. pH_{in}=4.0, 10.1). Excretion of metabolic endproducts at the gills may allow fish to survive in acidic or basic conditions that might otherwise damage gill epithelia. The present results provide no evidence that CO_2, titratable base, or ammonia transfers at the gills are actively manipulated by the fish for their protective value during short term exposures to acidic or basic conditions. Indeed, endproduct transfers at the gills were remarkably constant over a wide range of inspired pH (Figs. 19, 20). Rather, it appears that the protective value of buffering the gill micro-environment is a fortuitous by-product of excretion, not the primary reason for endproduct release at the gills.

The present experimental observations and predictive model are important for fish toxicant studies in general, and for studies of Al in particular. Any gill contaminant whose toxicity varies with pH may be more or less toxic than expected from bulk water chemistry alone. For example, ammonia's toxicity is pH dependent, and Lloyd and Herbert (1960) were correct in equating the observed pH dependence of ammonia toxicity with CO_2 transfer at the gills. Szumski et al. (1982) were able to construct curves to predict toxic concentrations of ammonia considering water pH, alkalinity, and temperature, and assuming CO_2-induced pH changes in branchial water, but these workers were hindered by not having

Fig. 26. Buffering of the gill micro-environment of rainbow trout held in soft water of various acidities. Buffering at the gills is good over the inspired pH range 4.8-9.3, but breaks down under more acidic or more basic conditions. 95% confidence limits are indicated about the mean expired pHs.


10
9
8
7
6
5
4
expired pH
4
5
6
7
8
9
10
inspired pH

direct measurements of the actual pH changes occurring at fish gills. Pagenkopf (1983) presented a gill surface interaction model to explain pH dependent and water chemistry dependent trace metal toxicity in fish, but did not consider water chemistry changes in the branchial micro-environment: incorporation of pH changes near the gills into such a model would make it more complete. In theory, by measuring pH changes near the gills using opercular catheters, or by calculating pH changes using measured CO_2, titratable base, and ammonia transfers and an appropriate titration curve, the toxicity of any pH dependent gill contaminant can be better predicted.

This approach is particularly important for the Al toxicity studies which form the major focus of this thesis. The solubility of Al varies exponentially with pH, reaching a minimun at about pH 5.8 (Fig. 1). Chemical speciation of Al is also dependent on pH (Fig. 2). It is possible that acidic water (pH<5.5) containing Al would become supersaturated with Al near the gills where the pH is higher. Chemical species of Al would certainly shift to the less charged or uncharged Al-hydroxides (Fig. 2). The presence of Al precipitating onto the gills, or the increased availability of certain Al species (eg. $Al(OH)_2^+$, $Al(OH)_3^o$) may cause the ionoregulatory and respiratory problems that have been documented (Neville 1985; Wood et al. 1988a; Chapter 2). These possibilities are investigated further in Chapters 4 and 5.

To summarise, the pH of water near rainbow trout gills is different than the bulk water in which the fish lives. The pH of the gill micro-environment is higher in acidic water, and lower in basic water, with a crossover point near pH 6. Carbon dioxide, base, and ammonia transfers at the gills adequately explain the experimentally observed pH changes. The gill micro-environment is essentially buffered over a wide range of environmental pHs, which perhaps allows fish to live in acidic or basic conditions which might otherwise harm gill epithelia. Knowledge of the pH near the gills is important when pH dependent gill toxicants such as Al are considered, because their toxicity may be different at the branchial surface than would be predicted from bulk water pH alone.

Chapter 4

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

Introduction

In Chapter 3 it was demonstrated that acidic inspired soft water (pH<6) is rendered more basic as it passes across the gills of rainbow trout. Conversely, inspired soft water of pH>6 is rendered more acidic as it passes across the gills (see also Randall and Wright 1989). 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 a fish breathes (Model, Fig. 24). 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 narrow range of acidic pH (i.e. pH 4.0-6.5; Fig. 1, 2). In addition, changes in Al species (eg. $Al(OH)_3^o + 3H^+ \rightleftharpoons Al^{3+} + 3H_2O$) can buffer pH.

Using the methodological approach developed in Chapter 3, 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. A third goal was to assess changes over time in gill micro-environment chemistry during prolonged (44 h) exposures to Al. Finally, effects of short and longer term exposures to Al and low pH on fish ventilation, oxygen consumption, and ammonia excretion were assessed. The pHs examined (pH 4.2-6.3) represent very acidic to circumneutral conditions, and the Al concentration used (93 $\mu g.L^{-1}$) is representative of concentrations commonly found in acidic soft water in the field (Dillon et al. 1984).

Materials and methods

Experimental animals and water

Adult rainbow trout of both sexes, weight = 297±7 g (mean ±1 SEM, n=71) were purchased from Spring Valley Trout Farm, New Dundee, Ont. Fish were held in dechlorinated Hamilton city tapwater at 15-20°C, and were fed floating trout pellets twice weekly. Trout were then acclimated for at least two weeks to the standard synthetic soft water before all experiments. Soft water acclimation conditions were identical to those of Chapter 3.

For the ventilation experiments, trout were fitted with latex ventilation masks and opercular catheters (Clay-Adams PE-190 polyethylene tubing) while under MS222 anaesthesia, as described in Chapter 3. Fish were then placed in one of 5 ventilation collection boxes of the design described by Cameron and Davis (1970; see Fig. 17). Well-aerated water flowed from a headtank 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 (>140 torr) and P_{CO_2} low (<1 torr). Aluminum was added by peristaltic pump as a concentrated solution ($AlCl_3.6H_2O$ (Sigma); 0.39 $g.L^{-1}$; pH~4.0) to water leaving the headtank. The standard Al concentration used was 93±2 $\mu g.L^{-1}$ (n=67); concentrations in the absence of added Al were about 5 $\mu g.L^{-1}$. 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 ventilation experiments, five fish at a time were exposed to one of 7 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 another 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 done at the end of the day, followed by a return to circumneutral pH overnight.

In a second set of ventilation 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 μequiv.L^{-1} (n=97), respectively, and experimental temperatures were 15-16°C. Fluoride concentrations were <1 μequiv.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. These fish had neither masks nor opercular catheters. 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.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 μg.L^{-1} Al, n=16), 14-15° C, for 2 h. Inflowing water Ca^{2+}

and Na^+ concentrations were 54±1 and 83±2 μequiv.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 (see Chapter 3 for details). 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, as described in Chapter 3. 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] minus inspired [ammonia])$\cdot\dot{V}_w$. Total aqueous Al concentrations were determined using the pyrocatechol violet method (Dougan and Wilson

1974). The difference between expired and inspired values are referred to as "transfer" or "Δ", eg. 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 5x their weight of 0.1 N reagent-grade H_2SO_4 for 8 h at 80°C. Samples were vortexed, centrifuged for 1 min, and the supernatant diluted 100x before analysis at 309.3 nm on a Varian AA-1275 atomic absorption spectrophotometer with GTA-95 graphite tube atomizer. Graphite furnace is more accurate than the pyrocatechol violet method of Al determination, and is less prone to interference by organic material. Standard additions were used to show there was no interference of Al measurements by the dilute solutions of gill digest. Ten μL of sample were introduced into the graphite tube with 20 μL of 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 of sample and 10 µL of 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 generally presented as means ±1 SEM (n). The "n" number represents the number of different fish contributing to the mean. 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 (7 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. 27A). Trout exposed to 93 $\mu g.L^{-1}$ 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. 27A). 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. 27B); $\dot{V}_w$ 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. 27B). Fish exposed to Al had especially high $\dot{V}_w$ at pH_{in}=6.3.

Oxygen consumption ($\dot{M}_{O_2}$) in fish exposed to acidity alone for 2-3 h was variable, but averaged about 1.6 $mmol.kg^{-1}.h^{-1}$ (Fig. 28A). 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

Fig. 27. 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 $\mu g.L^{-1}$ 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 $\pm$1 SEM are indicated; significant differences between ΔpH of Al-exposed fish and ΔpH of fish exposed to acidity alone are indicated by * ($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. 27A.

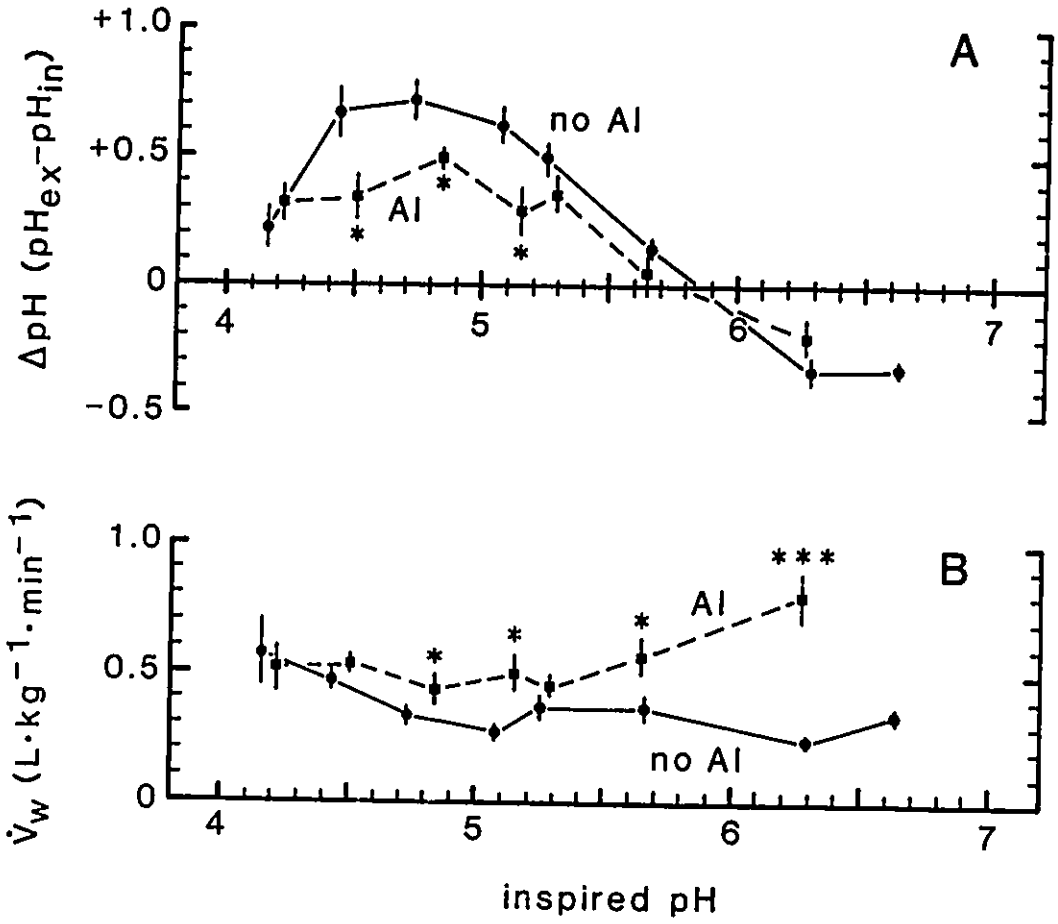
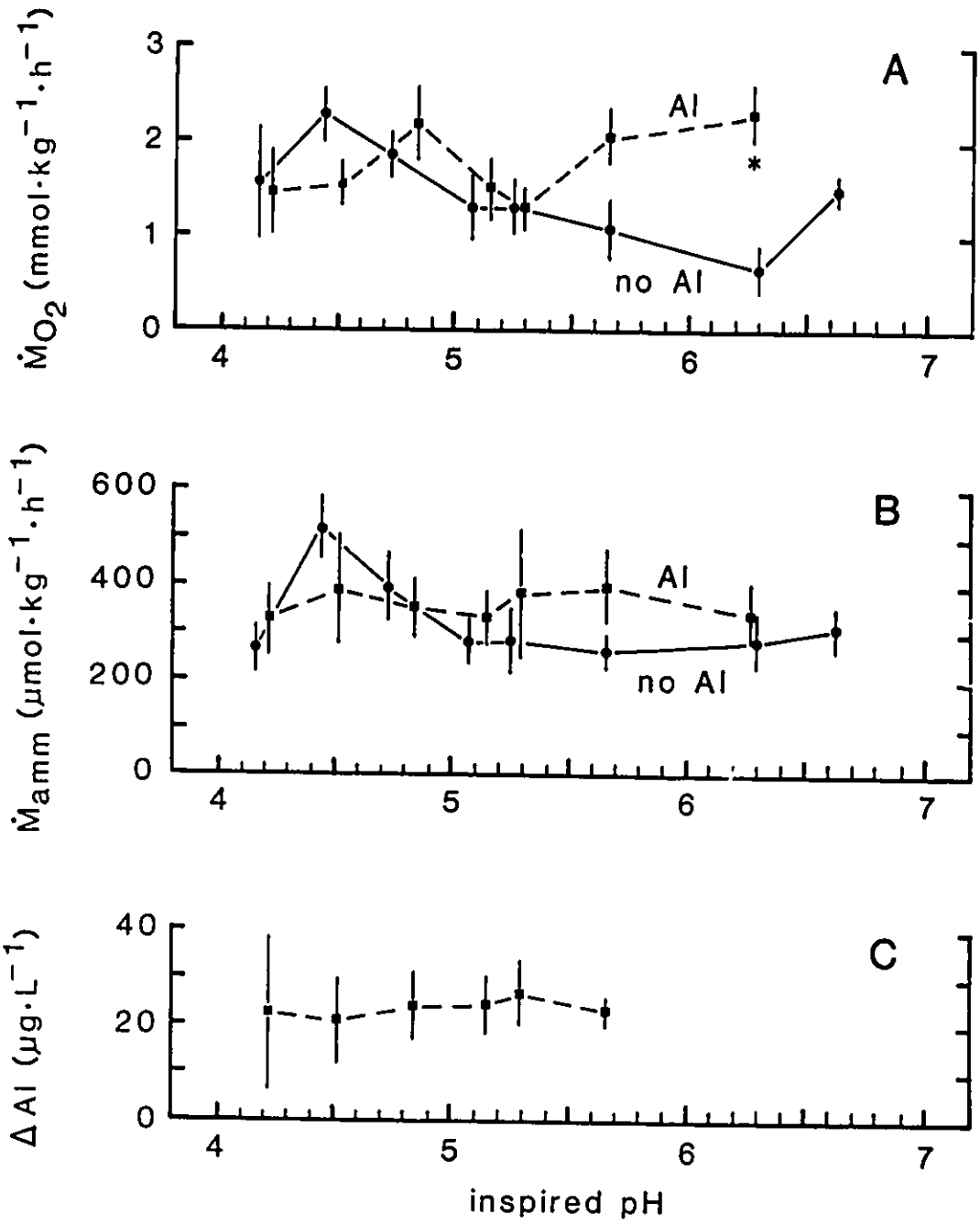

+1.0
+0.5
0
-0.5
ΔpH (pHex-pHin)
A
no Al
Al
4
5
6
7
1.0
0.5
0
V̇w (L·kg⁻¹·min⁻¹)
B
Al
no Al
***
inspired pH

Fig. 28. 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 $\mu g.L^{-1}$ Al. Except at pH_{in}=6.3 (asterisk) there were no significant differences in $\dot{M}_{O_2}$ between Al-exposed fish and fish exposed to acidity alone ($P<0.05$, 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. 27A.

C) The differences between inspired and expired Al concentrations (ΔAl) for some of the fish exposed to 93 $\mu g.L^{-1}$ 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.


A
B
C
Al
no Al
*
$\dot{M}_{O_2}$ (mmol·kg^{-1}·h^{-1})
$\dot{M}_{amm}$ (µmol·kg^{-1}·h^{-1})
ΔAl (µg·L^{-1})
0
1
2
3
200
400
600
20
40
4
5
6
7
inspired pH

alone (Fig. 28A). Only at pH_{in}=6.3, the point of maximum elevation in $\dot{V}_w$ in the presence of Al (Fig. 27B), was $\dot{M}_{O_2}$ significantly elevated. Ammonia excretion ($\dot{M}_{amm}$) was also variable, but averaged about 350 $\mu mol.kg^{-1}.h^{-1}$ for both treatments (Fig. 28B). For the Al-exposed trout, Al removed from water passing over the gills was approximately constant (ΔAl=20-30 $\mu g.L^{-1}$) between pH_{in}=4.2 and 5.7 (Fig. 28C).

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. 29A). 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 exposure (Fig. 29B), coincident with the gradual increase in $\dot{V}_w$ (Fig. 29A). 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. 27A) this is not

surprising. It is the change in the new pH_{ex} with time (asterisks on Fig. 29B) 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. 30A). This pattern follows that of $\dot{V}_w$ (Fig. 29A). Ammonia excretion ($\dot{M}_{amm}$) was also fairly constant throughout 44 h exposure to pH_{in}=5.2 and 4.8 (Fig. 30B), 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 $\mu g.L^{-1}$ Al caused large increases in fish ventilation (almost quadrupling by 20 h, Fig. 29C), and 4 of 5 fish died by 40 h (Fig. 29D, 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. 29C), 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

Fig. 29

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 the greatest 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 at 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. 29A. Mean pH_{in}=6.62±0.01 (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 $\dot{V}_w$. Expired pH was <u>always</u> lower after acid additions started than before the additions (usually P<0.01); crosses have been left off all points (Fig. 29B, 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 2-3 h later (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. Statistical conventions as in Fig. 29A.

D) Expired pH of the same 15 fish presented in Fig. 29C. 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. Mean pH_{in}=6.50±0.03 (15) before start of acid exposures (white arrow). Crosses in circles indicate fish deaths.

