Effects of Aluminum and Low pH on Net Ion Fluxes and Ion Balance in the Brook Trout (*Salvelinus fontinalis*)

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Adult brook trout (*Salvelinus fontinalis*) were exposed for up to 11 d to one of a matrix of 18 Al, low pH, and Ca^{2+} combinations, chosen as representive of acidified softwater environments in the wild. Reduction in water pH led to pH-dependent net losses of Na^+ and Cl^- exacerbated by the presence of Al in the water and reduced by elevating Ca^{2+}. Any animal losing more than 4% of its total body Na^+ over the first 24 h of Al exposure had a greater than 90% likelihood of eventual mortality. Na^+ losses arose from inhibition of influx and stimulation of efflux. The inhibition was persistent and pH dependent. Addition of Al to acidified water had a slight further inhibitory effect on Na^+ influx and a large stimulatory effect on efflux. The latter was dependent on Al concentration, was the main cause of initial ion losses and mortality, and declined with time in surviving animals. All Al-exposed fish accumulated Al on their gills, but this was apparently mainly surface or subsurface bound, since no internal Al (plasma or liver) could be detected. Nonsurviving fish had substantially higher gill Al levels than survivors.

Pendant des périodes allant jusqu'à 11 d, on a exposé des ombles de fontaine (*Salvelinus fontinalis*) adultes à 18 différentes combinaisons de teneurs en Al et en Ca^{2+} et de faibles pH, caractéristiques des conditions naturelles des eaux douces acides. Une réduction du pH de l'eau a mené à des pertes nettes de Na^+ et de Cl^- liées au pH, aggravées par la présence d'Al dans l'eau et réduites par une augmentation de la teneur en Ca^{2+}. Les individus qui avaient perdu lus de 4 % de la teneur corporelle totale en Na^+ pendant les 24 premières heures d'exposition à l'Al étaient plus susceptibles de mourir dans plus de 90 % des cas. Les pertes de Na^+ proviennent de l'inhibition de son entrée, constante et dépendante du pH, et de la stimulation de sa sortie. L'apport d'Al aux eaux acides a un autre léger effet inhibiteur sur l'entrée du Na^+ et un grand effet stimulateur sur la sortie. En plus de dépendre de la teneur en Al, ce dernier effet est la principale cause des pertes initiales d'ions et de la mortalité et diminue en fonction du temps chez les individus survivants. Tous les poissons exposés à l'Al l'ont accumulé dans leurs ouïes mais il semble que cette liaison se soit surtout effectuée à la surface ou sous la surface, étant donné qu'aucune trace d'Al n'a été observée dans le plasma ou le foie. On a noté des teneurs plus élevées d'Al dans les ouïes des poissons morts que chez les survivants.

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The physiological responses of freshwater fish to environmental acidity have been studied extensively over the past 15 yr (see reviews by Fromm 1980; Wood and McDonald 1982; McDonald 1983b; Wood 1987). Considerable evidence from field and laboratory studies now points to ionoregulatory failure as the key factor leading to fish death in acidified waters, although the toxic effect may be exerted through disturbances to a host of physiological functions including fluid volume distribution, hematological and acid–base homeostasis, and oxygen uptake and transport (Packer 1979; Ultsch and Gros 1979; McDonald et al. 1980; Ultsch et al. 1981; Milligan and Wood 1982).

Two important environmental factors that influence the survival of fish in acidified water are water hardness and elevated concentrations of trace metals. Numerous studies have shown that increasing the concentration of external Ca^{2+} (the major component of water hardness) enhances the survival of fish in acidified water. In fact, Wright and Snekvik (1978) showed, from a survey of 700 softwater lakes in southern Norway, that water Ca^{2+} was at least as important as pH in determining fish population status. For acidic lakes and streams, there is mounting evidence that elevated concentrations of metals such as Al, Cd, Cu, Pb, Mn, Ni, and Zn, resulting from either atmospheric deposition or leaching of soils, may contribute to fish mortality (see reviews by Spry et al. 1981; Baker 1982; Campbell and Stokes 1985; McDonald et al. 1987). Of these metals, Al has caused the greatest concern because of its ubiquitous presence in soils and rocks and its widespread elevation in acidified waters. Indeed, Schofield and Trojnar (1980) found Al concentration to be the most important among 12 water quality parameters (more important than either pH or Ca^{2+}) in deter-

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TABLE 1. Chemical composition of water used to hold fish (Hamilton tap water) or acclimate fish (artificial soft water). For soft water the maximum ranges are reported. The large variation in [Cl^-] is the result of using $CaCl_2$ to make up the artificial soft water. Experimental water was prepared by adjusting pH of artificial soft water to pH 4.4, 4.8, or 5.2 and Al concentration to 111, 333, or 1000 μg/L. Over the course of an experiment, variations in experimental pH and Al were typically within 0.3 unit and 10%, respectively, of initial values.

	Hamilton tap water (μequiv/L)	Artificial soft water (μequiv/L)
Na^+	540	34–50
Cl^-	700	10–411
K^+	20	2–3
Ca^{2+}	2100	25 or 400
pH	7.8	6.3–6.6

mining brook trout (*Salvelinus fontinalis*) population status in 53 Adirondack lakes.

The chemistry of Al in natural waters is very complex; organic and inorganic ligands (e.g. humic acids, fluoride) play an important role in Al bioavailability, and water pH has a profound effect upon Al speciation and solubility (Driscoll et al. 1980; LaZerte 1984). Minimum Al solubility occurs at pH 6.0–7.0 and there is a greater than 7000-fold increase in solubility over the pH range from 6.0 to 4.0. At pH 4.0 the free aquo ion (i.e. Al^{3+}) is the predominant (>90%) monomeric species, while at pH 6.0, monovalent and divalent hydroxides predominate. Similarly complex is the toxicity of Al; several studies indicate that maximum toxicity occurs over the pH range 5.0–5.5 and decreases at lower and higher pH's (Freeman and Everhart 1971; Muniz and Leivestad 1980; Schofield and Trojnar 1980). Indeed, some authors have shown that low levels of Al can have a protective effect at pH's below about 4.4 (Muniz and Leivestad 1980; Schofield and Trojnar 1980; Baker and Schofield 1982).

Not surprisingly, given this complexity, much is still unknown concerning the mechanism of Al toxicity to fish. In general, the mechanism of Al toxicity appears in many respects to resemble that of H^+ toxicity; the main target of Al appears to be the gills (Schofield and Trojnar 1980; Muniz and Leivestad 1980; Buergel and Soltero 1983). Al accumulates on the gills in a concentration- and pH-dependent fashion (Neville 1985; Karlsson-Norrgren et al. 1986a, 1986b), thereby producing epithelial damage (Schofield and Trojnar 1980; Chevalier et al. 1985; Karlsson-Norrgren et al. 1986a, 1986b) and impairment of normal gill function (Staurnes et al. 1984; Witters 1986) resembling that seen with low pH alone (see McDonald 1983a for review). Furthermore, the physiological effects of Al appear to be similar to those of low pH in that ionic disturbances are prominent (Muniz and Leivestad 1980; Neville 1985) although there is some evidence to indicate that Al may also cause respiratory distress (Neville 1985). Finally, Ca^{2+} appears to ameliorate Al toxicity, at least to juvenile fish, in much the same way as it does acid toxicity (Muniz and Leivestad 1980; Brown 1983).

To further clarify the mechanism of Al toxicity to fish, we have undertaken a study of the interactive effects of Al, pH, and Ca^{2+} on adult brook trout. We were specifically interested in the following: the differences in toxic action of H^+ and Al, particularly in relation to their effects on branchial ion regulation and plasma ion balance; the nature of the protective effect of calcium, and the interrelations amongst ion losses, tissue accumulation of Al, and mortality. The levels of pH (4.4–5.2), Al concentration (0–1000 μg/L), and Ca^{2+} concentration (0.5–8 mg/L, 25–400 μequiv/L) used in this study were chosen to represent a range of conditions likely to be encountered by brook trout populations in acidified softwater lakes in the northeastern United States and eastern Canada, a region where acid precipitation is considered to be a serious threat to aquatic ecosystems (Harvey et al. 1981; Schofield 1982; Kelso et al. 1986).