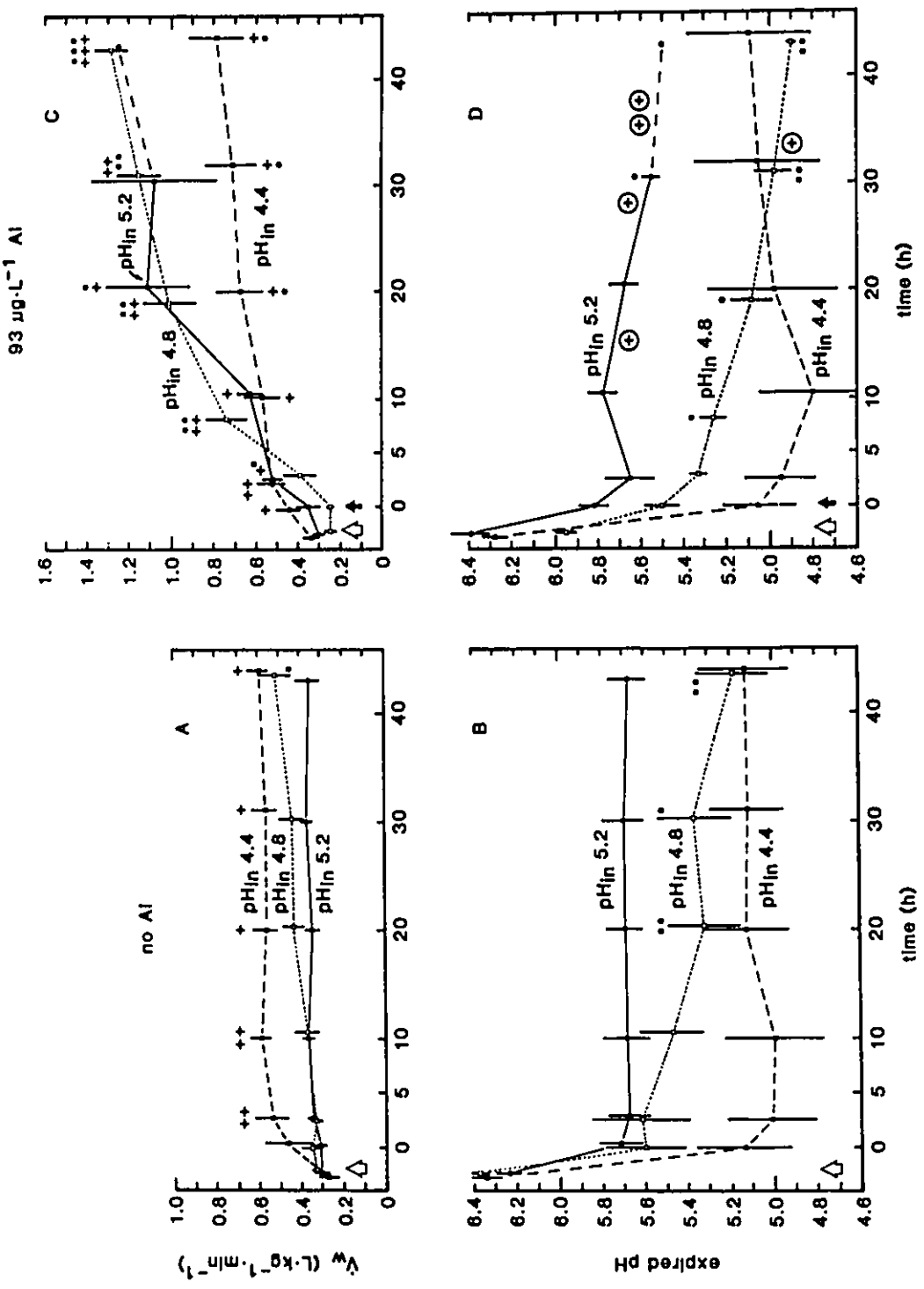

A
no Al
pHin 4.4
pHin 4.8
pHin 5.2
B
pHin 5.2
pHin 4.8
pHin 4.4
C
93 µg·L⁻¹ Al
pHin 5.2
pHin 4.8
pHin 4.4
D
pHin 5.2
pHin 4.8
pHin 4.4
time (h)
time (h)
V̇w (L·kg⁻¹·min⁻¹)
expired pH

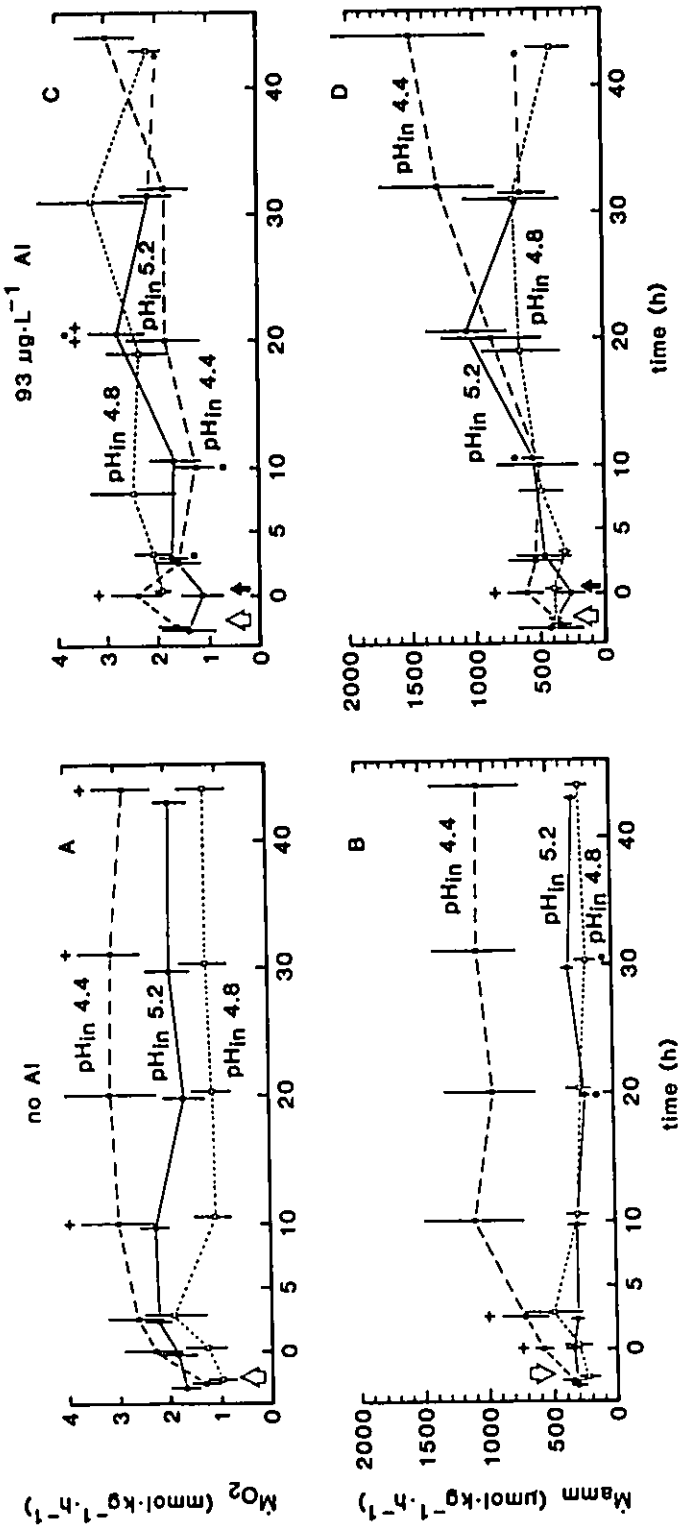
Fig. 30

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. 29A, 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. 29A). See Fig. 29A for details of statistics used.

B) Ammonia excretion ($\dot{M}_{amm}$) of the same 15 fish presented in Fig. 30A. $\dot{M}_{amm}$ 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. 29C, D. White arrow represents start of acid addition; black arrow, start of Al addition 2-3 h later (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. 30A). 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. Statistical conventions as in Fig. 29A.

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.


no Al
93 µg·L⁻¹ Al
A
B
C
D
pHin 4.4
pHin 5.2
pHin 4.8
ṀO2 (mmol·kg⁻¹·h⁻¹)
Ṁamm (µmol·kg⁻¹·h⁻¹)
time (h)

during the pH_{in}=4.8 plus Al treatment (Fig. 29D). 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. 29B). In the pH_{in}=4.4 plus Al exposure there were no significant changes in pH_{ex} over time, in accord with smaller increases in $\dot{V}_W$, 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. 29C), there were only small increases in $\dot{M}_{O_2}$ (<2-fold), significant at only a few exposure times in the various groups (Fig. 30C). 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. 30D). 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 $\mu g.L^{-1}$ 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 Δammonia decreased during the Al-induced hyperventilation.

Deposition of Al onto the gills

Measured Al accumulation on the gills was a small fraction of Al removed from water passing over the gills. Aluminum removed from the water passing over rainbow trout gills during the three, 44 h Al treatments is given in Table 3. Usually ΔAl was between 5 and 20 $\mu g.L^{-1}$, slightly lower than the ΔAl values (~20 $\mu g.L^{-1}$) measured during the 2-3 h exposures (Fig. 28C). 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. $\dot{V}_w \cdot$ 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 $\mu g.L^{-1}$ 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 44 h exposures to 93 $\mu g.L^{-1}$ Al at pH 4.4 and 4.8 (Table 3). The one remaining fish at the end of the pH 5.2 exposure was not sampled for Al. Background filament Al concentration (from the pH 4.4, no Al 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

Table 3. Gill Al concentrations and Al extraction (ΔAl; inspired [Al] minus expired [Al]) at gills of rainbow trout exposed for 44 h to 93 $\mu g.L^{-1}$ Al in soft water at pH 5.2, 4.8, and 4.4. Means $\pm$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 ($\mu g.g^{-1}$ wet tissue)	ΔAl ($\mu g.L^{-1}$) 2-3 h	10 h	20 h	31 h	44 h	estimated cumulative Al deposition (mg)	measured gill Al deposition (mg)
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

$\mu g.g^{-1}$ 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 the 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 3). 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).

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. 31, where the observed ΔpHs at the gills of fish exposed to Al for 2-3 h (from Fig. 27A) 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 (Fig. 31). According to the bulk water solubility curve, 93 $\mu g.L^{-1}$ Al should start to precipitate from water at pH-5.3, the pH where 93 $\mu g.L^{-1}$ Al intersects the bulk water solubility curve. However, because of the more basic

conditions at the gills relative to inspired water, 93 $\mu g.L^{-1}$ Al will begin to precipitate onto gill epithelia when the inspired soft water is just pH 4.8 or greater (Fig. 31). It should be noted that the Al solubility curve for microcrystalline gibbsite (Roberson and Hem 1969) was used in these 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 to 6.0 (Fig. 31). If Al precipitation resulting from these pH changes were the cause of Al deposition on the gills, maximum deposition of Al would be predicted at highest pH (5.2), and lowest deposition (theoretically none) at lowest pH (<4.4). In order to test these predictions a separate experiment was run 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 $\mu g.L^{-1}$ 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 3), 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. 32). 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. 27B, 28C, Table 3). This result indicates that even during relatively short exposures most of the deposited Al was removed from the gills.

Fig. 31. 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 $\mu g.L^{-1}$ Al (Fig. 27A). 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 $\mu g.L^{-1}$ Al. Solubility curve used is for microcrystalline gibbsite (from Roberson and Hem 1969). Note that the Al solubility scale is logarithmic. See text for more details.

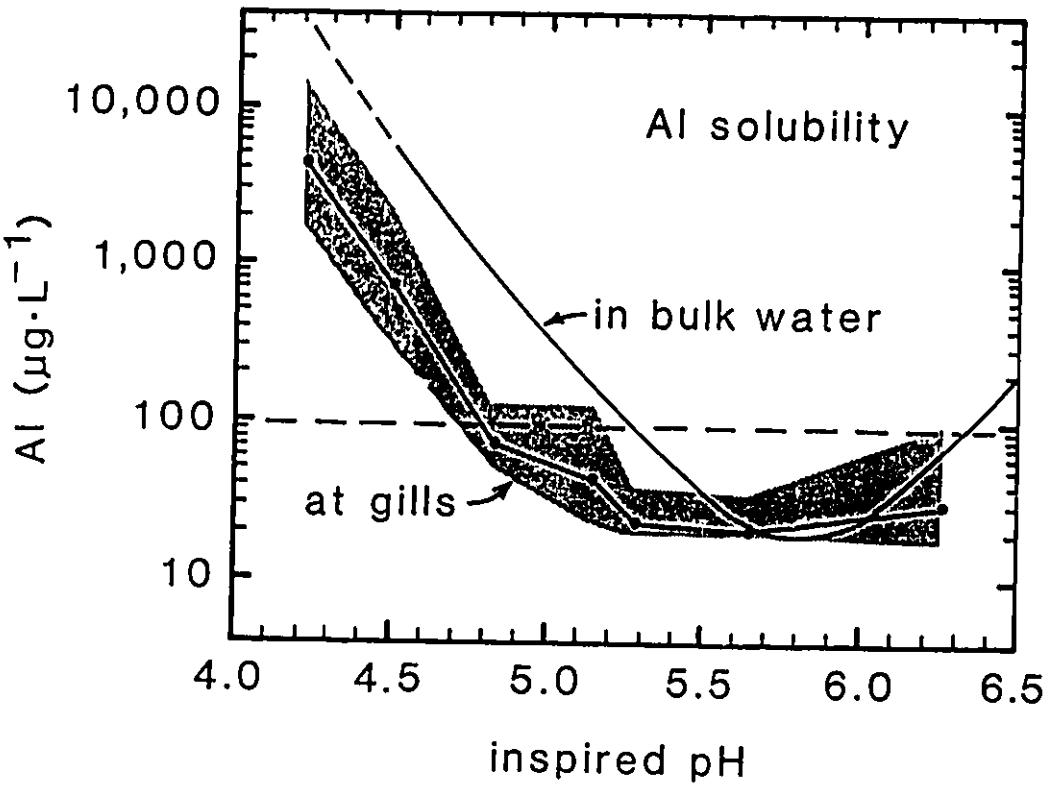
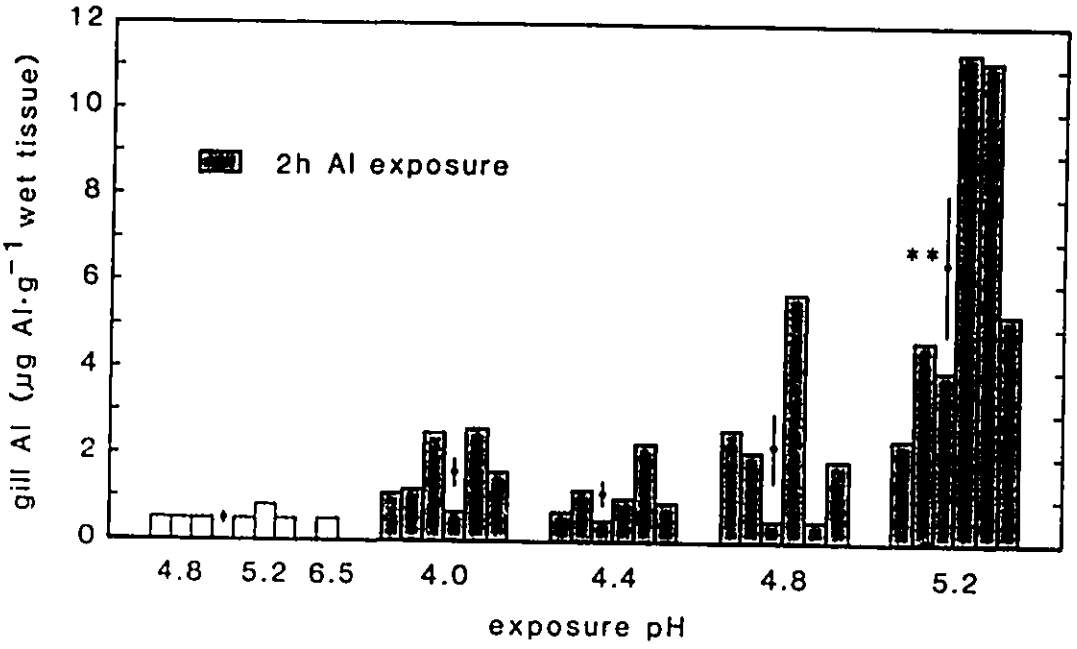

10,000
1,000
100
10
Al (μg·L⁻¹)
Al solubility
in bulk water
at gills
4.0
4.5
5.0
5.5
6.0
6.5
inspired pH

Fig. 32. Aluminum concentrations on gills of rainbow trout held for 2 h at the indicated pHs, in the absence (clear bars) or presence (98 $\mu g.L^{-1}$; 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 $\pm$1 SEM for each exposure group are also indicated.


12
10
8
6
4
2
0
gill Al (μg Al·g⁻¹ wet tissue)
2h Al exposure
**
4.8
5.2
6.5
4.0
4.4
4.8
5.2
exposure pH

Discussion

Water pH and Al chemistry in the gill micro-environment

By means of opercular catheters and latex ventilation masks, it was shown 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. 27A), in agreement with results of Chapter 3. In the pH_{in} range 4.5 to 5.2 in the presence of 93 $\mu g.L^{-1}$ Al, the observed ΔpHs near the gills were lower than observed in the absence of Al (Fig. 27A). Lower ΔpH of the Al-exposed trout can be partly explained by their higher ventilation at pH_{in}=4.8 to 6.3 (Fig. 27B), because approximately the same amount of base, ammonia (Fig. 28B), and CO_2 were released at the gills, but into a larger volume - higher flow - of water (see Chapter 3).

In addition, chemical species of Al change with pH, from mostly Al^{3+} at pH<4.5 to mostly $Al(OH)_3^o$ near pH 6.0 (Fig. 2), which buffers pH by consuming OH^- ions. Ninety-three $\mu g.L^{-1}$ Al^{3+} represents about 10.5 $\mu equiv.L^{-1}$ of consumed base if the Al^{3+} is converted completely to $Al(OH)_3^o$; over the observed ΔpHs, actual base consumed was only about 3 $\mu equiv.L^{-1}$. In the soft water, 3 $\mu equiv.L^{-1}$ 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 Chapter 3). 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.

Changes in ventilation and gill water chemistry over time

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. 29A, B; 30A, 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. 29C, 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 the earlier study at comparable pHs and Al concentrations (Chapter 2).

The continuous increase in $\dot{V}_w$ over time at pH_{in}=4.8 contrasts with the results of Walker et al. (1988b) where brook trout exposed to 330 $\mu g.L^{-1}$ 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. (1988b) attributed the later 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. Large increases in ventilation resulted in only small increases in $\dot{M}_{O_2}$ in the present study (Fig. 30C), 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 the previous cannulation study on Al-exposed rainbow trout (Chapter 2).

Gill Al accumulation

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 $\mu g.L^{-1}$ 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. 27A). 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. 31). Precipitation of Al from a 93 $\mu g.L^{-1}$ Al solution onto the gills was predicted, from Fig. 31, 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 $\mu g.L^{-1}$ Al (Fig. 32).

Over longer exposures, expired pH decreased enough (as $\dot{V}_w$ increased) to alleviate Al precipitation at the gills only in the pH_{in}=4.8 plus Al exposure. The decrease in expired pH from 5.3 to

4.9 (Fig. 29D) theoretically increased the amount of Al which could be held in solution, from about 70 to about 500 $\mu g.L^{-1}$ (from Fig. 31). 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 would likely cause 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; Mueller et al., submitted). In turn, gill damage would result in the ionoregulatory and respiratory disturbances (Neville 1985; Wood et al. 1988; Chapter 2), which ultimately kill the fish.

Differences in Al solubilities between inspired water and the water near fish gills (Fig. 31) 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 to 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, Chapter 2). 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. 31). 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. 27A) would now

keep Al solubility low. This precipitation of Al could well explain the very high ventilation of trout exposed to Al at pH_{in} 6.3 (Fig. 27B).

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, there are two important caveats. First, it remains to be demonstrated whether precipitation reactions occur quickly enough, relative to the contact time of water at the gills, for this explanation to be feasible. This question is addressed experimentally in Chapter 5. Second, precipitation as the sole explanation of gill Al deposition may well be an oversimplification.

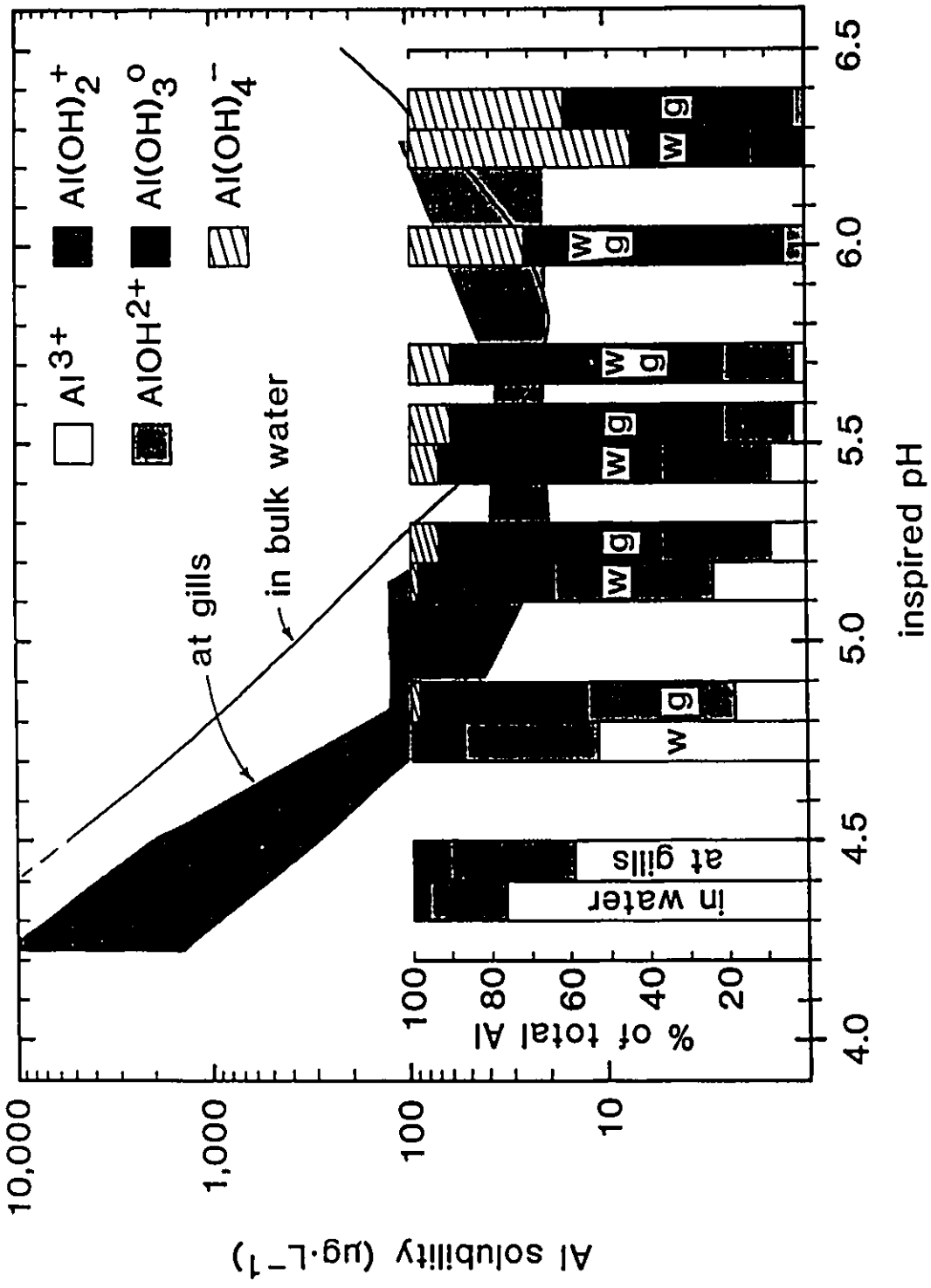
For example, explanations are needed as to why Al is accumulated on gills of fish exposed to Al for 2 h at pH_{in}=4.0 and 4.4 (Fig. 32) or 44 h at pH_{in}=4.4 (Table 3): in these instances, measured pH near the gills was so low that no Al should have precipitated from solution (i.e. Fig. 31). Three plausible explanations are (i) alkalinization near the gills has been underestimated, thereby underestimating Al precipitation, (ii) changes in Al speciation are important in 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 discussed in Chapter 3. 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. 33) on the basis of the measured ΔpHs of Al-exposed fish (Fig. 27A), 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 the soft water (<1 μM) and the use of a one pass, flow through system. In the absence of data regarding rates of Al species shifts and precipitation rates, I assumed that these reactions can occur during water transit time at the gills; clearly it would be useful to determine these reaction rates (see Chapter 5).

Four important conclusions arise from Fig. 33. First, below pH_{in}~5.2, changes in Al solubility are much larger than changes in Al speciation as water passes over the gills. For example, at

Fig. 33. 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. 31). Aluminum species are given as per cent of total Al, taken from Dyrssen (1984; Fig. 2). Here, total Al=93 $\mu g.L^{-1}$ (=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.


Al solubility (μg·L⁻¹)
10,000
1,000
100
10
Al³⁺
Al(OH)₂⁺
AlOH²⁺
Al(OH)₃°
Al(OH)₄⁻
at gills
in bulk water
% of total Al
100
80
60
40
20
in water
at gills
w
g
4.0
4.5
5.0
5.5
6.0
6.5
inspired pH

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}~5.2 and 6.3, so the two effects could be of equal importance here. Note however that Al solubility at these pHs is so low that supersaturated conditions existed in both inspired and expired water in the tests outlined in Fig. 27A.

Third, 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. 32). Aluminum hydroxides are generally thought to be the more toxic forms of Al; presumably these species adsorb to the gill surface and polymerize (i.e. 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. 33). Finally, whatever the relative importance of the different Al species, the present analysis illustrates that previous efforts to determine which Al species is most toxic to fish (eg. Sadler and Lynam 1987; Palmer et al. 1989) should be reconsidered (eg. Neville and Campbell 1988). Due to pH changes in the gill micro-environment, 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, a conclusion supported by more detailed and accurate studies in Chapter 5. 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 could be explained by 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, and Chapter 5) 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., submitted). 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, cannot be determined at present.

To summarise, the pH near rainbow trout gills was higher than in acidic inspired soft 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 vs 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. The toxic effects of Al could be 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.

Chapter 5

Mechanisms of Al extraction and accumulation at fish gills

Introduction

The results of Chapter 4 indicated that pH changes in the branchial micro-environment could be important in Al deposition on fish gills. As acidic water containing Al is rendered more basic by ammonia and base released at the gills, Al could theoretically precipitate from solution onto the branchial surface as the solubility of Al is exceeded. Shifts from one Al species to another could also be responsible for Al accumulation; the various positively charged species might be expected to bind with differing affinities to negatively charged gills. Branchial surfaces are presumed to be negatively charged because of carboxyl groups on mucus (Satchell 1984). In addition, shifts to neutral species, such as $Al(OH)_3^o$, might favour the formation of Al precipitates. Water breathed by fish is only in contact with the gills for 0.4-2 s (Randall 1970), so the processes involved in Al deposition - whether adsorption, complexation, or precipitation - must be fast.

The present study was designed to examine in detail the mechanisms of Al deposition, by frequently measuring expired pH and Al extraction at the gills at three different inspired pHs (pH

5.1, 4.7, and 4.1) over short (6 h) exposures to Al. As in Chapter 4, calculated Al deposition at the gills could be compared to actual gill Al accumulation, to indirectly determine sloughing of Al. The accuracy of calculated deposition was improved relative to Chapter 4 by making more frequent measurements of ΔAl and ventilation volume. A methodological comparison of three rinsing protocols for gills sampled for Al was also done during this part of the study.

Once collected, accurate values of Al extraction and deposition could be compared with inspired or expired pH to examine their pattern of variation with respect to water pH. Expired pH could also be used to calculate Al solubility and speciation, to which extraction and deposition of Al could be compared. These analyses had the potential to determine whether Al solubility changes were responsible for Al extraction at fish gills. If speciation changes were important, the Al species most likely involved in Al extraction and deposition could be determined.

Finally, the question whether Al can precipitate from solution fast enough to account for Al deposition on gills was addressed. In the past, researchers working with Al chemistry have usually been interested in geological rather than biological time scales, and have often measured Al precipitation from concentrated Al solutions over hours to days (eg. May et al. 1979). The fastest reported time for Al precipitation is 1 min,

for 540 or 1080 $\mu g.L^{-1}$ Al solutions at 2-20°C (Tipping et al. 1988). The object of this part of the study was to measure Al precipitation over a time scale approximating water residence time at fish gills, in acidic soft water of low Al concentration, with an alkalinization of the water similar to that measured at fish gills.

Material and methods

In vivo experiments

Thirty rainbow trout from Spring Valley Trout Farm, New Dundee, Ont., were used in the opercular catheter and ventilation experiments (227±8 g). Fish were acclimated at 15-16°C for at least two weeks to synthetic soft water before all experiments; soft water was produced as described in Chapter 3. Acclimation and experimental water composition was: Ca^{2+} ~50 μequiv.L^{-1}, Na^{+} ~50 μequiv.L^{-1}, Cl^{-} ~100 μequiv.L^{-1}, titratable alkalinity to pH 4.0 ~130 μequiv.L^{-1}, pH ~6.5.

Fish were fitted with latex ventilation masks (see Chapter 3), but were fitted with two opercular catheters each, one per gill, to double the number of expired water samples. Trout were allowed to recover for 24 h before an experiment was started. Six separate experiments were run: five fish at a time were exposed to pH 5.1, 4.7, or 4.1 in the presence (138±1 μg.L^{-1}) or absence (1 μg.L^{-1}) of Al for 6 h. Measurements of ventilation volume ($\dot{V}_w$), and inspired and expired Al were taken approximately hourly, using methods given in Chapter 4. The difference between inspired and expired Al (ΔAl) is referred to as Al extraction at the gills. Water Al was measured by graphite furnace (see next section). Expired pH was measured ~0.5 h before the experiments and at 3 and 5.5 h during the experiments (see Chapter 3 for procedure). In addition, inspired and expired water was assayed for protein using

a modified micro Lowry method (Lowry 1951) and Sigma reagents, to check for mucus release from the gills. One mL aliquots of sample were added to 2 mL of 50:1 Na_2CO_3:$CuSO_4$ solution, and 100 µL of 2 N Folin and Ciocalteu's phenol reagent were added. Protein samples were read after 1 h against diluted 400 $\mu g.mL^{-1}$ BSA standard (Sigma) on a Pye Unicam PU 8600 spectrophotometer at 750 nm.

At the end of each experiment, fish were removed from their boxes without anaesthetic and killed with a blow to the head. A 2.3 cm diameter circle of Whatman 3 MM qualitative filter paper was placed on the left gills with forceps for ~15 s to collect surface accumulations of Al and mucus. Filter papers were placed in 7 mL vials of experimental water (pH 5.1, 4.7, or 4.1, as appropriate; no Al), shaken for 5 s, then left standing overnight at 4°C. Filter papers were removed from the vials the next day, water samples were frozen (-20°C), and the residual protein (mucus) and Al contents of the 7 mL water were later measured. Hemoglobin content was also measured (colorimetric cyanmethemoglobin method of Blaxhall and Daisley (1973); Sigma Drabkin's reagent) to check the contribution of blood contamination to the protein measurements.

After the left gills were blotted, portions of the third right gill were removed for accumulated Al analysis. To assess sampling protocol effects on the results of Al analysis, one gill portion was not rinsed, a second portion was placed in 7 mL of

experimental water (appropriate pH, no Al) for one minute, and a third portion was held with forceps and agitated in 3 successive 7 mL rinses of experimental water (no Al), 20 s agitation per rinse. Gill portions were stored frozen at -20°C, later thawed, digested in 0.05 M H_2SO_4, diluted 100x (Chapter 4), and analysed for Al by graphite furnace (next section). Finally, the complete left gill basket was removed from each fish and weighed.

Aluminum precipitation experiments

Increases in water pH caused by base and ammonia release at fish gills could theoretically cause reductions in Al solubility in the gill micro-environment, resulting in Al deposition. However, Al precipitation must occur quickly to be an important cause of Al accumulation on fish gills, because gill water residence time is <2 s. To determine the speed of Al precipitation from soft water, a series of experiments were run in which water chemistry changes at the gills were mimicked.

Preliminary experiments on Al precipitation rates used a Hach 2100A turbidity meter, a Pye Unicam PU 8600 spectrophotometer, and a glass 300 mL filtering funnel (Millipore) with 0.22 or 0.45 μm membrane filters (Millipore). For these preliminary experiments, Al concentrations were measured using the pyrocatechol violet method (Dougan and Wilson 1974).

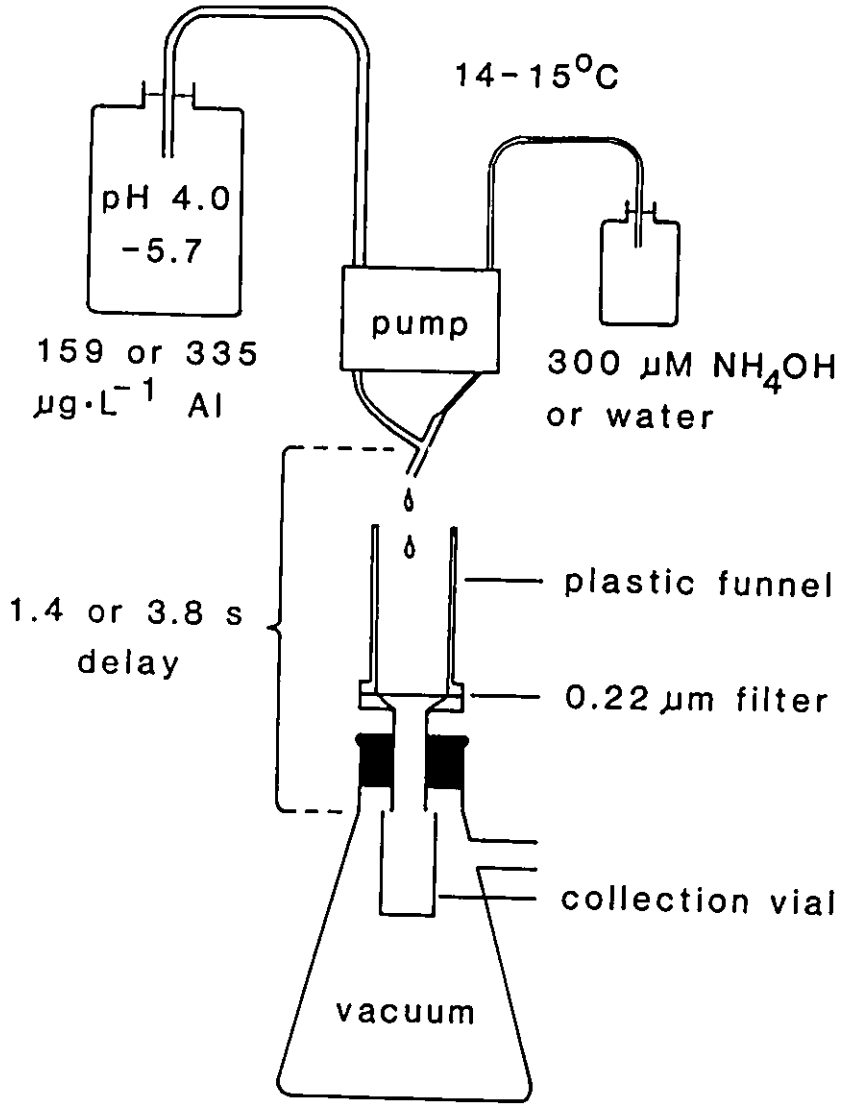
Final experiments used a peristaltic pump (Gilson Minipuls 2) to mix the test Al solution (7.2 $mL.min^{-1}$) with deionised water or

300 μM NH_4OH (0.7 mL.min^{-1} for both). The dilution resulted in about 30 μM base addition, the amount released at rainbow trout gills into acidic water (Chapter 3). Deionised water addition was the control situation. All solutions were kept at 14-15°C. Initial pH of the Al solution was adjusted with dilute H_2SO_4. Final solution pHs (after mixing with base or water, but before being filtered) were measured in 8 mL polyethylene vials (stirred) using a Radiometer PHM82 meter and Radiometer GK2401C combination electrode.