Materials and Methods

Experimental Animals

Adult brook trout of both sexes (100–300 g) were obtained from Goosen's Trout Farm in Otterville, Ontario. The fish were transported to McMaster University where they were maintained for at least 2 wk in flowing, dechlorinated Hamilton tap water (see Table 1) at 7–18°C. Prior to use, the fish were held in batches of 20–25 in 500-L green polyethylene tanks for 2–4 wk and acclimated to artificial soft water (Table 1) with a Ca^{2+} concentration of either 25 or 400 μequiv/L (0.5 or 8.0 mg/L) at circumneutral pH (6.0–6.5) and a temperature of 10 ± 1°C. Soft water was prepared by adding NaCl and either $Ca(NO_3)_2$ or $CaCl_2$ (see Results) to deionized water obtained from a Culligan Aquacleer MP-500 reverse osmosis system. Water in the acclimation tanks was recirculated through a polyester wool/activated charcoal filter and was replaced at 2- to 4-d intervals. Fish in the holding tanks and the softwater acclimation facilities were fed commercial trout pellets every other day, but were not fed for 1 wk before an experiment.

Experimental Protocol

Fish acclimated to soft water were placed individually in darkened Plexiglas flux chambers (McDonald and Rogano 1986) of nominal 2.7-L volume and supplied with flowing soft water (~0.5 $L \cdot kg^{-1} \cdot min^{-1}$) with the same levels of Ca^{2+} and pH as in the acclimation tank. Water temperature in the flux chambers was maintained at 10 ± 1°C and continuous aeration ensured thorough mixing of the water. After a 24- to 48-h period of recovery from the stress of handling, fish (N = 108) were exposed for 24 h to low-pH water (pH 4.4, 4.8, or 5.2) obtained by adding 0.1 N H_2SO_4 to 1500-L reservoirs of soft water supplying the flux chambers. Following this initial low-pH challenge, fish were then either maintained at the same pH for up to 10 d (controls, N = 36) or were exposed to elevated concentrations of Al (111, 333, or 1000 μg/L) at the same pH (N = 72). Al was added to the low-pH soft water as either $AlK(SO_4)_2$ or $AlCl_3$ (see Results). The chemical composition of the experimental water is given in Table 1. Water was never recirculated, but rather was run to waste after leaving the fish boxes.

The chemistry of aqueous Al is complex (Fig. 1). Water pH has profound effects on both Al speciation (Fig. 1A) and solubility (Fig. 1B), and even though the test concentrations of Al we employed were well below the solubility limits of Al in "new" solutions (see data points on Fig. 1B), the concentration of Al solutions tends to gradually decline with time as insoluble aluminum hydroxides form (Smith and Hem 1972). To avoid these speciation and solubility problems the following precautions were taken. First, batches of soft water containing Al (1500 L) were made up daily and used immediately. Consequently, Al-containing water did not "age" for more than 24–36 h. Second, the accumulation of waste products (e.g. ammonia) in the flux chambers was avoided by maintaining a

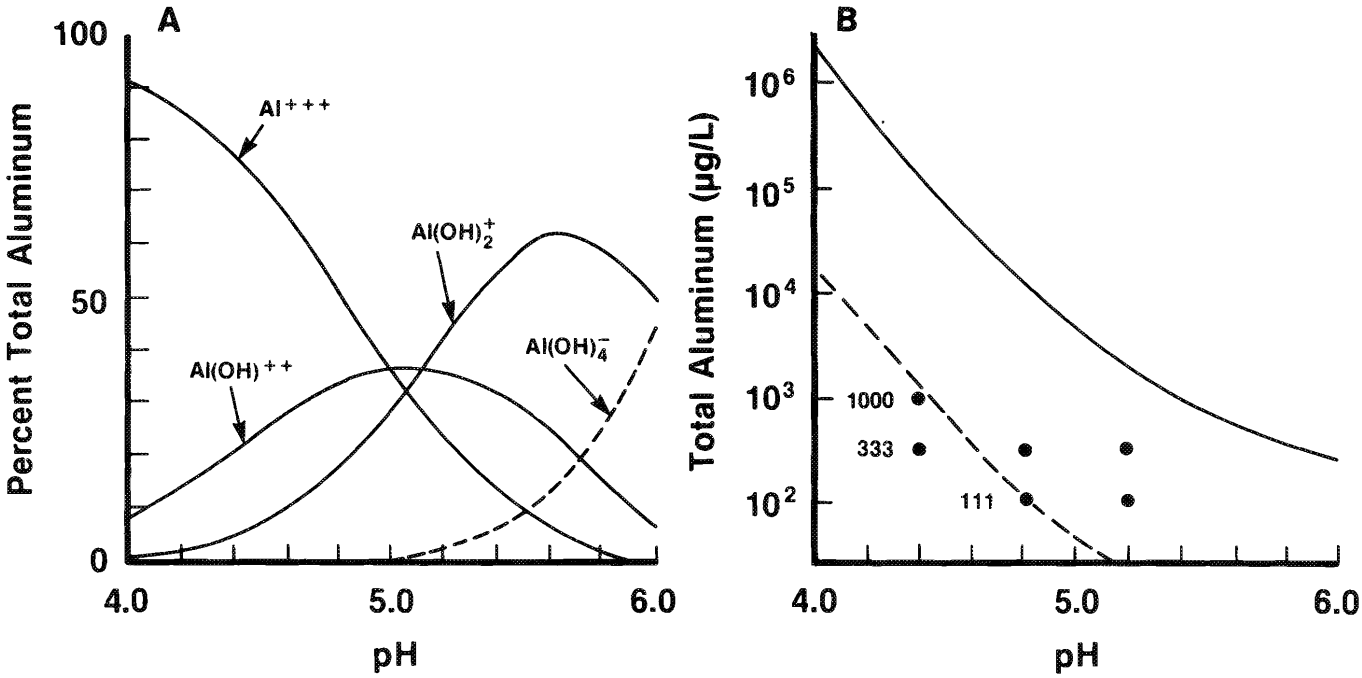

FIG. 1. Al chemistry in low ionic strength waters. Calculations based on thermodynamic equilibrium constants for 25°C and zero ionic strength from Johnson et al. (1981). (A) Al speciation diagram; relative concentrations of Al species at 25°C in freshwater. (B) Total soluble Al as a function of pH from the solution of amorphous $Al(OH)_3$ (solid line). Shown is the experimental matrix employed in this study (see Fig. 5). In natural waters, in equilibrium with gibbsite (crystalline $Al(OH)_3$), Al solubility would be as described by the broken line (Harvey et al. 1981).

continuous flow through the chambers and by discarding the water outflow. Third, the rates and pH of water flowing through the flux chambers were periodically adjusted so that the pH rose by less than 0.3 pH unit in transit through the chambers and the average of the inflow and outflow pH values equalled the desired experimental pH.

At the termination of an experiment the fish were killed by a sharp blow to the head, a terminal blood sample was obtained by caudal puncture, and the gills and liver were excised, blotted, and frozen, as was the remainder of the carcass, for later analysis of Al levels. Fish that died during an experiment were removed as soon as discovered and the gills and liver were excised as above. In addition, to establish control plasma ion levels, blood samples were taken from a group of fish acclimated for 18 d to soft water (Ca^{2+} = 25 µequiv/L) at cirumneutral pH.

Ion and Ammonia Flux Measurements

Net exchanges of ions and ammonia between the animal and its environment were assessed by measuring the concentrations of Na^+, Cl^-, Ca^{2+}, K^+, and ammonia in water entering and leaving the flux chambers. At all sampling times (see Fig. 2 for sampling schedule), the total outflow of each chamber was collected for 2 min and the volume determined by weighing. Immediately afterward, a 150-mL sample of inflow was collected. About 60 mL of each sample was saved, preserved by addition of 20 µL of concentrated HNO_3 (analytical grade), and set aside for later analysis (see below). Net flux rates (J_{net}) were calculated from measured concentrations by the Fick principle:

$$J_{net}^{X}(\mu\text{equiv}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}) = \frac{(\text{input}[X] - \text{output}[X]) \times \text{water flow (L/min)}}{\text{fish mass (kg)}}$$

where $[X]$ is the concentration (microequivalents per litre) of substance X. Flux measurements were made at various times during the control period and during exposure to low pH and elevated Al. In some experiments, unidirectional Na^+ flux rates (J_{in} and J_{out}) were measured by stopping water flow to and from the flux chambers, adding 1 µCi of $^{22}Na^+$ (New England Nuclear) to each chamber, and monitoring the disappearance of the radioisotope from the water over a 30-min period. Na^+ influx (microequivalents per kilogram per hour) was calculated according to the following formula:

$$J_{in}^{Na+} = \frac{(\ln Q_i^* - \ln Q_f^*)}{t \times W} \times Q_{out}$$

where Q^* is the total amount of radioactivity in the medium at the beginning (i) and end (f) of the sampling interval (t), Q_{out} is the average amount of Na^+ in the medium, t is the sampling interval (hours), and W is the body weight (kilograms) (after McDonald et al. 1983). Na^+ efflux (J_{out}) was calculated as the difference between J_{in} and J_{net}. During these static flux measurements, water samples for ion and radiotracer analysis were taken from the flux chambers at 10-min intervals. At the same time the pH of the water was checked and adjusted to the desired level by the addition of either H_2SO_4 or KOH.

Analytical Procedures

Concentrations of Na^+, Ca^{2+}, and K^+ in the water and blood plasma were measured with a Varian AA-1275 atomic absorption spectrophotometer after appropriate dilution. Water and plasma Cl^- concentrations were measured by coulometric titration (Buchler–Cotlove chloridometer). Water ammonia concentration was determined by the salicylate–hypochlorite method of Verdouw et al. (1978). Al concentrations in the water, plasma, and tissues were measured on the Varian AA-1275 using an automated graphite furnace attachment (Varian GTA 95). Prior to analysis, tissues were digested in 2 volumes of concentrated HNO_3 and then diluted 1:105 with deionized

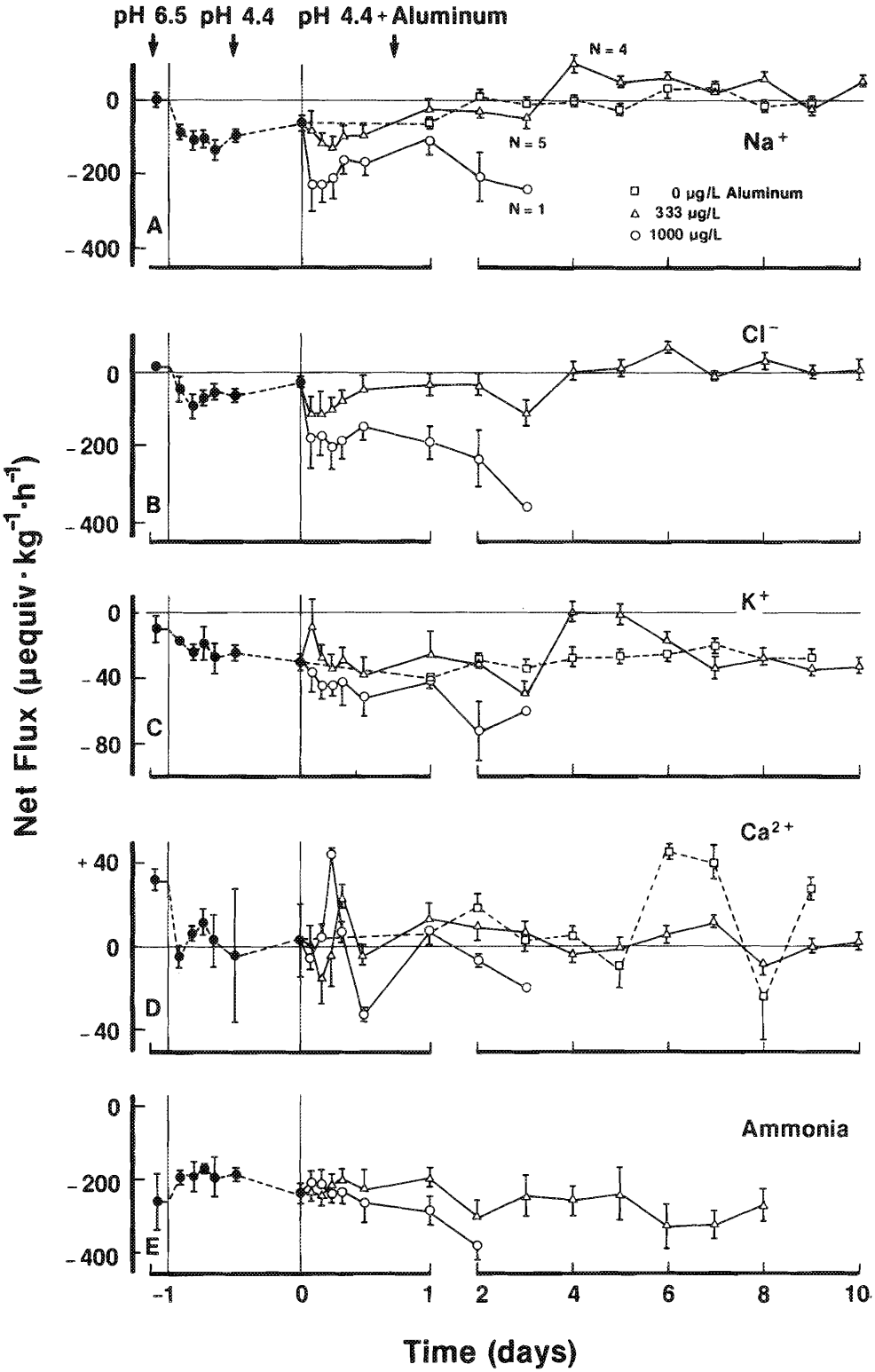

FIG. 2. Net flux rates (means ± 1 SEM) for (A) Na^+, (B) Cl^-, (C) K^+, (D) Ca^{2+}, and (E) ammonia at pH 4.4 and 0 and 333 and 1000 µg Al/L. Water Ca^{2+} concentration = 25 µequiv/L. $N = 6$ for each treatment except where noted in Fig. 2A; reduction in N is due to Al-induced mortality. Ion flux rates of initial 24 h period (pH 4.4) are pooled means for the three treatment groups (i.e. $N = 18$). Al exposure started on day 0. Note expanded scales for the net fluxes of K^+ and Ca^{2+} and note change in time scale at day 1.

water. Tissue Al concentrations were determined by standard additions of known quantities of Al to the unknown samples. Levels of $^{22}Na^+$ in 5-mL water samples were measured either by gamma counting in a well-counter (Nuclear Chicago Model 1085) or by scintillation counting (LKB-Wallac). For scintillation counting the water samples were added to 10 mL of

TABLE 2. Summary of fish mortality at various combinations of pH, Al, and Ca^{2+}. Each cell started with six fish. Significant differences between high- and low-Ca^{2+} cells were assessed using either a chi-square test (all high- and low-Ca^{2+} cells combined) or Fisher's exact probability test (individual cell comparisons). Asterisks indicate significant effects of Ca^{2+} ($p < 0.05$).

pH	Al (μg/L)	Mortalities at Ca^{2+} concentration (μequiv/L) 25	400
5.2	0	0	0
	111	0	0
	333	6*	0
4.8	0	0	0
	111	0	0
	333	5*	1
4.4	0	0	0
	333	2	0
	1000	6	6
Combined		19*	7

Aqueous Counting Scintillant (Amersham). Hematocrit was determined by centrifugation at 5000 × *g* for 5 min.