The Al solution and base (experimental) or Al and water (control) met at a polyethylene "T", then flowed along either 2 or 16 cm of polyethylene tubing, which took 0.8 or 3.2 s after mixing, respectively (Fig. 34). The solution then dripped onto a 0.22 μm Nuclepore filter under vacuum (700 mm Hg) and was collected in an 8 mL polyethylene vial suspended beneath the filtering apparatus. Total delay between mixing and the arrival of a drop in the vial <u>after</u> filtration was estimated to be about 1.4 or 3.8 s. The filter funnel assembly was plastic (polycarbonate; Nuclepore), with a rubber O-ring and silicone gasket. The funnel was disassembled completely between each sample run, rinsed in 5% HCl, distilled water, then with deionised water.

The re-assembled filtering apparatus and fresh 0.22 μm filter were rinsed with two, 100 mL volumes of 5% HCl under vacuum, then with two, 100 mL volumes of soft water acidified to pH-4.3 with

Fig. 34. Illustration of the mixing and filtration apparatus used in the final Al precipitation experiments. The plastic filtering apparatus and 0.22 μm filters were rinsed between each sample run (see text for details).


14-15°C
pH 4.0
-5.7
159 or 335
µg·L⁻¹ Al
pump
300 µM NH4OH
or water
1.4 or 3.8 s
delay
plastic funnel
0.22 µm filter
collection vial
vacuum

dilute H_2SO_4, and finally rinsed for 2 min with the experimental or control solution dripping onto the filter, again under vacuum. The third or fourth minute of filtered Al solution was the sample collected for analysis; the filters did not clog in this time. This protocol eliminated Al contamination between samples.

For all water Al analyses, samples (7 mL) were acidified with 20 µL concentrated HCl, then analysed without dilution for total Al using a Varian AA-1275 atomic absorption spectrophotometer with GTA-95 graphite tube atomizer. Ten µL of water sample - or diluted gill sample - were analysed against standards at 309.3 nm. Operating parameters were as follows: 5 s at 80°C, 35 s at 95°, 10 s at 110°, 12 s at 1200°, and 2.7 s at 2500°C, N_2 gas.

Statistical analysis

To determine whether Al solubility or species changes were responsible for deposition of Al at fish gills, ΔAl and gill Al were first compared with inspired pH (pH 5.1, 4.7, 4.1), then with measured expired pH (variable pHs). For each opercular catheter, the mean of the 2 and 3.5 h ΔAl values (Fig. 36) was compared to pH_{in} or pH_{ex} taken at 3 h; likewise, for each catheter the mean of ΔAl for 5 and 6 h was compared to the 5.5 h pH_{ex}. Gill Al for each fish was compared to the mean pH_{ex} of both gills over the course of the experiment (3 or 4 pH_{ex} values); the 3 rinsing protocols were considered separately. Expired pH was then used to calculate Al solubility, using the pH-solubility relationship for

micro-crystalline gibbsite given by Roberson and Hem (1969), and Al species using the speciation scheme of Dyrssen (1984).

Regression analysis (least squares method; analysis of variance) was used to analyse the relationships between extraction or deposition of Al and inspired pH. Correlation coefficients were calculated to determine the degree of association between ΔAl or gill Al with pH_{ex}, log Al solubility, Al oversaturation, and the five Al species. Correlation coefficents were calculated because expired pH, from which all these comparisons were based, was varied indirectly by changing inspired pH (i.e. pH_{ex} was a dependent variable), and because pH_{ex} can be affected by Al deposition, through fish ventilation changes (Fig. 27, Chapter 4). That is, Al extraction and pH_{ex} are sometimes interdependent.

Other statistical methods used were unpaired Student's t-tests for analysis of fish ventilation and Al filtration data. Duncan's Multiple Range test was used to compare gill Al data. Unless indicated otherwise, the level of significance for all tests was $P<0.05$.

Results

In vivo experiments: Al deposition on fish gills

A total of 30 rainbow trout fitted with latex masks and opercular catheters were exposed for 6 h to acidic soft water (pH 5.1, 4.7, or 4.1) in the presence (138±1 $\mu g.L^{-1}$) or absence of Al. Ventilation volumes ($\dot{V}_w$) of the fish did not change in response to moderately acidic conditions alone (pH 5.1, 4.7), but doubled in 2 h and tripled by 6 h during the pH 4.1 exposure (Fig. 35A). One fish died at about 3.5 h at pH 4.1 (cross in circle). In contrast, in the presence of Al, $\dot{V}_w$ generally increased over the first 2 h of exposure at pH 5.1 and 4.1, then stayed constant (Fig. 35B). Ventilation did not increase as much during the pH 4.1, Al exposure as during the pH 4.1, no Al exposure: Al apparently reduced the irritating effects of extreme acidity.

Aluminum extraction at the gills (ΔAl), the difference between inspired and expired Al concentrations, was highest in the pH 5.1 plus Al exposure, intermediate for pH 4.7, and lowest for pH 4.1 (Fig. 36). There was a tendency for ΔAl to decrease over 6 h, probably a result of increased $\dot{V}_w$. Mean estimated Al deposition at one set of gills was calculated for each exposure, by multiplying ΔAl by one-half the measured volume of water passing over the gills (i.e. $\dot{V}_w$·fish weight), then by the elapsed time from the previous sample. Mean calculated Al depositions per set of gills over the 6 h exposures to 138 $\mu g.L^{-1}$ Al were 0.73 mg

Fig. 35. A. Ventilation volumes ($\dot{V}_w$) of rainbow trout exposed to pH 5.1, 4.7, and 4.1 soft water for 6 h. Five fish per experiment. Clear arrow = start of acid additions. i, i_2 = 1.5, 0.5 h before start of acid additions. $\dot{V}_w$ only increased during the pH 4.1 exposure. Cross in circle = 1 fish death. *, **, *** = $P<0.05$, $P<0.01$, $P<0.001$, paired t-test compared to $\dot{V}_w$ at time i_2.

B. As above, but in the presence of 138 ± 1 $\mu g.L^{-1}$ Al. Dark arrow = start of acid and Al additions. $\dot{V}_w$ increased with time in all three exposures.

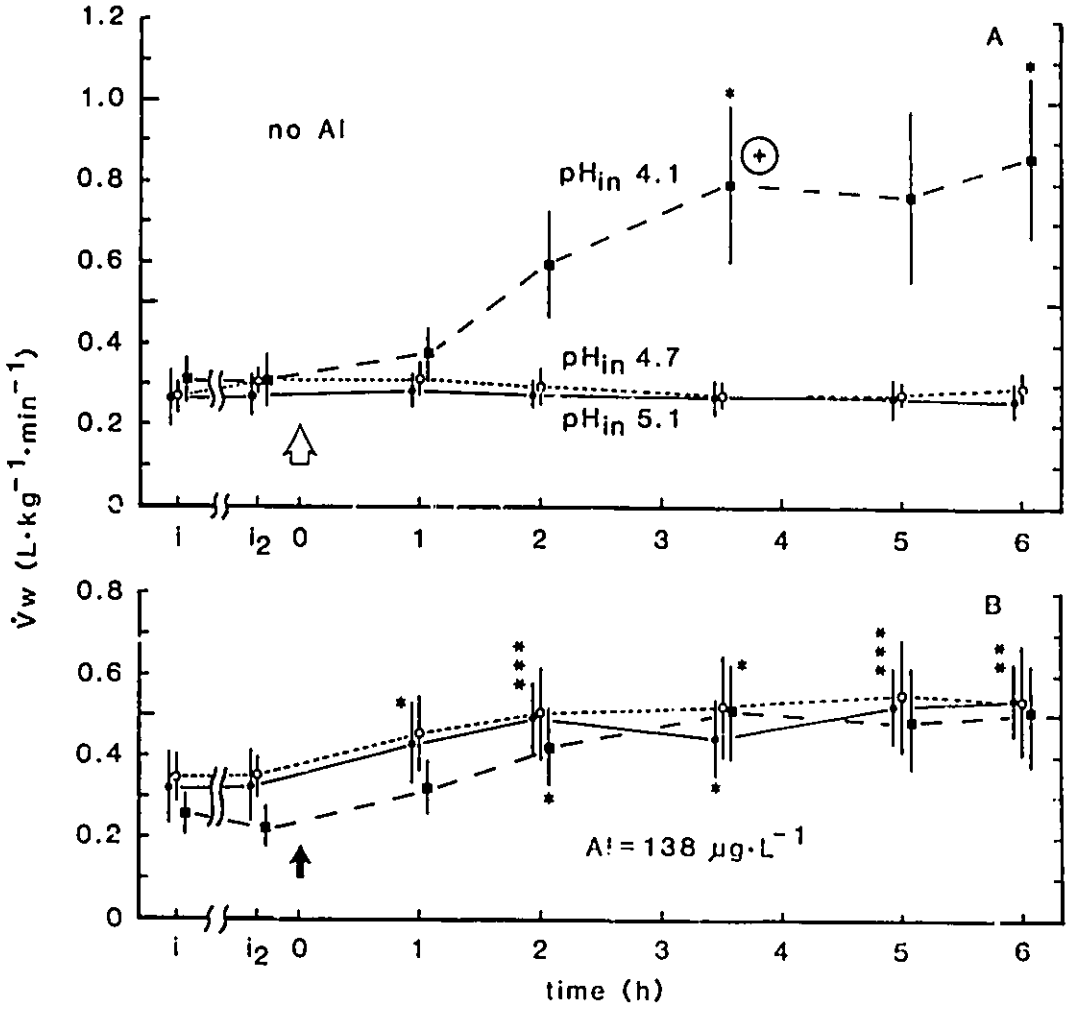

A
no Al
pH$_{in}$ 4.1
pH$_{in}$ 4.7
pH$_{in}$ 5.1
B
Al = 138 µg·L^{-1}
$\dot{V}_w$ (L·kg^{-1}·min^{-1})
time (h)
i
i$_2$
0
1
2
3
4
5
6

Fig. 36. Mean Al extraction ($\Delta Al = [Al]_{in}$ minus $[Al]_{ex}$) for rainbow trout exposed to 138±1 $\mu g.L^{-1}$ Al for 6 h. ΔAl was highest for pH_{in} 5.1, lowest for pH_{in} 4.1 exposures. ΔAl for pH_{in}=5.1 at 1 h was low because $[Al]_{in}$ was only 105±6 $\mu g.L^{-1}$ at that time.

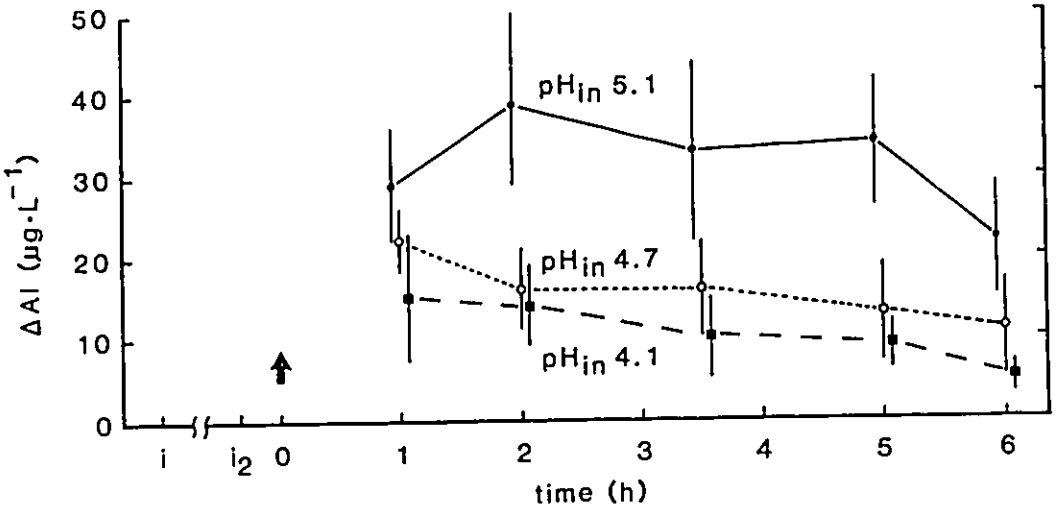
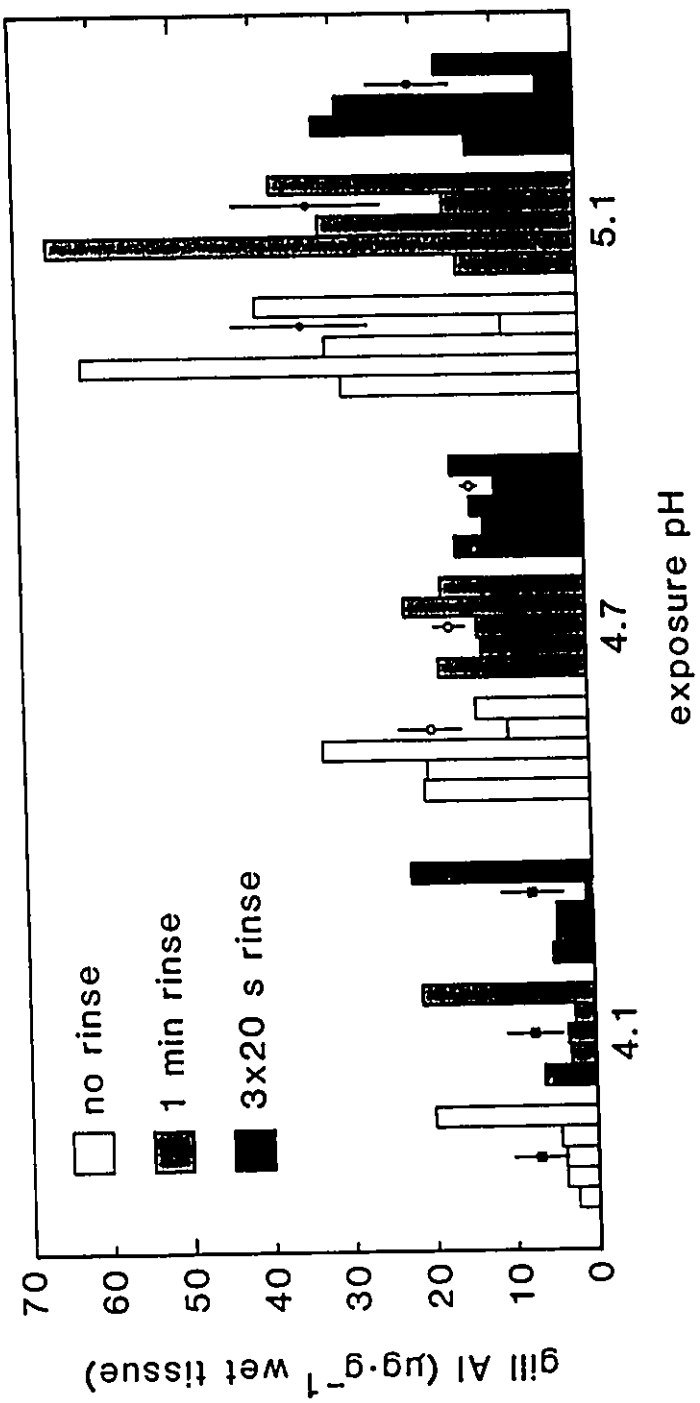

50
40
30
20
10
0
ΔAl (μg·L⁻¹)
pH$_{in}$ 5.1
pH$_{in}$ 4.7
pH$_{in}$ 4.1
i
i$_2$
0
1
2
3
4
5
6
time (h)

Table 4. Calculated and measured Al deposition on gills of rainbow trout exposed for 6 h to 138±1 $\mu g.L^{-1}$ in soft water at pH 5.1, 4.7, or 4.1. Means ±1 SEM (n). Cumulative Al deposition onto one set of gills was estimated from ΔAl, $\dot{V}_w$, and fish weight. Actual Al accumulations per set of gills for each fish were calculated directly from measured gill Al concentrations (Fig. 37; combined data from all rinse protocols) and individual, whole gill basket weights (mean = 2.4 g). Mean gill Al concentrations (all rinse protocols, all fish) are given in the Table, as well as surface Al removed by filter paper discs and released into 7 mL water. See text for more details.

exposure pH	calculated cumulative Al deposition (mg)	measured gill Al deposition (mg)	measured gill Al concentration ($\mu g.g^{-1}$ wet tissue)	surface Al blotted from gills (μg)
5.1	0.73±0.29 (5)	0.06±0.01 (5)	29.4±7.3 (5)	0.13±0.03 (4)
4.7	0.31±0.09 (5)	0.04±0.01 (5)	16.8±1.1 (5)	0.20±0.02 (5)
4.1	0.18±0.08 (5)	0.02±0.01 (5)	7.3±3.5 (5)	0.11±0.02 (5)

Fig. 37. Measured Al accumulation on the gills of rainbow trout exposed to 138±1 $\mu g.L^{-1}$ Al at pH 4.1, 4.7, and 5.1 for 6 h. Gill portions were handled with 3 different rinsing protocols (see text for details). For each pH, values from five individual fish are shown, as well as the means ±1 SEM. In each case, the order of the gills from the five fish treated 3 ways is preserved left to right. There was no effect of rinsing protocol on gill Al concentrations (Duncan's Multiple Range test, $P>0.05$). Within each rinse protocol, gill Al concentrations for the pH 5.1 exposure were greater than for the 4.1 exposure ($P<0.05$), but not significantly different from the pH 4.7 values (Duncan's Multiple Range test). Mean gill Al for fish exposed to acidity alone was 0.1 μg $Al.g^{-1}$ (range: 0.0-1.3, all rinse protocols; n=14).


gill Al (μg·g⁻¹ wet tissue)
0
10
20
30
40
50
60
70
no rinse
1 min rinse
3x20 s rinse
4.1
4.7
5.1
exposure pH

Al for pH 5.1, 0.31 mg for pH 4.7, and 0.18 mg Al for the pH 4.1 exposure (Table 4).

Expired pH for all fish was measured before the start of acid or Al additions (time i_2 on Figures) and at 3 and 5.5 h after the exposures started. At ambient pH (6.54±0.01, n=30), expired pH for all fish was 6.22±0.02 (n=57). Without added Al, expired pHs were approximately constant during the exposures, averaging pH 5.81±0.06, pH 5.35±0.10, and pH 4.29±0.04 for pH_{in} 5.1, 4.7, and 4.1, respectively. For fish exposed to Al, expired pHs were also constant during the exposures, averaging 5.60±0.06, 5.08±0.07, and 4.42±0.06 for pH_{in} 5.1, 4.7, and 4.1, respectively. Inspired and expired protein was measured before and during all exposures, as an index of mucus glycoproteins released at the gills. Protein released at the gills (i.e. expired-inspired) showed no pattern in any of the exposures, averaging 0-1.0 μg throughout the experiments. The protein concentrations measured were at the detection limit of the Lowry method used, so are not very reliable.