Statistical Analysis

Data are reported throughout as means ± 1 SEM. Differences in mortalities between high- and low-Ca^{2+} cells were evaluated using a 2 × 2 contingency table and either a chi-squared test (for $N > 20$) or Fisher's exact probability test for smaller sample sizes (Siegel 1956). Differences between groups were evaluated using the Student's unpaired *t*-test. Interrelationships between ion losses and water pH (at constant or zero Al) or ion losses and Al (at constant pH) were evaluated using least squares regression analysis.

Results

Al Toxicity

In low-Ca^{2+} water (25 μequiv/L), there was increasing mortality with increasing Al and virtually 100% mortality at the highest Al concentration at each pH level (Table 2). The toxicity of 333 μg Al/L at low Ca^{2+} decreased with a decrease in pH; mortality was 100% at pH 5.2 but only 33% at pH 4.4. Water Ca^{2+} had a significant protective effect ($p < 0.05$, Table 2). In low-Ca^{2+} waters, the various pH/Al combinations caused 35% mortality overall (19 fish out of 54). In contrast, the mortality in high-Ca^{2+} water was only 13% (7 fish out of 54) for the same pH/Al combinations. All fish deaths occurred between 1 and 5 d after exposure to Al, but in low Ca^{2+} the majority of the deaths (79%) occurred within 2 d whereas in high Ca^{2+}, only two out of the total of seven deaths had occurred by this time. There were no differences in either mortality or net ion fluxes in fish exposed to the same pH/Al/Ca^{2+} combinations using $Ca(NO_3)_2$ and $AlK(SO_4)_2$ versus $CaCl_2$ and $AlCl_3$. Thus, results with the different salts have been combined.

Net Ion Fluxes

Fish exposed to reduced water pH with and without Al suffered marked disturbances to internal ionic balance, as indicated by high rates of Na^+ and Cl^- loss to the ambient water (Fig. 2–4). The patterns of ion loss were qualitatively similar for all 18 combinations of pH and Al. Therefore, rather than present each treatment individually, the results of three sets of experiments, that of pH 4.4, and 0, 333, and 1000 μg Al/L at low Ca^{2+} (25 μequiv/L), are shown to illustrate typical responses (Fig. 2).

In fish at circumneutral pH, acclimated to the flux chambers for 24–48 h, the mean net flux rates for Na^+ and Cl^- were not significantly different from zero ($p < 0.05$, paired *t*-test), indicating that the animals were in ion balance. Sudden exposure to pH 4.4 caused a substantial net efflux of Na^+ and Cl^- within 2–3 h (Fig. 2A, 2B). Peak rates of Na^+ and Cl^- loss occurred after 4–6 h at pH 4.4 and were of approximately equal magnitude. The rates of Na^+ and Cl^- loss subsequently declined, although after 24 h at pH 4.4, they had not returned to zero.

In those fish maintained at pH 4.4 without Al exposure, net Na^+ and Cl^- flux rates returned to zero approximately 48–72 h after the start of low pH exposure and remained at this level for the duration of the experiment (Fig. 2A, 2B; Cl^- flux rates not shown). No mortality occurred in the six fish kept in these conditions.

Upon exposure to 333 μg Al/L after 24 h at pH 4.4, fish showed increased rates of Na^+ and Cl^- loss for several hours followed by gradually decreasing ion losses over the next 48–72 h (Fig. 2A, 2B). However, two of the six fish died during Al exposure, one within 72 h and another within 120 h. The survivors appeared to recover some of their initial Na^+ and Cl^- losses, as indicated by periods of net Na^+ and, to a lesser extent, net Cl^- uptake. For the remaining 3–4 d of the experiment, net Na^+ and Cl^- flux rates were not significantly different from zero.

When fish were exposed to 1000 μg Al/L after 24 h at pH 4.4, Na^+ and Cl^- efflux rates increased rapidly to peak levels approximately twofold higher than losses from fish at 333 μg Al/L (Fig. 2A, 2B). Over the next 48 h, the fish all continued to lose Na^+ and Cl^- at relatively high rates and all died within 72 h of the initial Al exposure.

Exposure to low pH and Al also caused brook trout to lose K^+ in a pattern similar to that for Na^+, with the highest rate of K^+ efflux occurring at the highest Al concentration (Fig. 2C). Although the patterns of Na^+ and K^+ loss were generally similar, the rate of K^+ loss was typically only about 20% of that for Na^+. Furthermore, K^+ balance remained negative throughout the 11-d exposure at pH = 4.4 and, Al = 0 μg/L. In contrast, low pH and Al exposure had no detectable effect on net Ca^{2+} flux rates, which fluctuated on either side of zero, even in dying fish (Fig. 2D).

There was little change in the rate of ammonia excretion in response to pH 4.4. However, after 1 – 2 d of exposure to 333 and 1000 μg Al/L, ammonia excretion rates had increased by 30–50% (Fig. 2E). This phenomenon is likely stress related, resulting from a cortisol-mediated increase in protein catabolism (cf. McDonald 1983a).

From the above results it is evident that net Na^+ and Cl^- flux rates were very sensitive indicators of low pH and Al stress. Since water Cl^- levels in these experiments were near the lower limit of detection by coulometric titration, measurements of Cl^- flux rates were subject to greater error than were Na^+ flux rates. Therefore, further analysis of ionic disturbances will largely concentrate on Na^+ fluxes.

Since the "instantaneous" Na^+ flux rates changed with time over the course of the experiments, they did not indicate the