At the end of all experiments, fish were removed from their ventilation boxes, killed, and the gills sampled. Left gill baskets weighed 2.4±0.2 g, wet weight (n=15).

The third right gill filaments were assayed for Al. These filaments were either not rinsed, were placed in experimental water (Al free) for 1 min, or were agitated in 3, 20 s rinses of

experimental water (Al free). Effects of exposure pH on accumulated Al on gills, and of the three rinsing protocols, were determined. Gill Al increased as inspired pH increased ($P<0.01$ for no rinse and 1 min rinse, $P<0.05$ for 3x20 s rinse), and the three rinsing protocols yielded the same results (Fig 37). The only instance where the rinsing protocol might have affected gill Al concentration was after the 3, 20 s rinses in the pH 5.1 exposure, but there was no significant difference between the three rinse protocols ($P>0.05$, Duncan's Multiple Range test). Total Al accumulations per set of gills, using individual gill accumulations (Fig. 37) and individual, whole gill basket weights, averaged 0.06, 0.04, and 0.02 mg Al for pH 5.1, 4.7, and 4.1 exposusures, respectively (Table 4; combined data from all rinse protocols).

Deposition of Al on gills was also assessed by blotting the surface of intact left gills. Filter paper discs from Al-exposed fish released 0.13 μg Al into 7 mL deionised water for the pH 5.1 exposure, 0.20 μg Al for the pH 4.7 exposure, and 0.11 μg Al for the pH 4.1 exposure (Table 4; 0.20 significantly different from 0.11, $P<0.05$). Aluminum released into 7 mL deionised water was 0.00-0.02 μg Al for the pH 5.1, 4.7, and 4.1 exposures without added Al. Analysis for protein (eg. mucus production) at the gills was unsuccessful, because measured protein on the gills was largely blood picked up by the filter paper discs: the

correlation coefficent (r) between protein and hemoglobin was 0.93, nearly a perfect correlation ($P<0.001$; $n=28$).

Expired pH and ΔAl values for both gills of the Al-exposed fish were used to better understand the mechanisms of Al accumulation on fish gills. Expired pH was dependent on inspired pH ($pH_{ex} = 1.19 \cdot pH_{in} - 0.55$, $P<0.001$, $n=57$; $r=0.87$), as was mean ΔAl ($\Delta Al = 20.9 \cdot pH_{in} - 79.3$, $P<0.01$, $n=60$; $r=0.38$). As inspired pH increased, so did ΔAl and expired pH. These two dependent variables were also associated, as shown by their correlation coefficients: as expired pH increased, so did ΔAl ($r=0.62$, $P<0.001$; Fig. 38). ΔAl correlated better with expired pH than with inspired pH ($r=0.62$, 0.38, respectively).

To dissect further the relationship between ΔAl, pH_{ex}, and Al, ΔAl was plotted against the log of Al solubility calculated for each measured expired pH. There was a highly significant, negative correlation between ΔAl and Al solubility ($r=-0.47$, $P<0.001$; Fig. 39A). That is, the lower the Al solubility, the higher the Al extraction at the gills. Similarly, ΔAl plotted against oversaturation of Al yielded a highly significant positive correlation ($r=0.46$, $P<0.001$; Fig. 39B). Here, oversaturation of Al was taken as the difference between Al solubility (Roberson and Hem 1969) and 138 $\mu g.L^{-1}$ Al, if Al solubility was <138 $\mu g.L^{-1}$. Precipitation of Al from oversaturated solution onto the gills is supported by these correlations.

Fig. 38. Mean extraction of Al (ΔAl) at rainbow trout gills plotted against corresponding measured expired pHs (at 3, 5.5 h exposure to 138$\pm$1 $\mu g.L^{-1}$ Al). n=57. There was a strong positive correlation between ΔAl and pH_{ex} i.e. ΔAl increased as expired pH increased. Line fitted by least squares linear regression.

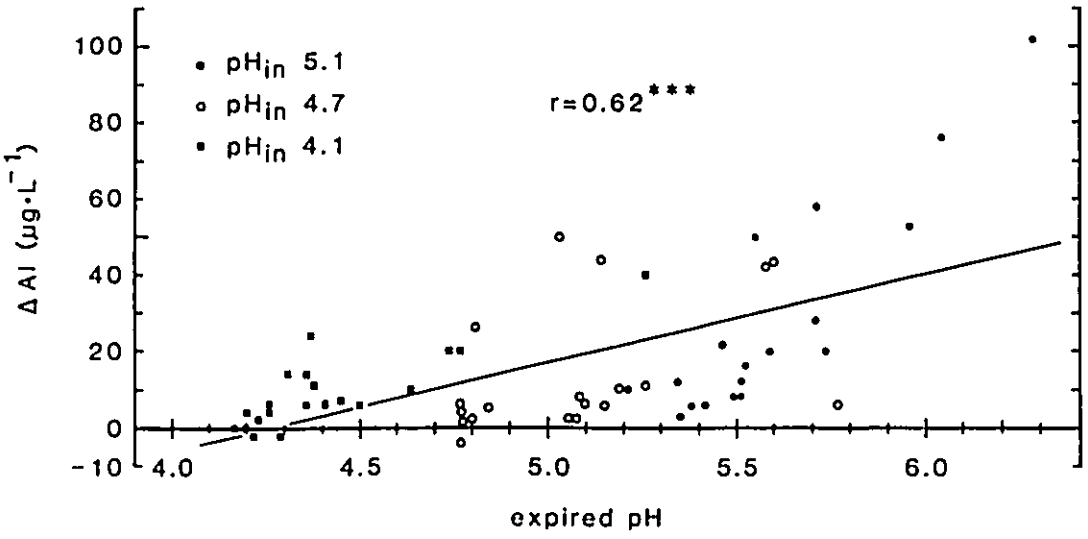
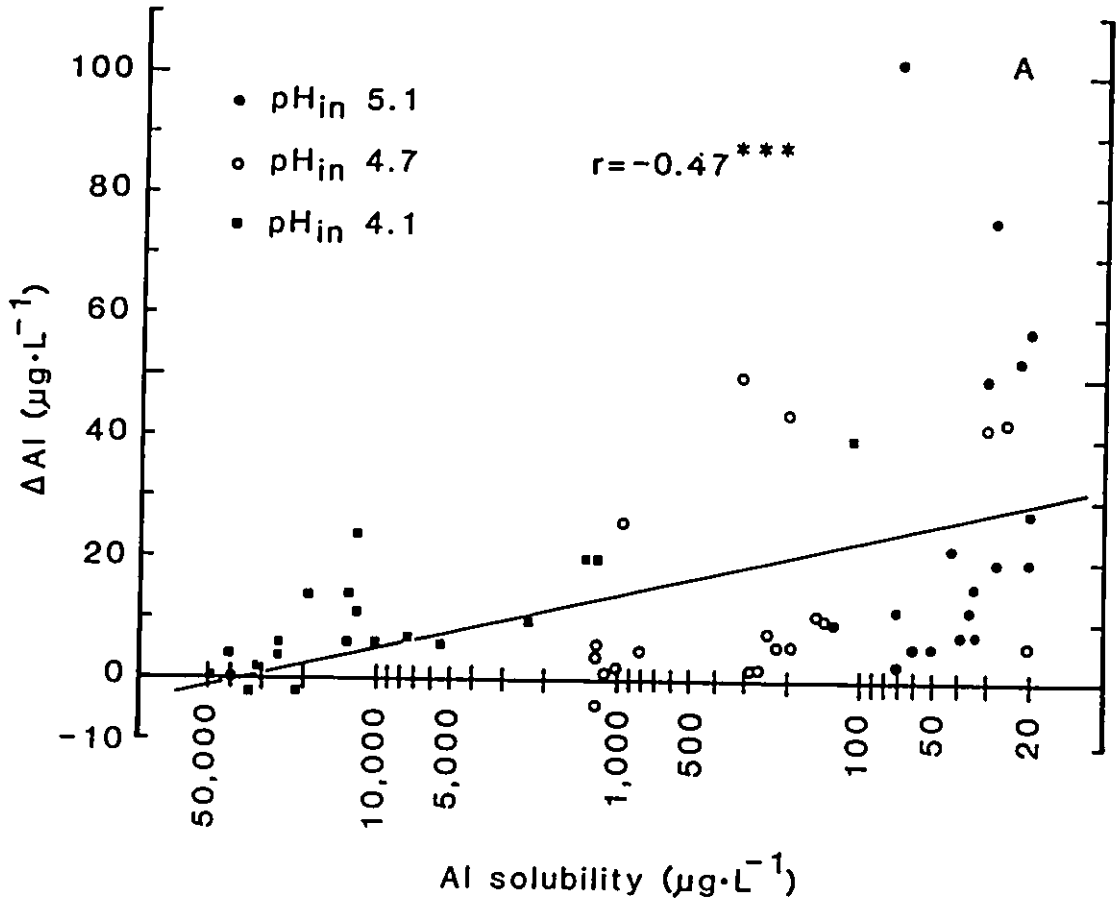

100
80
60
40
20
0
-10
4.0
4.5
5.0
5.5
6.0
ΔAl (µg·L⁻¹)
expired pH
pHin 5.1
pHin 4.7
pHin 4.1
r=0.62***

Fig. 39. A. Mean ΔAl at rainbow trout gills *versus* Al solubility (log scale) for the same data set in Fig. 38. Total Al=138$\pm$1 $\mu g.L^{-1}$. Solubility of Al was calculated for each expired pH (see Fig. 38), using the solubility diagram of Roberson and Hem (1969). There was a strong, negative correlation between ΔAl and log Al solubility. n=57.

B. Mean ΔAl at rainbow trout gills *versus* calculated oversaturation. There was a strong positive correlation. See text for details.


A
• pH_{in} 5.1
o pH_{in} 4.7
▪ pH_{in} 4.1
r=-0.47***
ΔAl (μg·L⁻¹)
100
80
60
40
20
0
-10
50,000
10,000
5,000
1,000
500
100
50
20
Al solubility (μg·L⁻¹)

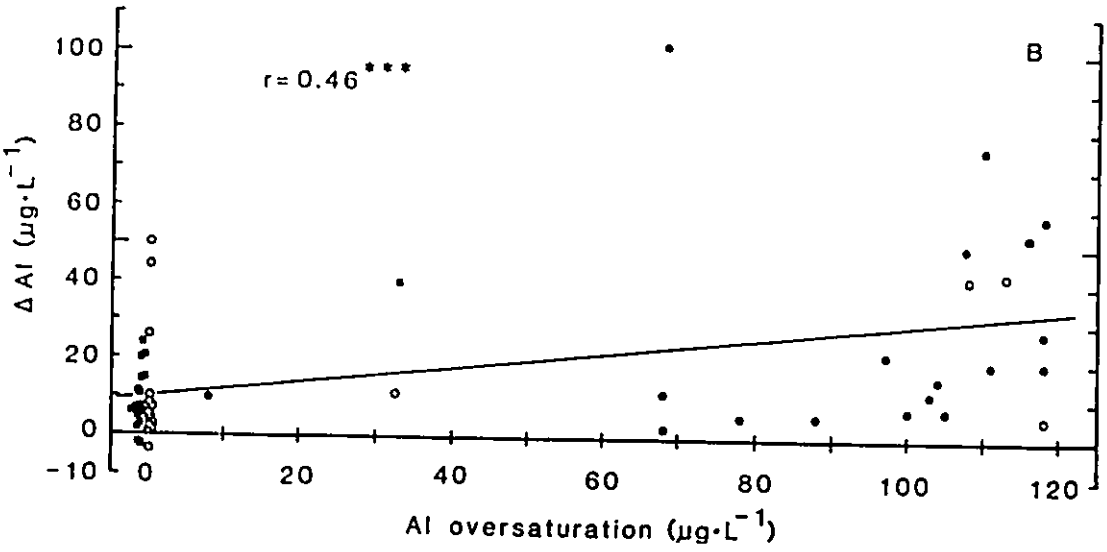
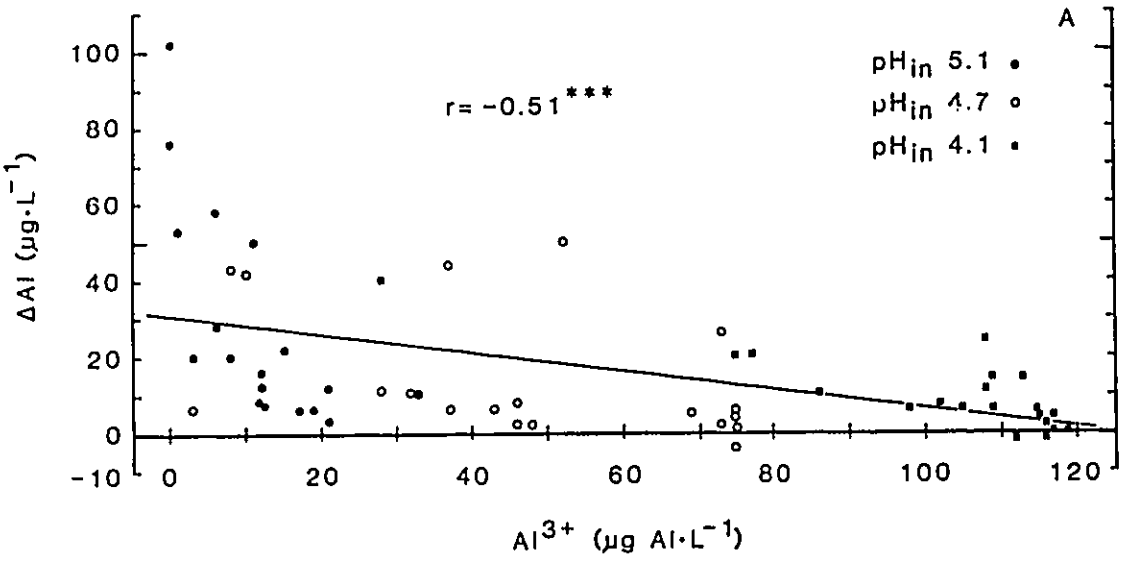

B
r=0.46***
ΔAl (μg·L⁻¹)
100
80
60
40
20
0
-10
0
20
40
60
80
100
120
Al oversaturation (μg·L⁻¹)

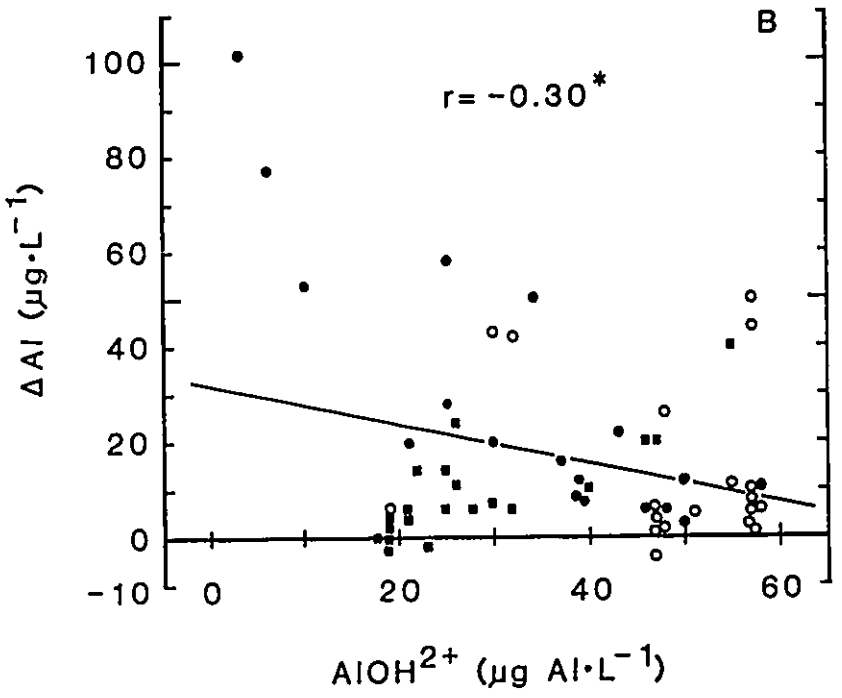
Fig. 40. Mean ΔAl at rainbow trout gills *versus* calculated concentrations of individual species of Al, from the same data set in Fig. 38. Species were determined from measured expired pHs and the speciation scheme of Dyrssen (1984). Total Al=138$\pm$1 $\mu g.L^{-1}$. n=57.

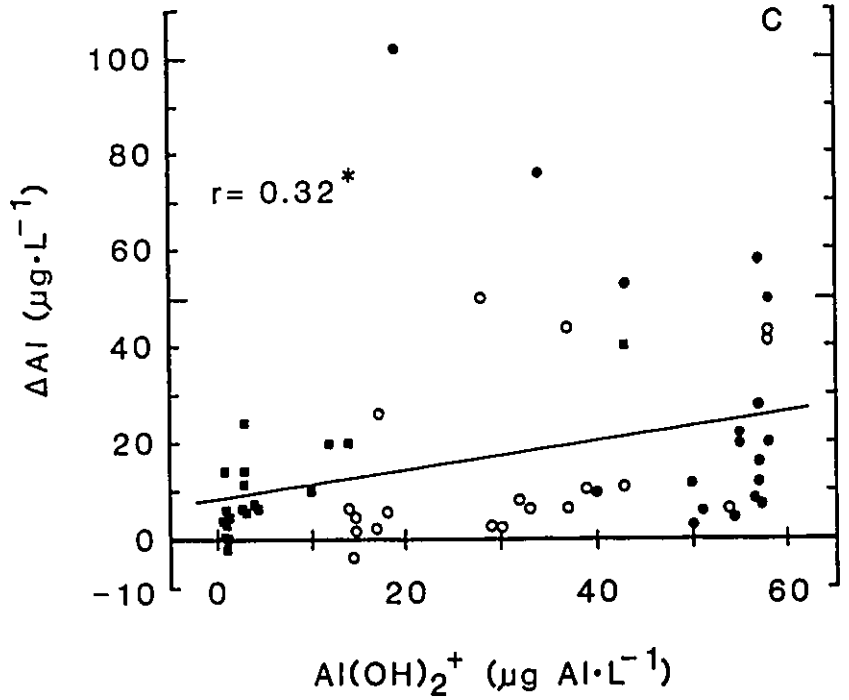
A. Mean ΔAl *versus* Al^{3+}. There was a strong negative correlation.