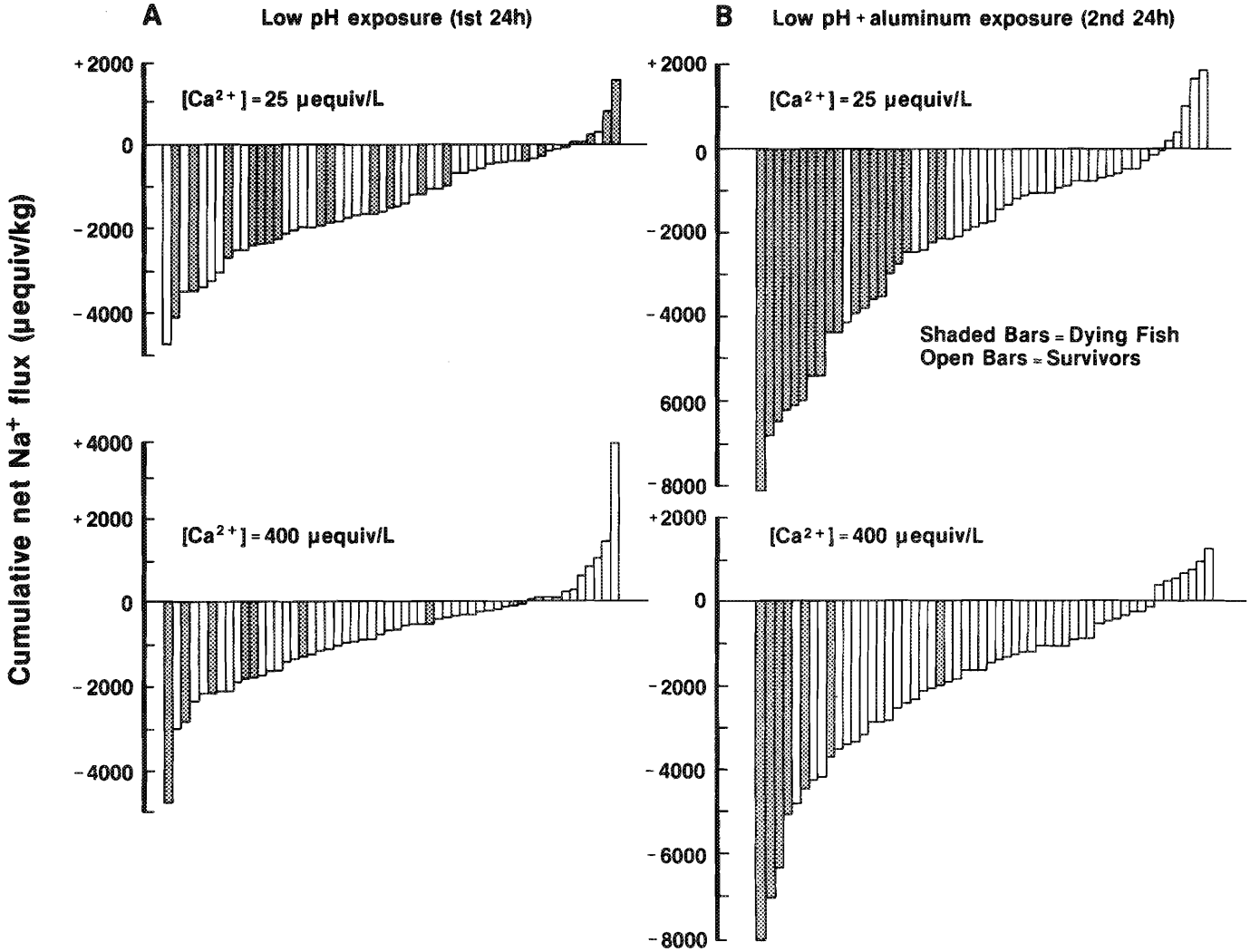

FIG. 3. Cumulative net Na^+ fluxes for individual fishes, ranked according to the magnitude of the flux: − = net loss; + = net uptake. Open bars indicate fish that survived for the duration of the experiment (i.e. 11 d) and shaded bars indicate fish that died subsequent to the first 24 h of Al exposure. (A) Na^+ fluxes over the first 24-h period of the experiment in which fish were exposed to low pH alone (pH 4.4, 4.8, or 5.2). (B) Na^+ fluxes over the second 24-h period in which fish were exposed to Al (0, 111, 333, or 1000 µg/L) plus low pH.

magnitude of the Na^+ losses incurred by the fish. The net Na^+ flux rates were therefore integrated over time to give cumulative net Na^+ fluxes. Cumulative net Na^+ fluxes were calculated individually for each fish for the first and second 24-h periods of the experiment, before any mortality occurred. This allows a comparison of Na^+ losses between fish which later died during the experiments (shaded bars, Fig. 3) and fish which survived (open bars).

There was considerable variation amongst fish in net Na^+ losses either when the fish were exposed to low pH (4.4, 4.8, or 5.2) over the first 24 h (Fig. 3A) or over the second 24 h (Fig. 3B), when exposed to low pH (4.4, 4.8, or 5.2) plus Al at various concentrations (0, 111, 333, or 1000 µg/L). Here, the protective effect of Ca^{2+} can clearly be seen in the generally smaller sodium losses at 400 versus 25 Ca^{2+} and the disruptive effect of Al by the greater Na^+ losses (Fig. 3A versus 3B). During the initial 24 h, there was no apparent relationship between the amount of Na^+ loss and eventual mortality. However, when fish were subjected to a combination of low pH and elevated Al in the second 24 h, there were significantly greater Na^+ losses in those fish which subsequently died. The lethal threshold of ion loss in low Ca^{2+} (for the second 24 h) was approximately 2000 µequiv Na^+/kg or approximately 4% of total body Na^+ (53 mequiv/kg, cf. Shearer 1984). Of the 19 fish that died in the low-Ca^{2+} group, all lost in excess of this amount and only five of survivors lost more. In the high-Ca^{2+} group, the apparent threshold was substantially higher, in excess of 3500 µequiv/kg. Six of the seven fish that died and only three of the 47 survivors in the high-Ca^{2+} group exceeded this level of loss. The marked difference between survivors and nonsurvivors is perhaps best illustrated by an example from one cell (pH 4.4, 333 µg Al/L, 25 µeq Ca^{2+}/L) which had two mortalities out of six fish. In this cell the survivors (240-h exposure) lost 1100 ± 220 µequiv Na^+/kg over the first 24 h of Al exposure whereas the two nonsurvivors (which died after 72 and 96 h) lost 5010 and 2300 µeq/kg, respectively, during this period.

The mean cumulative net Na^+ fluxes for the second 24-h period (low pH plus Al) were used to assess the interactive effects of pH and Al on Na^+ balance (Fig. 4). Considering the low-Ca^{2+} group first, in the absence of Al, fish showed a net uptake of Na^+ at pH 5.2 while sustaining net losses of Na^+ at the two lower pH levels (Fig. 4A). At each of the three pH levels tested, there was a significant direct correlation between

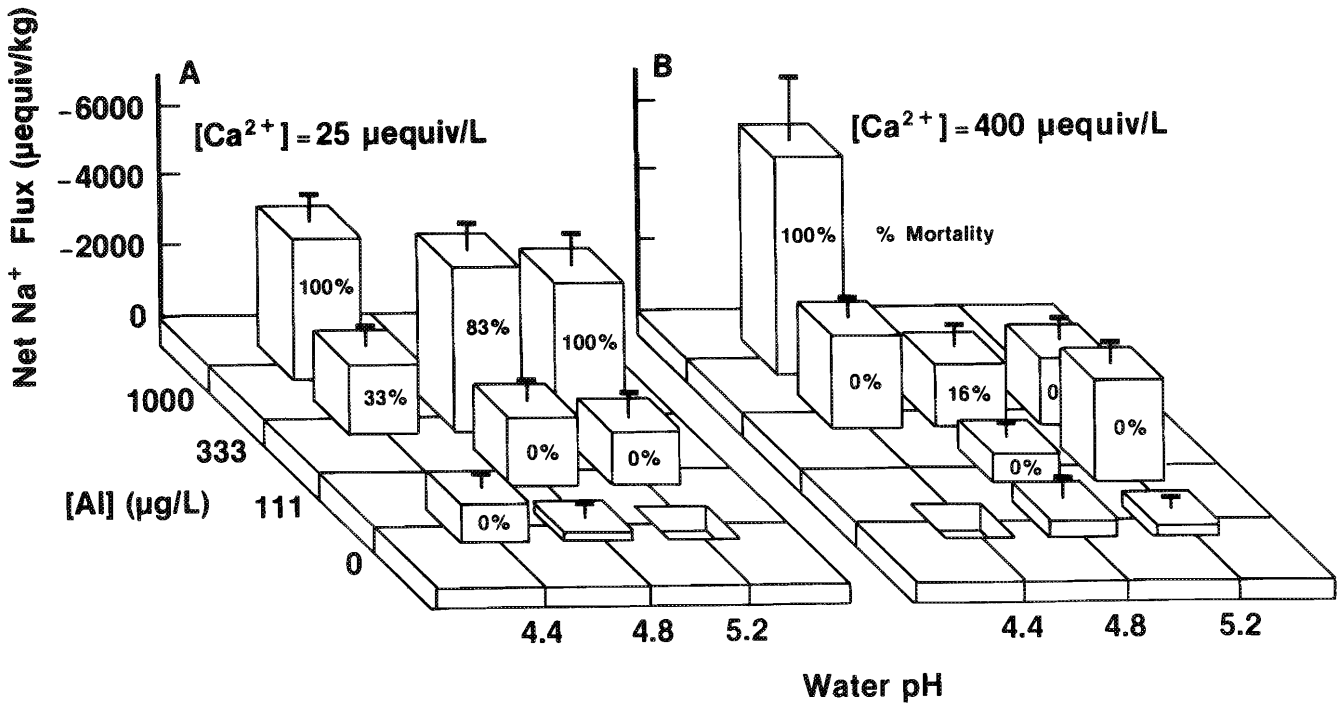

FIG. 4. Effects of pH, Al, and Ca^{2+} on cumulative net Na^+ fluxes (means ± 1 SEM, $N = 6$ for each cell) over the first 24 h of Al exposure (follows 24 h at same pH without Al). Exposure conditions that were not employed are indicated by the absence of a bar (e.g. 111 µg Al/L at pH 4.4).

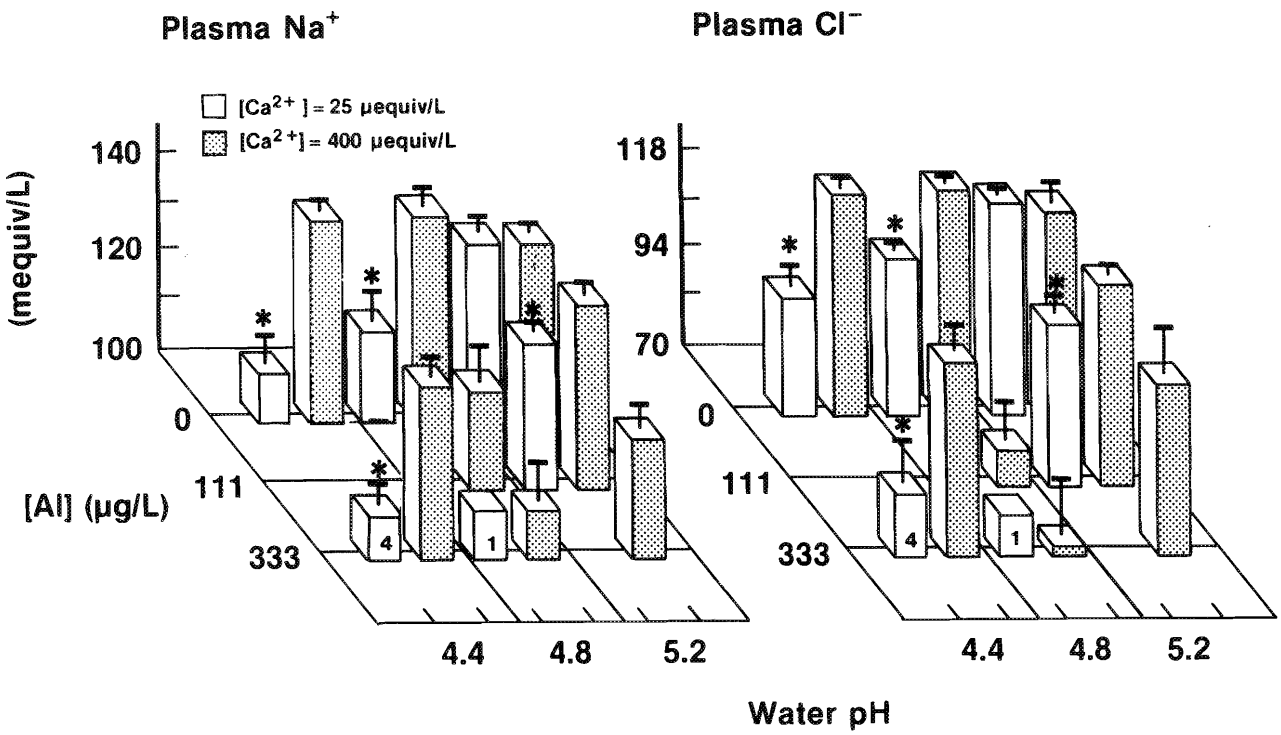

FIG. 5. Effects of water pH, Al, and Ca^{2+} on plasma concentrations of Na^+ and Cl^-. Terminal samples, survivors only, $N = 6$ except where noted. Asterisks indicate significant differences ($p < 0.05$, unpaired t-test) between high- and low-Ca^{2+} animals at the same pH/Al exposure.

Al concentration and net Na^+ efflux ($r = 0.670$, 0.851, and 0.789 for pH 4.4, 4.8, and 5.2, respectively), with the highest Na^+ losses associated with high mortality (Fig. 4). Also, at 333 µg Al/L, Na^+ losses were significantly higher at pH 4.8 than at pH 4.4 ($p < 0.01$, unpaired t-test).

In high-Ca^{2+} water, the significantly lower mortality (Table 2) was associated with lower and more variable cumulative net Na^+ fluxes (Fig. 4B). Nonetheless, in all cases except pH 4.8 and 111 µg Al/L, Al caused significantly greater ion losses than low pH alone ($p < 0.01$, unpaired t-test).

Plasma Ion Concentrations

Ion levels (Fig. 5) in terminally collected blood samples (taken from surviving fish at the end of the experiments)

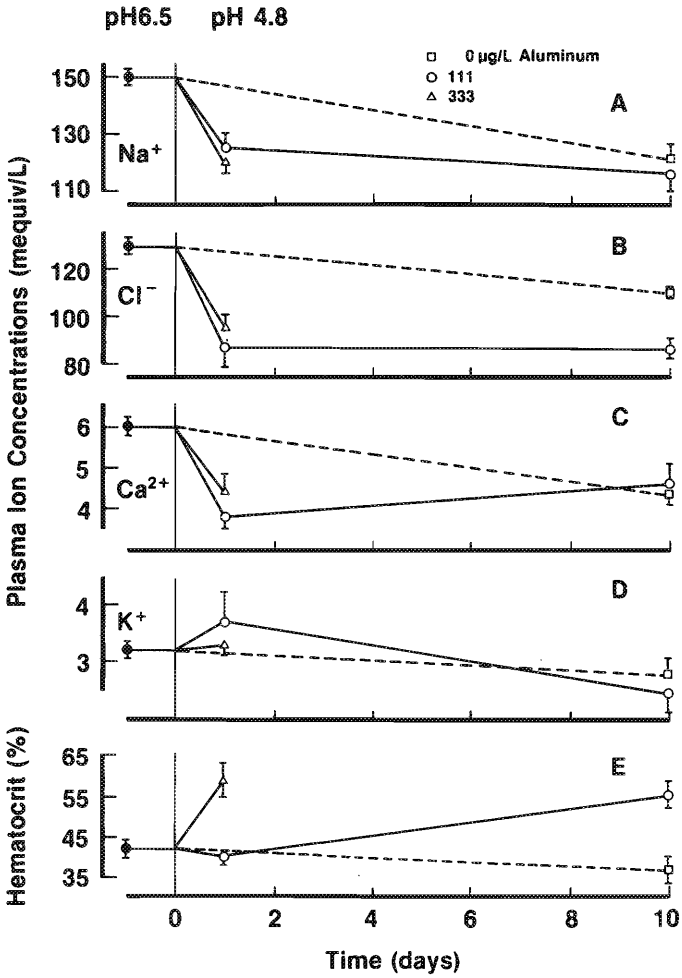

FIG. 6. Effects of pH 4.8 and 0 and 111 and 333 µg Al/L on (A–D) plasma ion concentrations (E) and hematocrit. Water Ca^{2+} concentration was 25 µequiv/L. Terminal samples, survivors only, $N = 6$ in acid-exposed fish. For fish at pH 6.5, $N = 10$; blood samples were drawn by caudal puncture after 18 d of acclimation to artificial salt water (Ca^{2+} = 25 µequiv/L).

reflected, to a large extent, the effects of net Na^+ and Cl^- losses to the medium. In all groups of fish, plasma Na^+ and Cl^- levels were reduced relative to control fish maintained at pH 6.5 and acclimated to soft water. In control fish, plasma [Na^+] and [Cl^-] were 149 ± 1 and 133 ± 1 mequiv/L, respectively ($N = 10$; data plotted on Fig. 6).

The blood ion data confirm the major protective effect of Ca^{2+}; in virtually every cell, plasma Na^+ and Cl^- levels were significantly higher in the high-Ca^{2+} survivors (shaded bars, Fig. 5) than in the low-Ca^{2+} survivors (open bars) for the same pH/Al exposure. These data also reveal some important effects of sublethal exposures not readily apparent from initial Na^+ losses (Fig. 4). First, in those low-Ca^{2+} animals not exposed to Al, plasma ion levels correlated well with pH ($r = 0.847$), with the lowest levels at pH 4.4. In contrast, no effect of low pH (without Al) was seen in the high-Ca^{2+} animals. Second, there was a clear effect of Al exposure on high-Ca^{2+} animals; at each pH (pH 4.4, 4.8, 5.2) there was a significant inverse correlation ($r \geq 0.545$) between Al concentration and plasma NaCl concentration. In contrast, no plasma ion/Al correlation was evident in low-Ca^{2+} animals despite the correlation between Al concentration and net ion losses in this group (Fig. 4). However, it should be noted that the plasma data are terminal samples for survivors only. Thus, animals that lost a large amount of NaCl at the higher Al concentrations (Fig. 4) had already died when the terminal samples were collected.