A
r= -0.51***
pH$_{in}$ 5.1 •
pH$_{in}$ 4.7 ○
pH$_{in}$ 4.1 ▪
ΔAl (μg·L^{-1})
100
80
60
40
20
0
-10
0
20
40
60
80
100
120
Al^{3+} (μg Al·L^{-1})

B. Mean ΔAl *versus* $AlOH^{2+}$. There was a weak negative correlation.

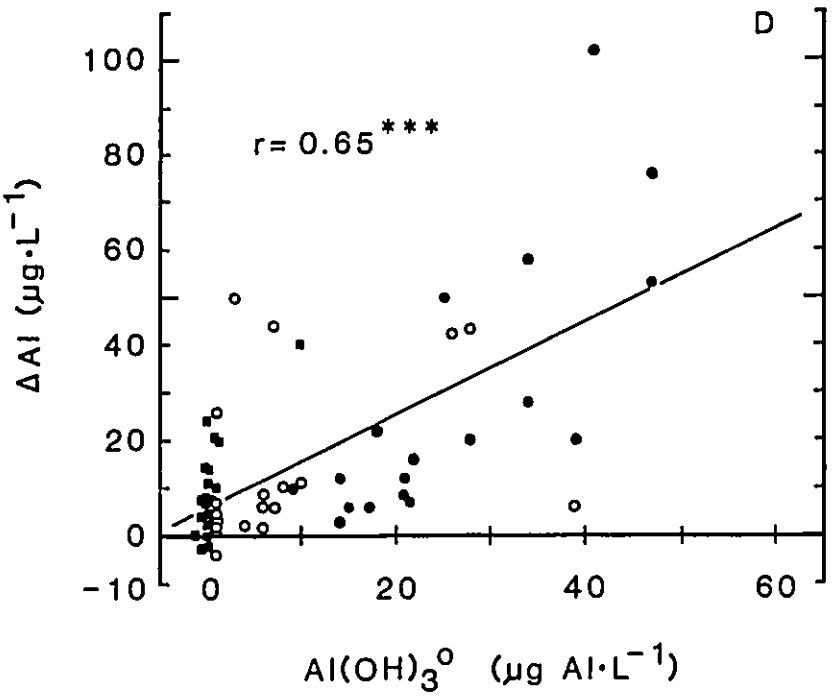
C. Mean ΔAl *versus* $Al(OH)_2^{+}$. There was a weak positive correlation.


B
r= -0.30*
100
80
60
40
20
0
-10
0
20
40
60
ΔAl (μg·L⁻¹)
AlOH²⁺ (μg Al·L⁻¹)


C
r= 0.32*
100
80
60
40
20
0
-10
0
20
40
60
ΔAl (μg·L⁻¹)
Al(OH)₂⁺ (μg Al·L⁻¹)

D. Mean ΔAl *versus* $Al(OH)_3^o$. There was a strong positive correlation.

E. Mean ΔAl *versus* $Al(OH)_4^-$. There was a strong positive correlation.


D
r= 0.65***
ΔAl (μg·L⁻¹)
100
80
60
40
20
0
-10
0
20
40
60
Al(OH)3° (μg Al·L⁻¹)

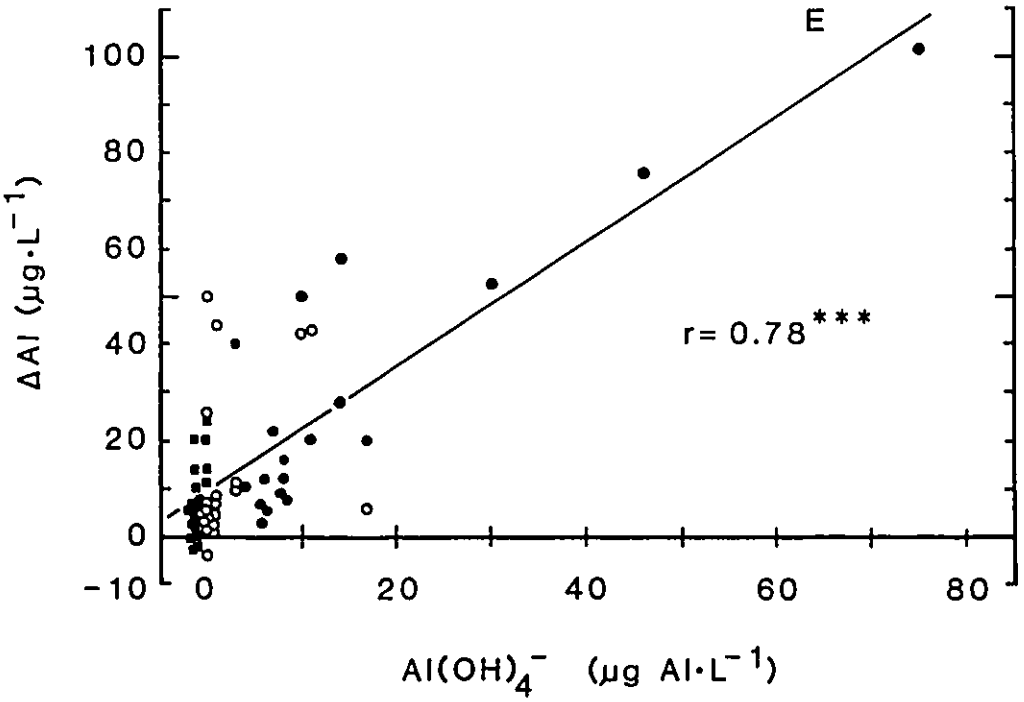

E
r= 0.78***
ΔAl (μg·L⁻¹)
100
80
60
40
20
0
-10
0
20
40
60
80
Al(OH)4⁻ (μg Al·L⁻¹)

Table 5. Correlation coefficients between measured gill Al accumulation after 6 h exposure to 138±1 $\mu g.L^{-1}$ Al (3 rinse protocols) and mean expired pH (measured), log Al solubility and oversaturation (calculated), and calculated concentrations of five Al species. Solubility of Al was calculated for each mean expired pH using the solubility diagram of Roberson and Hem (1969). Concentrations of Al species were calculated using the speciation scheme of Dyrssen (1984). n=15 for each comparison.

	correlation coefficient							
gill Al accumulation	mean pH_{ex}	log Al solubility	over-saturation	Al^{3+}	$AlOH^{2+}$	$Al(OH)_2^+$	$Al(OH)_3^o$	$Al(OH)_4^-$
no rinse	0.60 *	-0.63 *	0.54 *	-0.61 *	0.11	0.58 *	0.50	0.29
1 min rinse	0.58 *	-0.66 **	0.65 **	-0.64 *	0.16	0.71 **	0.46	0.14
3 x 20 s rinses	0.54 *	-0.60 *	0.50	-0.57 *	0.16	0.57 *	0.44	0.16

However, higher ΔAl at higher expired pH could also be a result of greater affinity of various species of Al for gill surfaces. To assess this possibility, concentrations of the five Al species of the speciation scheme of Dyrssen (1984) were calculated from measured expired pHs, and ΔAl was compared to these values. Total Al for the calculations was 138 $\mu g.L^{-1}$. Mean ΔAl showed a strong negative correlation with Al^{3+} ($r=-0.51$, $P<0.001$), and a weaker negative correlation with $AlOH^{2+}$ ($r=-0.30$, $P<0.05$), suggesting that Al extraction at the gills was not related to either of these Al species (Fig. 40A, B). Mean ΔAl showed a weak positive correlation with $Al(OH)_2^+$ ($r=0.32$, $P<0.05$), and a very strong, positive correlation ($r=0.65$, $P<0.001$) with the neutral $Al(OH)_3^o$ species (Fig. 40C, D). There was also a very strong correlation($r=0.78$, $P<0.001$) between ΔAl and the Al anion, $Al(OH)_4^-$ (Fig. 40E). However, this species represents Al going into solution in alkaline conditions, and is unlikely to bind to gill surfaces because of its negative charge, so may not be important in Al deposition on fish gills (see Discussion).

Recall that gill Al increased as inspired water pH increased (Fig. 37). Measured gill Al accumulations can also be compared to expired pH, and to calculated Al solubilities and Al species at those pHs. These comparisons for all three gill rinse protocols are summarised in Table 5. Gill Al correlated positively with expired pH (more gill Al at higher pH_{ex}), was negatively correlated with log Al solubility (more gill Al at lower Al

solubility), and was positively correlated with oversaturation (more gill Al at greater oversaturation). Gill Al accumulations showed a negative correlation with Al^{3+} and a positive correlation with $Al(OH)_2^+$ (Table 5). There were non-significant correlations with $AlOH^{2+}$, $Al(OH)_3^o$ ($P>0.05$, <0.10), and $Al(OH)_4^-$.

In general, these analyses indicated that reduced Al solubility near the gills is a possible explanation of Al extraction at the gills. Al^{3+}, $AlOH^{2+}$, and $Al(OH)_4^-$ are unlikely to be responsible for Al deposition at the gills. $Al(OH)_2^+$ and $Al(OH)_3^o$ are the Al species most likely to accumulate on the gills.

Aluminum precipitation experiments

One major concern of the preceding analysis is whether Al precipitation from solution can occur quickly enough to be an important factor in Al extraction and accumulation on fish gills. To address this problem, a series of filtering experiments were run.

Initial experiments demonstrated that Al precipitated quickly from concentrated solutions brought to near neutral pH. For example, a 44 $mg.L^{-1}$ Al solution brought from pH 4.0 to pH 6.4 with 1 N KOH (at room temperature) turned cloudy within 3 min. On the turbidity meter, turbidity increased by 1.2 FTU units 25 s after base addition. Absorbance (291 nm) on the spectrophotometer increased by 0.006 units about 15 s after base addition. However,

it became clear that these methods were not sensitive enough or quick enough to measure fast Al precipitation in dilute solutions, so filtering was tried.

Filtering a concentrated Al solution (44 $mg.L^{-1}$) 10 min after partial neutralisation (pH 4.0 brought to pH 5.8 with 1 N KOH) removed 90-100% of the Al, wheareas filtering the same solution at pH 4.0 removed only about 4% of total Al. A glass filtering apparatus and 0.45 μm membrane filter were used. With a more reasonable concentration of Al (505 $\mu g.L^{-1}$ at pH 5.3, 15°C), filtration through a 0.45 or 0.22 μm membrane removed about 25 and 36% of total Al, respectively. About 93% of the Al was removed when the same solution was brought to pH 6.4 and filtered within 3-5 min. However, for lower concentrations of Al, it became apparent that Al was being removed from solution by the glass filtering apparatus and glass porous filter support, as well as by the filters.

Washing the glassware in 10% HCl, washing the filter with 200 mL deionised water under vacuum, and collecting filtered water into polyethylene vials suspended under the filtering apparatus, were tried in an attempt to reduce the amount of Al removed from acidic, theoretically under-saturated solutions. Using this rinsing protocol, a 108 $\mu g.L^{-1}$ Al solution at pH 4.8 had about 30% of its Al removed when filtered through a 0.45 μm filter, whereas the same solution brought to pH 6.5 with 1 N KOH had about 74% of its Al removed when filtered 3 min after the pH was raised. Using

similar methods, a 305 $\mu g.L^{-1}$ Al solution at pH 5.2 showed 38% removal when filtered, whereas Al removal increased to 70% when the same solution was filtered 15 s after raising its pH to 6.2. This pattern was reproducible, with 30-40% of total Al removed if solution pH was low, and twice that amount removed if the pH was brought above pH 6.

Removal of approximately one-third of total Al from theoretically under-saturated solutions at low pH still suggested interactions of Al and glass, so a plastic filtering apparatus was tried. A peristaltic pump was used to reduce the time between base addition (NH_4OH) and filtering. Now, little Al was removed if the final Al solution was <pH 5.3 (~0-10% removed; 0.22 μm filter), and about 40% was removed if final solution pH was 5.6-5.7 (150 $\mu g.L^{-1}$ Al, 1-2, 2-3, or 15 s after mixing). Removal of Al by the filtering apparatus was reduced by replacing glass with plastic, but now sometimes the opposite problem occurred: apparent Al release into the system (i.e. contamination between samples). To reduce contamination, silicon tubing after the mixing "T" was replaced with polyethylene tubing, and a better rinsing protocol was developed, which included complete dismantling of the filtering apparatus between samples.

The final experiments are summarised in Figure 41. For a 159 ± 2 $\mu g.L^{-1}$ Al solution in soft water, ~10% of total Al was removed by filtering if the final pH of the solution was ≤ 5.3. If final solution pH was 5.7, or was raised to that pH by 30 μM NH_4OH

addition, the amount of Al removed by filtering increased to about 35% of total Al. This effect was seen just 1.4 s after the pH rise induced by NH_4OH addition. There was no indication that the amount of Al removed from solution depended on the delay between base and Al solution mixing and filtration, therefore results from the 1.4 and 3.8 s delay tests have been pooled in Figure 41.

To see if the same pattern of Al removal occurred from a more concentrated solution of Al at comparable pHs, experiments with a 335 ± 5 $\mu g.L^{-1}$ Al solution were run. The pattern of increased Al removal from solution after base was added was still evident (Fig. 42), and, if the amounts removed are expressed as concentrations, more Al was removed from the more concentrated Al solution at a given pH. However, the trend of greater Al removal at higher pH was less pronounced, partly because of the small number of replicates run, but also because of smaller increases in pH as 30 μM NH_4OH was added, presumably because of greater pH buffering by the higher concentration of Al.

Fig. 41. Effects of solution pH on the amount of Al filtered from a 159 $\mu g.L^{-1}$ Al soft water solution, expressed as a percentage of total Al. 14-15°C. Clear bars: percent of total Al removed from solution when filtered through a 0.22 μm filter 1.4 or 3.8 s after mixing with deionised water (control). Combined data from 1.4 and 3.8 s tests. Adjacent dark bars: percent of total Al removed when solution pH was raised from control values by mixing with base (30 μM NH_4OH). Combined data for 1.4 and 3.8 s tests. About 35% of total Al was removed by filtration if final solution pH was 5.7; only about 10% was removed if pH was $\leq$5.3. *** = $P<0.001$ (t-test), vs pH 5.3 (control). For solution pHs $\leq$5.0, n=6; for pH 5.3, n=16; for pH 5.7 (dark bar) n=18; and for pH 5.7 (clear bar) n=10. Error bars: 1 SEM.

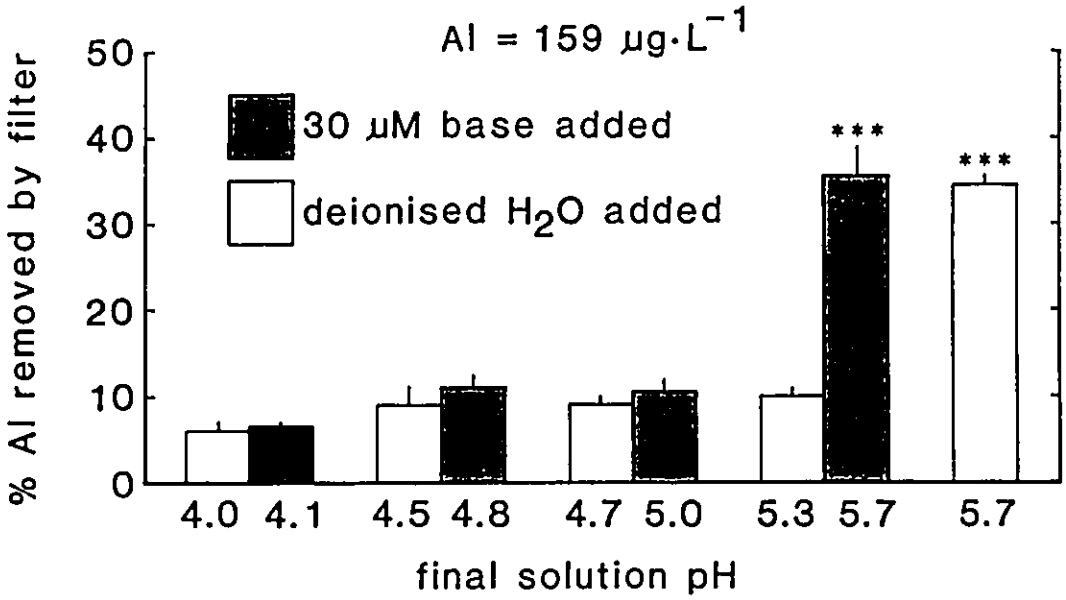
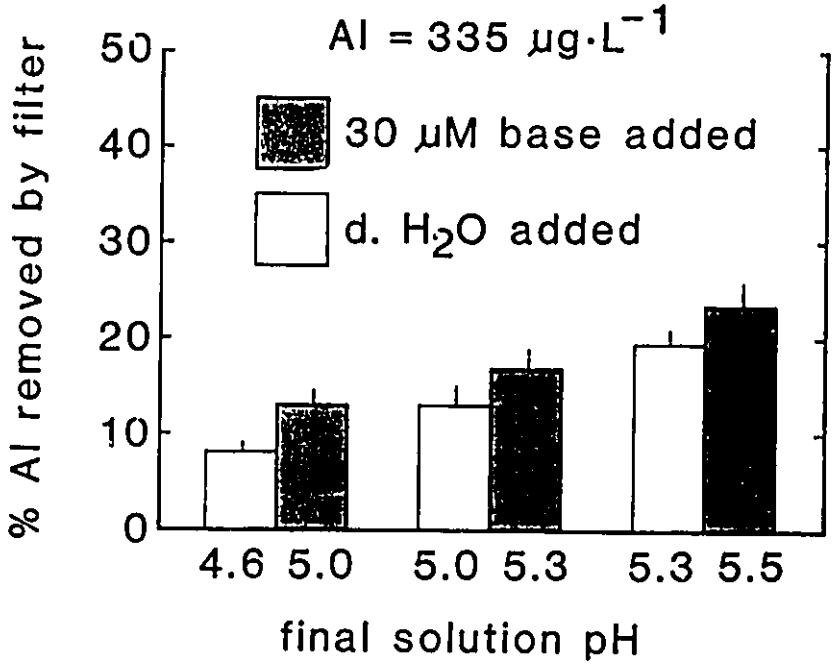

Al = 159 µg·L^{-1}
30 µM base added
deionised H_2O added
% Al removed by filter
50
40
30
20
10
0
***
***
4.0 4.1
4.5 4.8
4.7 5.0
5.3 5.7
5.7
final solution pH

Fig. 42. Effect of solution pH on the amount of Al filtered from a 335 $\mu g.L^{-1}$ Al soft water solution, expressed as a percentage of total Al. Delay after base or water addition to the solution and filtering was 1.4 s. Other details as in legend of Fig. 41. The trend was more Al removed as solution pH increased. For all bars, n=3.