Figure 6 gives an indication of the time course of the plasma ion disturbances. These data are for fish exposed to pH 4.8 and Al concentrations of 0, 111, and 333 µg/L, and in this case, blood samples were collected in two experimental series, one terminated after 1 d of Al exposure and the other after 10 d of Al exposure. These data show that most of the plasma NaCl depressions resulting from pH and Al exposure occurred in the first 24 h (Fig. 6A, 6B). Little difference was seen between 111 and 333 µg/L in terms of plasma NaCl loss, although the increase in hematocrit (Fig. 6E), which is typically secondary to ion loss (cf. Milligan and Wood 1982), was much larger at 333 µg/L than at 111 µg/L. Furthermore, five out of the six fish at 333 µg/L died whereas the six fish at 111 µg/L survived. Fish exposed to 111 µg Al/L showed a significant decrease in plasma Ca^{2+} concentration within 24 h (Fig. 6C). However, at the end of the 11-d experiment, Ca^{2+} concentration was not significantly different from control values in all fish (unpaired *t*-test). Little variation was seen in plasma K^+ (Fig. 6D) despite persistent net K^+ losses from the animals (Fig. 2C). This is likely due to the substantial intracellular reserve of K^+.

Unidirectional Na^+ Fluxes

At periodic intervals, the whole-animal net Na^+ fluxes were partitioned into their influx and efflux components by means of Na^+ radiotracer disappearance from the medium (Fig. 7). Since these measurements were for 30 min only, they were, in effect, "snapshots" and did not have the resolution of the cumulative Na^+ losses (Fig. 3, 4) or the terminal plasma measurements (Fig. 5, 6). For example, based on these measurements, there was virtually no effect of water Ca^{2+} concentration on either influx or efflux (although the net flux data and plasma ions indicate that such effects should exist). Nor were there any consistent differences between those animals that were exposed to low pH only and those exposed to sublethal levels of Al (111 µg/L at pH 5.2, 333µg/L at pH 4.4). Nevertheless, the results (Fig. 7) do provide some insights into the specific disturbances caused by H^+ and Al. To simplify the presentation of these results, the data for high and low Ca^{2+} and zero and sublethal Al have been pooled at each pH.

The initial effect of low pH and/or Al exposure was invariably a combination of influx inhibition and a marked efflux stimulation (Fig. 7A, 7B). While the pH (alone) effect was mainly on influx (Fig. 7A), the effect of Al at low pH was on both influx and efflux (Fig. 7B), inhibiting the former and increasing the latter. The efflux effect was larger and therefore probably the main reason for the initial ion losses (Fig. 3), but it was time dependent, being most pronounced in any one treatment over the first 2–8 h of exposure and then subsequently declining. Efflux typically returned to near control levels within 24 h (Fig. 7C) in all but the acutely lethal exposures. In contrast, the effects of low pH and sublethal Al exposure on Na^+ influx were quite persistent. In all fish at low pH alone, influx remained significantly inhibited relative to controls at pH 6.5, with means varying between 20 and 60% of initial values (Fig. 7C). Since these fish were, for the most part, nearly in Na^+ balance (e.g. Fig. 2) from day 3 onward, their Na^+ effluxes must have been consistently less than initial values as well.

The acute effects of Al on Na^+ fluxes were similar to but greater than those of low pH alone. The main initial effects were

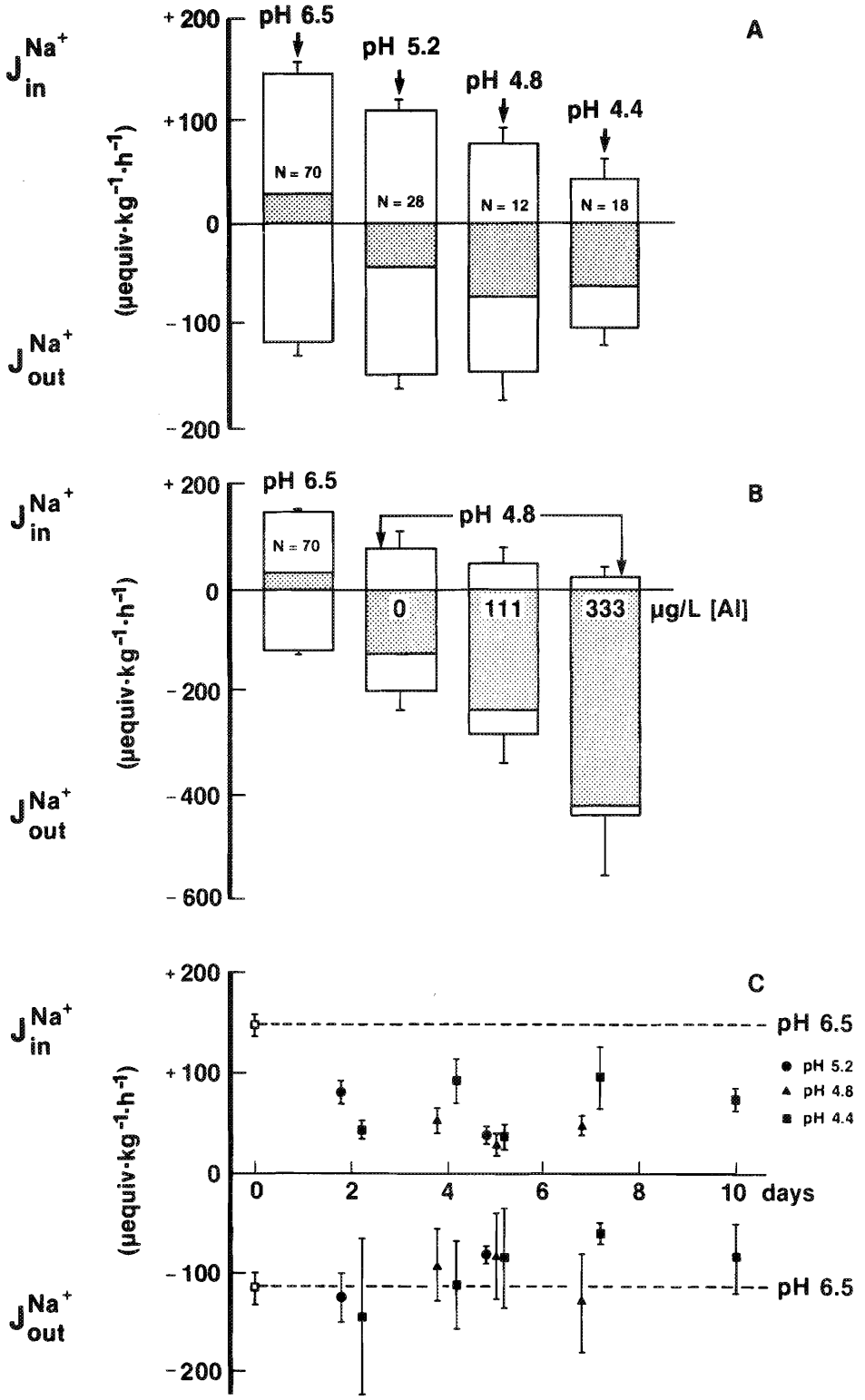

FIG. 7. Unidirectional Na^+ fluxes (J_{in} and J_{out}) as determined by radiotracer disappearance from the water over 30 min. Net flux (J_{net}) is indicated by the shaded areas. Data from experiments on low-Ca^{2+}-acclimated animals have been pooled with those from high-Ca^{2+}-acclimated animals. (A) Acute effect of low pH. For pH 5.2, 4.8, and 4.4, measurements were made after 8 h of low pH exposure. Means ± 1 SEM, N as indicated. (B) Acute effect of Al exposure. Measurements were made after 6 h of exposure to Al. Means ± 1 SEM, $N = 6$ except where noted. Note different scale from Fig. 7A. (C) Effect of duration of low pH exposure. Measurements made at pH 5.2 have been pooled with those made at pH 5.2 and 111 μg Al/L; measurements made at 4.4 have been pooled with those made at pH 4.4 and 333 μg Al/L. Means ± 1 SEM, N = 6–24.

a concentration-dependent stimulation of efflux above that caused by low pH alone and a slight further inhibition of Na^+ influx (Fig. 7B). With time, these effects abated in survivors to the point that the influxes and effluxes in sublethally Al-exposed fish (111 μg/L at pH 5.2, 333 μg/L at pH 4.4) were indistinguishable from fish exposed only to low pH (Fig. 2).