Al = 335 µg·L⁻¹
30 µM base added
d. H2O added
% Al removed by filter
50
40
30
20
10
0
4.6 5.0
5.0 5.3
5.3 5.5
final solution pH

Discussion

Mechanisms of gill Al deposition

Rainbow trout fitted with ventilation masks and opercular catheters showed elevations in ventilation during 6 h exposures to 138 $\mu g.L^{-1}$ Al (Fig. 35B), similar to those seen previously (Chapter 4). Ventilation increases were lower in the presence of Al than in its absence during the pH 4.1 exposures: this was the only example of reduction by Al of H^+ ion effects during any of my experiments. It is likely the Al^{3+} cation reduced the effects of the very acidic conditions by competing with H^+ ions for binding sites (eg. Pagenkopf 1983).

Aluminum extraction at the gills (Fig. 36) and accumulation of Al on the gills (Fig. 37) was highest at inspired pH 5.1, intermediate at pH_{in} 4.7, and lowest at pH_{in} 4.1. As expected from the results of Chapters 3 and 4, expired pH was also dependent on inspired pH. In turn, extraction and accumulation of Al showed positive correlations with expired pH measured at the gills (Fig. 38, Table 5). The solubility of Al decreases exponentially as pH increases from pH 4.0 to pH 5.8 (Roberson and Hem 1969), and Al chemistry changes from predominantly Al^{3+} at pH 4.0 to a mixture of Al-hydroxides and the $Al(OH)_4^{-1}$ anion near pH 6 (eg. Dyrssen 1984). Comparing ΔAl or measured gill Al with calculated Al solubility and Al species yielded some insights into likely mechanisms involved in Al extraction and accumulation at

the gills. It should be remembered that the opercular catheter method may underestimate alkalinization near fish gills, as discussed in Chapter 4.

Aluminum extraction and accumulation showed significant, negative correlations with Al solubility (Fig. 39A, Table 5), and positive correlations with Al oversaturation (Fig. 39B, Table 5). That is, extraction and accumulation of Al at the gills increased as Al solubility decreased and oversaturation increased. These relationships support the idea that increased pH in the gill micro-environment, reducing Al solubility, results in Al precipitation onto the gills (Fig. 31, Chapter 4). Aluminum precipitating onto the gills would then cause the severe ionoregulatory and respiratory disturbances that resulted in higher mortality at pH 5.2 than at pH 4.8 or 4.4 (Chapter 2).

However, some Al extraction and Al accumulation on the gills occurred even at acidic expired pHs when Al solubility was still presumably high. Using expired pH to calculate Al species expected to exist near the gills is useful in determining which Al species might be responsible for Al accumulation on gills. From such calculations, Al^{3+} and $AlOH^{2+}$ were unlikely to be responsible for much of the Al deposition on the branchial surface, judging by their negative or non-significant correlations with ΔAl and gill Al (Fig. 40A, B; Table 5). The small amount of Al extraction and accumulation at gills observed for inspired pHs below about pH 4.5 may be related to adsorption of these positively charged species

(together perhaps with the small fraction of $Al(OH)_2^+$ at this pH) to negatively charged gill surfaces and mucus (Satchell 1984).

Although data are hard to come by, it is probable that the actual shifts in Al species are rapid (a few milliseconds; Wakeman 1986), well within water transit time at fish gills. However, it not known whether adsorption of positively charged species to the negatively charged gill surfaces can occur within the contact time of water with gills (<2 s). Aluminum adsorption onto negatively charged clays is complete in <30 s (Walker et al. 1988a). Complexation of Al with carboxyl groups of humic and fulvic acids (Lewis et al. 1988; Bache 1986; Plankey and Patterson 1987) may have reaction half times of as little as 5 s (Mak and Langford 1982). From these studies, it is likely that adsorption to gills can occur in <2 s, but there is a clear need for more data on this topic.

Two prime candidates for Al species responsible for greater ΔAl and gill Al accumulations at higher expired pHs are $Al(OH)_2^+$ and $Al(OH)_3^\circ$. These species showed significant to highly significant, positive correlations with ΔAl (Fig. 40C, D), and significant or nearly-significant correlations with gill Al (Table 5). The mechanism of deposition of neutral $Al(OH)_3^\circ$ is undoubtedly different from the deposition of Al^{3+} and $AlOH^{2+}$, while that of $Al(OH)_2^+$ may or may not be different. In any event, $Al(OH)_2^+$ and $Al(OH)_3^\circ$ probably would not be adsorbed to negatively charged gill surfaces to the same degree as the more positively

charged species. Instead, the mechanism of their deposition may be a precipitation phenomenon, especially for neutral $Al(OH)_3^o$, where repulsion between Al species is minimal and polymerisation could occur (eg. Dentel and Gossett 1988).

If $Al(OH)_3^o$ is considered the species responsible for Al precipitation from solution, then the correlation of $Al(OH)_3^o$ with ΔAl (Fig. 40D) is equivalent to the (negative) correlation between ΔAl and the solubility of Al, and the positive correlation with oversaturation (Fig. 39A, B). Aluminum deposition could also occur initially as a precipitation phenomenon, and, once in contact with the gills, a positively charged Al species such as $Al(OH)_2^+$ could interact with negative charges on the branchial surface.

The Al anion, $Al(OH)_4^-$, showed no correlation with gill Al accumulation (Table 5), but showed a highly significant correlation with ΔAl (Fig. 40E). $Al(OH)_4^-$ represents the major form of dissolved Al in alkaline conditions, and its surprising correlation with ΔAl may be an artifact of the many acidic expired pHs (with low ΔAl) having calculated concentrations of $Al(OH)_4^-$ of 0 $\mu g.L^{-1}$ (Fig. 40E). In addition, 2 or 3 data points have a disproportionate effect on the correlation. $Al(OH)_4^-$ is unlikely to contribute substantially to Al deposition on fish gills, because charge repulsion would prevent its adsorption to the negatively charged gill surfaces.

Whatever the processes involved in Al deposition on fish gills, actual accumulation of Al on the gills was much less than accumulation calculated using Al extraction and fish ventilation. Calculated Al depositions per set of gills were 0.18-0.73 mg for the 6 h exposures to Al, whereas measured Al accumulations per gill set were 0.02-0.06 mg (Table 4). Overall, Al deposition per set of gills estimated from gill Al measurements were only about 11% the Al deposition calculated from Al extraction at the gills. This result agrees well with similar but incomplete and less accurate calculations in Chapter 4 (Table 3).

Sloughing of Al from the gills was assumed previously to explain the large differences between calculated and measured Al deposition at the gills. Blotting gills with filter paper discs collected 0.11-0.20 μg Al from gill surfaces (Table 4), less than 1% of the total measured Al deposition. The fact that the three rinsing protocols did not affect the amount of Al measured on the gills also suggests that most Al retained on the gills was bound intimately. Presumably, periodic sloughing of mucus at the gills removes most of the Al accumulating on the gills (i.e. nearly 90% of the total Al extracted at the gills). Sloughed Al was not often sampled by opercular catheters, possibly because mucus that was binding Al fell to the bottom of the opercular cavity and was

exhaled there. It is proposed that Al initially collects on gills through precipitation phenomena and is mostly sloughed off with mucus, and that the small proportion of Al remaining is positively charged Al (eg. $Al(OH)_2^+$) bound to negative charges on branchial surfaces.

Protein analysis of expired water did not indicate increased protein production (eg. mucus glycoprotein production) by gills of fish exposed to acidity and Al. This negative result may also have been because of mucus dropping out the bottom of the opercular cavity, but could have been a result of the method used, which was pushed to its limit of sensitivity. Perhaps expired water samples could in future be filtered or freeze concentrated to concentrate proteins released at the gills.

Rate of Al precipitation

Overall, it appears that extraction and accumulation of Al at the gills could be related to decreased solubility of Al in the more alkaline environment near the gills, or is related to Al species likely to polymerise and precipitate from solution. But is precipitation of Al from solution fast enough to explain Al deposition on fish gills? Initial experiments demonstrated precipitation of Al from concentrated, acidic solutions within minutes of partial neutralisation.

In more refined experiments, the amount of 0.22 μm filterable Al increased greatly within 1.4 s when a 159 $\mu g.L^{-1}$ Al solution in 14-15°C soft water was raised from pH 5.3 to 5.7 by the addition of 30 μM NH_4OH (Fig. 41). The additional filtered Al is presumably Al precipitate formed once the solubility of Al is exceeded due to the rise in solution pH. For the 159 $\mu g.L^{-1}$ Al solution, the solubility of Al would theoretically not be exceeded until pH $\geq$5.3 (Fig. 31). This prediction was supported by the present data, where percent filtered Al was >10% only when final solution pH was 5.7 (Fig. 41).

The exact mechanisms leading to the increases in filterable Al are not known. As pH increases and Al solubility is exceeded, monomers of Al may polymerise and form filterable complexes, or Al monomers may adsorb to the filter surface, polymerise, and form a precipitate (Dentel and Gossett 1988). These processes presumably

occur at fish gills, as suggested by Baker and Schofield (1982). The increase in pH of ~0.4 units is about the difference between inspired and expired water pH seen in rainbow trout in similar soft water (Fig. 27A), caused by the release of 30 μM base and ammonia at the gills (Chapter 3). This rise in pH is large enough to theoretically result in Al precipitation if initial Al concentration is >100 $\mu g.L^{-1}$ (Fig. 31). The residence time of water at the gills is 0.4-2 s (Randall 1970), and may be longer because of unstirred and boundary layers, so the time scale of the filtration experiments was appropriate.

Background removal of Al by filtration was about 10% of total Al. Presumably this amount represents Al adsorbing to negatively charges on the filter paper (Bisio et al. 1980; Johnson et al. 1989). Campbell et al. (1983) used filtration to separate particulate Al from dissolved, and had a similar loss of Al from a synthetic standard filtered through a rinsed, 0.4 μm filter using a polycarbonate filter apparatus. Polycarbonate filtering devices clearly are required to avoid the large removals of Al from solution that were found with glass. Thorough rinsing of the polycarbonate filter apparatus is needed to avoid contamination between samples.

The solubility of Al is lower, and reaction rates faster, in water of low ionic strength because activity coefficients are greater at lower ionic strength (eg. Bache 1986). The low ionic strength of the soft water used (~10^{-4} M) would be expected to

maximise Al precipitation rates. Aluminum filtered from solution was about 10% of total Al at pH $\leq$5.3, increasing to 35% whether the solution was brought to pH 5.7 just seconds before filtration, or was prepared at pH 5.7 and filtered minutes later (Fig. 41). In contrast, Chappel and Birchall (1988) reported only about 20% filtration of Al at pH 5.7 compared to about 10% at pH 5.3 (20 h after solutions were prepared, 0.22 μm filters, ~3 mg.L^{-1} Al solution). These workers, however, used water of ionic strength about 10^{-2} M, which would have an Al activity coefficient about half that in the soft water used here (extended Dubye-Hückel equation; from Stumm and Morgan 1981). This could explain their apparently higher Al solubility even after 20 h.

The 14-15°C conditions used in the experiments would have slowed Al precipitation rates compared to rates at room temperature. However, Al precipitation as determined by filtration still occurred within seconds. Aluminum precipitation in very cold water (eg. 0-4°C), which would represent conditions encountered by fish during springmelt pulses of Al and acidity, was not assessed. Aluminum precipitation would be slowed further at 0-4°, but it is not known whether pH changes at the gills would be the same at these temperatures as at 15°. For example, fish not eating at cold temperatures might release more ammonia at the gills due to catabolism of endogenous protein, which would worsen Al precipitation. Alternatively, they might release less ammonia

due to lowered metabolic rates. Clearly this question deserves more attention.

As expected, more Al was filtered on an absolute basis from the 335 $\mu g.L^{-1}$ Al solution at a given pH compared to the 159 $\mu g.L^{-1}$ Al solution (Fig. 42). According to the Al solubility curve for microcrystalline gibbsite (Fig. 31), 335 $\mu g.L^{-1}$ is close to saturation at pH 5.0: certainly at pH $\geq$5.3 the Al solution is well above saturation, as indicated by the 17-24% removal of Al by filtration (Fig. 42). For the 335 $\mu g.L^{-1}$ Al solution at pH 5.3, the addition of 30 μM base raised its pH to 5.5, whereas for the 159 $\mu g.L^{-1}$ Al solution, pH increased to 5.7. This difference was likely due to the extra pH buffering of more Al in solution, because the formation of Al-hydroxides consumes base.

In summary, Al extraction from acidic solutions and Al accumulation on rainbow trout gills were compared to measured expired pH, then to Al solubility and Al species calculated from expired pH. Overall, Al deposition correlated best with decreased Al solubility in the more alkaline gill micro-environment, or with Al species such as $Al(OH)_2^+$ or $Al(OH)_3^o$ that are most likely to form Al polymers and precipitate from solution. The small proportion (~10%) of Al deposition that remains on the gills may be positively charged Al species left bound to negatively charged branchial surfaces after most of the precipitated Al is sloughed off with mucus. Filtration experiments showed that Al precipitation occurs quickly (within 1.4 s) in soft water at

14-15ºC, which supports the scenario that initial Al accumulation is related to precipitation. Precipitation of Al onto the gills is probably most important at inspired water pH 5-6, where gill Al accumulation is greatest. Below pH 5, other processes such as adsorption to gill surfaces and mucus probably better explain the smaller amount of Al accumulating on fish gills.

Chapter 6

Model of physiological effects of Al at fish gills

Introduction

The overall goal of this thesis was to better define the effects of Al and acidity on fish through toxicological, physiological, and mechanistic studies. Toxicological and physiological effects of Al have been localised at the gills, so a model explaining the actions of Al must concentrate on this target organ. A summary of the main results to be incorporated into such a model of Al toxicity is presented first, then the model is described, and finally the limitations and other applications of the model are discussed.

Summary of thesis results

Simple mortality data from cannulated rainbow trout showed that the toxicological effects of 66 h exposures to 105 $\mu g.L^{-1}$ Al in soft water were worst at pH 5.2, intermediate at pH 4.8, and least at pH 4.4 (Fig. 4). Increased water Ca concentrations tended to reduce the toxic effects of Al. These results suggested that there was a fundamental difference between the effects of Al at moderately acidic pH than at very acidic pH in soft water.

Physiological results from cannulated fish showed that exposure to Al caused losses of Na^+ and Cl^- from blood plasma at pH 5.2 and 4.8, where there were no ion losses in the absence of Al (Fig. 7). In contrast, Al did not add to ionoregulatory disturbances already caused by acidity at pH 4.4. Calcium reduced the ionoregulatory effects of acidity but not those of Al.

Dramatic decreases in arterial O_2 tension and increases in CO_2 tension and blood lactate were seen in fish exposed to Al at pH 5.2 and 4.8 (Fig. 11, 16). These disturbances were never attributable to acidity alone. Calcium reduced the respiratory effects of Al at pH 4.8, but not at pH 5.2. Acid-base disturbances were a combination of respiratory acidosis, due to CO_2 build-up, and metabolic acidosis, due to entry of acidic equivalents from the acidic water (Fig. 11, 16). The respiratory and ionoregulatory results again demonstrated very different effects of Al at pH 5.2 and 4.8 than at pH 4.4.

Experiments with rainbow trout fitted with ventilation masks and opercular catheters showed that acidic soft water was rendered more basic as it passed over fish gills, and basic inspired water was made more acidic (Fig. 18). These effects were adequately explained by ammonia, base, and CO_2 released at the gills (Fig. 24).

Acidic soft water containing Al was also alkalinized in the gill micro-environment during both short (2-3 h) and longer term

(44 h) exposures to Al (Fig. 27A, 29D, respectively), although the increases in pH were moderated by the buffering action of Al. The increases in pH near the gills were still large enough that the theoretical solubility limit of Al would be exceeded, possibly resulting in Al precipitation from solution onto the gills (Fig. 31). For Al~100 $\mu g.L^{-1}$, this precipitation of Al from solution was predicted to begin at inspired pH~4.8, a prediction supported by gill Al accumulation data (Fig. 32, 37). More gill Al was found at higher exposure pH than at lower exposure pH. Fish ventilation volumes were greatly elevated in the presence of Al at higher pH (Fig. 27B, 29C), in accord with the blood gas disturbances documented earlier. These respiratory effects were likely a result of the greater deposition of Al on gills at higher pH. A mechanism to explain Al deposition and accumulation on fish gills, and thereby the effects of Al, was developing.

Measurements of Al extraction at the gills of rainbow trout were made using opercular catheters, in an attempt to determine precise mechanisms involved in Al accumulation at the gills. Aluminum extraction (ΔAl) at trout gills was greater at higher inspired pH (Fig. 36) and, more importantly, at higher expired pH (Fig. 38). ΔAl correlated well with calculated Al solubility and oversaturation near the gills (Fig. 39), and with Al species (eg. $Al(OH)_3^o$) likely to precipitate from solution (Fig. 40). Aluminum precipitation experiments indicated that precipitation of Al from solution was fast enough to occur during the short (<2 s)

residence time of water at the gills (Fig. 41). Together, these results supported the idea that Al deposition on fish gills is a precipitation phenomenon.

Measured accumulation of Al at the gills was much lower than deposition of Al at the gills calculated from ΔAl and ventilation volumes (Table 3, 4), suggesting extensive sloughing of Al from the gills. Gill Al accumulation correlated with expired pH, Al solubility, Al oversaturation, and the $Al(OH)_2^+$ species (Table 5). Overall, from Al extraction and accumulation data, it appears that deposition of Al on fish gills occurs initially through a precipitation phenomenon, but most of this Al is sloughed off (possibly with mucus). The small proportion of Al remaining on the gills could be positively charged Al species (eg. $Al(OH)_2^+$) bound to negative charges on the branchial surface.

Model of Al interactions at fish gills

A model of the effects of Al at fish gills must integrate mechanisms of deposition and accumulation with the observed respiratory and ionoregulatory effects of Al. The model presented in Figure 43 attempts to do this; it is modified from the model of Wood and McDonald (1987). In brief, respiratory effects of Al are assumed to be due to Al precipitation on the gills and to associated gill responses. Ionoregulatory disturbances are assumed to be caused by interactions of charged Al species with the branchial surface.

In detail, for fish in circumneutral soft water, active uptake of Na^+ and Cl^- across the branchial epithelium compensates for ion losses from blood to water, presumably through paracellular channels (Fig. 43A). Oxygen diffuses across the gill membrane into the blood, and CO_2 diffuses out. Carbon dioxide acidifies water in the branchial micro-environment, catalysed by carbonic anhydrase on the external surface of the gills. Ammonia also diffuses through the branchial membrane, tending to alkalinize water next to the gills. The extent of net acidification or alkalinization depends on inspired water pH.

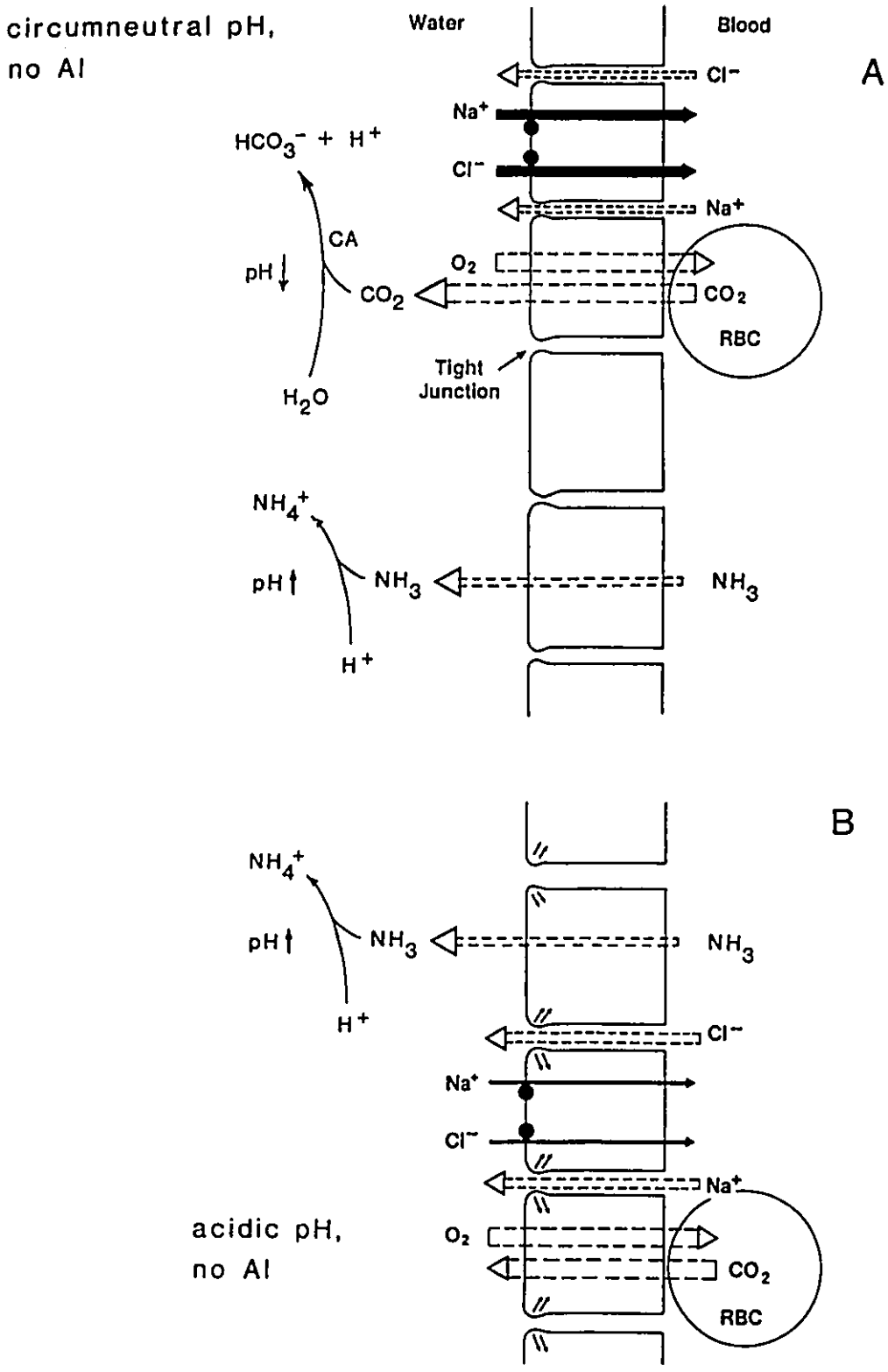
In acidic water (eg. pH 4.4), H^+ ions reduce the active uptake of Na^+ and Cl^- (Fig. 43B), and increase their passive efflux, possibly by displacing Ca^{2+} from tight junctions (McDonald 1983a). If paracellular pathways are negatively charged, Cl^- ion losses through them could be constrained; perhaps Cl^- ions also leak through epithelial cells, as occurs in secretory cells (eg. Petersen and Maruyama 1984; Turner et al. 1986). Transfers of O_2 and CO_2 are unaffected by acidity alone. Release of CO_2 no longer acidifies water near the gills, so the alkalinization due to NH_3 transfer predominates.

In the presence of 100-200 $\mu g.L^{-1}$ Al in moderately acidic water (eg. pH 4.8, 5.2), the pH rise in the gill micro-environment (due to NH_3) causes Al deposition on the gill surface (Fig. 43C). This deposition is due to decreased Al solubility (simplest explanation), or to changes in Al chemistry to Al species that

Fig. 43. Model of Al interactions at fish gills, modified from Wood and McDonald (1987). The branchial surface is represented, with soft water on the left and blood on the right. RBC = red blood cell. Width of arrows depicts relative transfers across the gill.

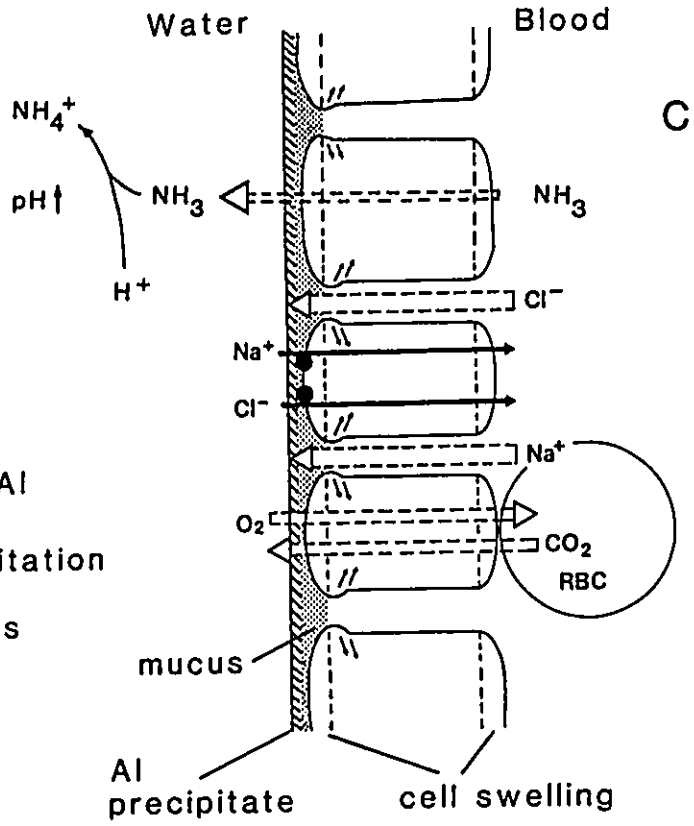
A. Circumneutral conditions in the absence of Al. Active ion uptake balances ion effluxes. O_2, CO_2, and ammonia diffuse through the gill, with a net acidification of the gill micro-environment due to CO_2 dissociation. Excretion of NH_4^+ is ignored in the model, because it does not affect water pH. C.A. = carbonic anhydrase.

B. Acidic conditions in the absence of Al. Active ion uptake is reduced, and ion effluxes increase. There is no interference with gas transfers, but now a net alkalinization of the gill micro-environment occurs because CO_2 no longer dissociates to HCO_3^- and H^+.


circumneutral pH,
no Al
Water
Blood
A
Cl⁻
Na⁺
Cl⁻
Na⁺
HCO3⁻ + H⁺
CA
pH
CO2
O2
CO2
RBC
H2O
Tight
Junction
NH4⁺
pH
NH3
NH3
H⁺
B
NH4⁺
pH
NH3
NH3
H⁺
Cl⁻
Na⁺
Cl⁻
Na⁺
acidic pH,
no Al
O2
CO2
RBC

C. Moderate acidity in the presence of Al. Alkalinization of the gill micro-environment causes decreased Al solubility and changes in Al species, resulting in Al deposition on the gill surface. Mucus production, cell swelling, reduced ion uptake, and increased ion effluxes result. Gas transfers are reduced at the gills because of increased diffusion distance.

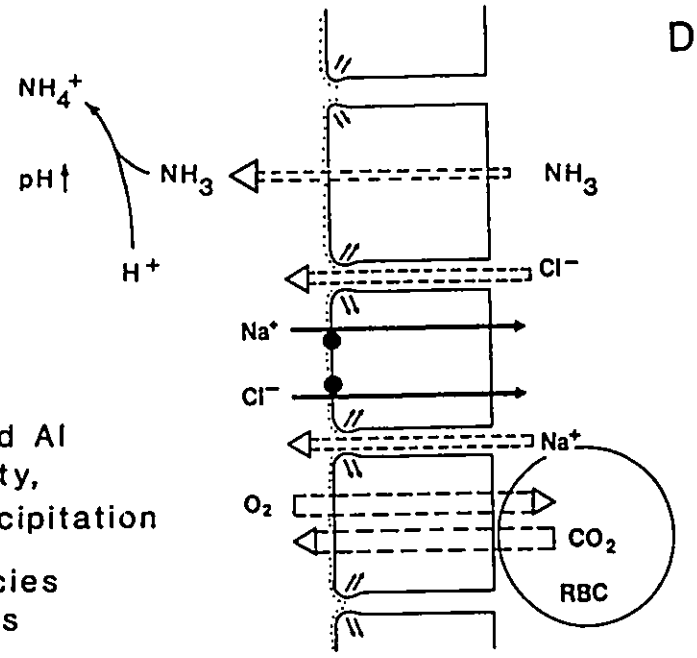
D. Very acidic conditions in the presence of Al. Alkalinization of the gill micro-environment does not result in Al deposition, because Al solubility is reduced but not exceeded. The small amount of Al binding to branchial surfaces does not add to the ionoregulatory disturbances caused by acidity alone (Fig. 43B), and may even reduce the toxic effects of H^+ ions through competition for binding sites.



moderate acidity, Al

pH↑ = reduced Al solubility → precipitation

= Al species changes



acidic pH, Al

pH↑ = reduced Al solubility, no precipitation

= Al species changes

better react with the branchial surfaces, or to a combination of the two mechanisms.

In the model, respiratory effects of Al (Fig. 43C) are a result of Al precipitation product itself, of excess mucus production stimulated by Al deposition (eg. Muniz and Leivestad 1980; Harvey and McArdle 1986; Karlson-Norrgren et al. 1986a; Jensen and Weber 1987), or of gill swelling, inflammation, and damage caused by Al deposition (eg. Chevalier et al. 1985; Karlsson-Norrgren et al. 1986a, b; Youson and Neville 1987; Mueller et al. submitted). These effects would all increase diffusion distances for blood gasses, resulting in decreased P_{aO_2} and increased P_{aCO_2}, which, in conjunction with ionoregulatory effects of Al, could kill the fish.

Sloughing of mucus continually removes Al precipitating on gill surfaces, but may not remove positively charged Al species bound to negative charges on the branchial surface itself. In the model, Al intimately bound to the gills is the portion of Al deposition that is responsible for the ionoregulatory effects of Al. Once bound on the branchial surfaces, Al reduces active ion uptake, possibly by reducing Na-K-ATPase activity (Staurnes et al. 1984), or through damage to chloride cells (eg. Chevalier et al. 1985; Youson and Neville 1987), or perhaps by interfering with ion channels. Increased ion efflux is possibly by the same action as H^+ ions in more acidic water, i.e. displacement of Ca^{2+} from branchial surfaces by positively charged Al species.

In the presence of 100-200 $\mu g.L^{-1}$ Al in very acidic water (eg. pH<4.4), deposition of Al on the gill surface is low. This situation prevails because, even though the solubility of Al is reduced in the more alkaline gill micro-environment, its solubility is not exceeded (Fig. 43D). As there is no precipitation of Al on branchial surfaces, respiratory toxicity of Al does not develop. Any Al accumulating on the gills is likely positively charged Al species binding to negative charges on the gills. However, the relatively small amount of bound Al does not add appreciably to the ionoregulatory disturbances already caused by H^+ ions. In extremely acidic conditions (pH ~4.0), Al^{3+} may compete with H^+ for binding sites (eg. at tight junctions), reducing the toxic effects caused by H^+ ions. Presumably, Ca^{2+} reduces some of the effects of Al and H^+ ions through similar competitive interactions.

Although this model effectively explains toxicity of Al at fish gills, it is far from definitive. Its main weakness is that conditions at the branchial surfaces are only approximated by measurements made using opercular catheters. For example, it is likely that opercular catheters underestimate pH changes at the trailing edges of gill lamellae, as discussed in Chapter 3, and yield an "average" expired pH. Higher ionic strength in water next to the gills could increase Al solubility there (Chapter 5), or alter CO_2 dissociation (Chapter 3), which would also increase Al solubility, by reducing the net alkalinization of the gill

micro-environment. Finally, mucus itself represents a different chemical environment than water (eg. Handy 1989), and would be expected to alter Al chemistry right next to the gills, for example, by forming complexes with Al. In spite of these problems, the model is useful in understanding Al toxicity in fish, and the extent of its inaccuracies remain to be seen.

Further applications of the model

The model of Al interactions at fish gills may explain short term Al toxicity in fish previously unexposed to Al, but can the model be extended to explain longer term fish survival and acclimation to Al? During short (1 h) experiments, Al accumulation on fish gills is approximately linear (Handy and Eddy 1989). Comparing between experiments presented in this thesis, gill Al accumulations were lowest after 2 h exposures to Al (Fig. 32), intermediate after 6 h exposures (Table 4, Fig. 37), highest after 44 h exposures (Table 3), and intermediate after 66 h (Table 1). These results suggest that linear deposition of Al at the gills does not continue indefinitely. McDonald et al. (submitted) reached a similar conclusion. Surviving fish may be more efficient at removing Al from the gills, or in preventing its accumulation in the first place. Gill micro-environment pH does not change much with time during Al exposures (Fig. 29D), so mechanisms other than alterations in NH_3 excretion must be important in reducing gill Al loads.

Acclimation to Al by fish (eg. Orr et al. 1986; Wood et al. 1988b,c; McDonald and Milligan 1988; McDonald et al. submitted; Reid et al. submitted) may be a general phenomenon of reduced Al accumulation. The mechanism of reduced Al accumulation remains obscure, but acclimation to Al by brook trout was probably related to a decrease in gill sialic acid, a component of mucus (McDonald et al. submitted). Decreased sialic acid content could have been a result of increased sloughing of mucus, or a change in mucus composition. In brook trout exposed continuously to Al, gill Al loads decreased with time after 24 h, presumably because of increased mucus turnover, or decreased affinity of mucus for Al (McDonald et al. submitted). Repair of initial Al damage to gills and proliferation of mucous cells occurred during the ~13 d acclimation process (Mueller et al. submitted). In addition, during similar experiments of acclimation to Al by rainbow trout, the gills developed increased affinity for Ca^{2+} and decreased affinity for Al; accordingly, Al was less able to displace Ca^{2+} from the gills (Reid et al. submitted).

These results of decreased gill Al accumulations during longer exposures to Al can be fitted into the present gill interaction model (Fig. 43C). Proliferation of mucous cells suggests increased mucus production in response to Al deposition. Increased mucus turnover rate would remove Al precipitating onto the gills, so that Al accumulation on the gills would not increase indefinitely. As long as mucus is continually sloughed off,

respiratory effects caused by Al would be reduced. Increased affinity of gills for Ca^{2+}, and decreased affinity for Al, would result in a reduction in ion effluxes at the gills that were originally caused by the displacement of Ca^{2+} from tight junctions by charged Al species. Repair of initial gill damage would partially restore gill processes such as active ion uptake, further reducing the ionoregulatory effects of Al. Note that in this model the alkalinization of the gill micro-environment is unchanged, so the Al solubility or speciation changes leading to Al deposition continue: it is the increased removal of Al that is responsible for recovery from the respiratory effects of Al, and the decreased affinity of the gill binding sites for charged Al species contributes to recovery from ionoregulatory disturbance.

To conclude, a model of interactions of Al with fish gills, taking into account water chemistry changes in the gill micro-environment, was developed to explain the results of this thesis. The model is versatile, and reasonably explains the respiratory and ionoregulatory effects of Al on fish in soft water. This approach, based on water chemistry in the gill micro-environment rather than on bulk water chemistry, potentially has wide application for any gill toxicant or contaminant whose toxicity varies with pH, as discussed in Chapter 3. It would be interesting to discover if other fish species which are more or less sensitive to Al than rainbow trout, and accumulate more or less Al on the gills (cf. Wood and McDonald 1987), have comparable or different pH changes at the gills.

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