Tissue Al Levels

All Al-exposed fish accumulated Al in their gills, but this was apparently mainly surface or subsurface bound, since no internal Al (plasma or liver) could be detected. In surviving fish, gill Al concentrations were relatively independent of water pH and Ca^{2+} but were dependent on water Al, averaging 235 ± 47 μg/g wet tissue (N = 24) at 111 μg/L and 413 ± 75 μg/g (N = 22) at 333 μg/L. In nonsurviving fish, gill Al levels were significantly higher ($p < 0.01$, unpaired t-test) than in survivors, averaging, for all Al cells combined, 837 ± 98 μg/g (N = 25).

Discussion

Toxic Action of Al

The results of the present study confirm previous findings (Muniz and Leivestad 1980; Neville 1985) in showing that Al exposure produces disturbances to ion balance that resemble those caused by exposure to low pH alone (reviewed by Wood 1987). Our study now clearly shows that transepithelial ionic fluxes and plasma ion concentrations become more severely disrupted with increasing concentration of either H^+ or Al and that the disturbance is primarily to NaCl balance and not to other body electrolytes. However, the major contribution of the present study is to offer new insights into the specific nature of the toxic action of Al upon gill ionoregulatory mechanisms and how that toxic action is modified by duration of exposure and by the pH and Ca^{2+} concentration of the external environment.

The results show that the ionoregulatory response of fish to Al/low pH exposure can be divided into two relatively distinct phases: an initial "shock" phase and a "recovery" phase. The shock phase is characterized by two responses: a pronounced reduction of ionic uptake which likely reflects an inhibition of the branchial transport ATPase's (Staurnes et al. 1984) and a large stimulation of passive ionic efflux which likely arises from an increase in the paracellular ionic permeability of the gill epithelium (McDonald 1983b; Marshall 1985). In this initial phase, passive ion efflux is the main contributor to net ion losses and in some animals can be of sufficient magnitude to cause death. The recovery phase, in contrast, is seen only in survivors and is incomplete, at least under the pH/Al conditions employed in this study. It is characterized by little, if any, recovery of uptake and by a reduction in efflux to control levels or less. In this phase, net ion losses arise largely from the persistent inhibition of uptake. In an earlier study (McDonald et al. 1983), we showed that rainbow trout (*Salmo gairdneri*) respond to acid stress in this fashion and also showed that the behavior of Cl^- fluxes was essentially the same as that of Na^+. In the present study, the failure of Na^+ influx (and by extension, Cl^- influx) to fully recover appears to be the main persistent effect of sublethal low pH and Al exposure. To defend ion content, or to recover initial ion losses, these fish must rely on a reduction of efflux across the gills. This, in turn, appears to be the major adaptive response to Al/low pH exposure (see below).

The initial shock response is apparently critical to survival, for the magnitude of the initial Na^+ loss is closely related to whether or not a fish survives a particular Al/low pH exposure (Fig. 3). The difference in ion loss between survivors and nonsurvivors, best illustrated by Fig. 3, is striking, particularly since a homogeneous population of brook trout was used (all from the same hatchery, all apparently in the same physiological state prior to use). A plausible explanation for this difference is that there is a critical threshold of initial disturbance to gill function. If this threshold is exceeded the result is the establishment of a positive feedback relationship between Al-induced epithelial damage and ion loss. In this circumstance, one can envision Al/H^+ interactions setting off a host of physiological and morphological reactions: displacing membrane-bound Ca^{2+}, weakening tight junctions, and deforming and lifting the lamellar epithelium (Chevalier et al. 1985), causing increased exposure of binding sites in the gills and further gill Al accumulation and acceleration of ion loss leading to death. This type of interaction would explain the observation of higher gill Al content in nonsurvivors compared with survivors. By the same reasoning, the initial damage in surviving fish would be below the critical threshold, allowing time for compensatory mechanisms to operate. The most useful function of these mechanisms would be the reduction of epithelial ion leakiness and/or protection of the epithelium from further surface effects of Al. The present results suggest that ion leakiness does seem to decline, based on the measurements of J_{out} (Fig. 7C versus 7A and 7B), but do not reveal how such changes could come about. A small contributing factor will be the reduction of the plasma to water NaCl diffusion gradient by the initial losses. However, the overall adaptive response may, in fact, be the result of a whole suite of adjustments: increased mucous secretion, cell volume changes that reduce paracellular channel permeability, the formation of stable and relatively innocuous polymeric Al precipitates, sequestering of Al in the intracellular compartment, repairing damaged cells, hormonal adjustments to epithelial permeability, and even changes in gill perfusion. Although there is an extensive literature on the morphological character of gill lesions induced by Al (Chevalier et al. 1985; Karlsson-Norrgren et al. 1986a, 1986b) and other toxicants (reviewed by Mallatt 1985), there is very little information on the morphological or physiological basis for the damage repair or adaptive mechanisms of the gills. This is clearly a very fruitful area for further research.

Interaction of Al and H^+

Although the toxicites of Al and H^+ resemble one another, they are not strictly additive, since, as demonstrated here and in previous toxicity studies, increasing acidity (at least over the pH range of 5–4) actually reduces the toxicity of Al. For example, Schofield and Trojnar (1980) showed that the median survival time (LT_{50}) of brook trout fry exposed to 1000 μg Al/L increased from 1.8 d at pH 4.9 to 5 d at pH 4.0. In the present study the relationship was similar but less straightforward; Al (at least at low Ca^{2+}) was about three times as toxic at pH 4.8 as at pH 4.4 (based on similar ion losses at 333/pH 4.8 and at 1000/pH 4.4, Fig. 5) but was either of similar or lower toxicity at pH 5.2 depending on water Ca^{2+} (similar initial ion losses at 5.2/333 and at 4.8/333 in low Ca^{2+}, Fig. 4; lower terminal plasma NaCl at 4.3/333 than at 5.2/333 in high Ca^{2+}, Fig. 5).

Underlying this complex interaction of Al and pH are two important aspects of Al chemistry in water; Al solubility drops

by a factor of 72 (Fig. 1B) over the pH range of 4.4–5.2 and is accompanied by major changes in speciation (Fig. 1A), from mostly trivalent Al at pH 4.4 to mostly monovalent and divalent hydroxides at pH 5.2. Another important factor in this context is the pH of the microenvironment at the gill surface. Although the gill surface is extremely difficult to characterize chemically, it is most likely alkaline relative to the immediately adjacent interlamellar water. Factors contributing to this condition are buffering by the polyanionic mucus layer anchored to the surface (Kirschner 1978) and the excretion of ammonia in the basic form, NH_3 (Wright and Wood 1985). Furthermore, the subsurface environment (extracellular fluids of the gill epithelium, and by extension the intracellular fluids) will contribute to this alkalinity, since it is likely to remain close to neutrality throughout Al exposure under even the most acutely lethal conditions (see Wood et al. 1988).

With this background in mind, two distinct possibilities can be suggested for a mechanism of Al toxicity, one based on solubility and the other based on speciation.

The first (as suggested by Schofield and Trojnar 1980) views Al toxicity as a function of the transformation of soluble monomeric Al species into either polymers and/or precipitates at the surface, this transformation being principally responsible for the gill damage that causes physiological disturbances. Increasing the water pH would enhance this process, providing, of course, that the pH of the surface microenvironment follows that of the surrounding water.

The second possible mechanism is based on differences in toxicity amongst the Al species. Here, the suggestion is that Al species vary in terms of their reactivity with surface binding sites and that polymerization and/or precipitation are secondary processes. This hypothesis would explain how Al could be more toxic at 4.8 than at 5.2, providing one assumes that the polycationic Al species (Al^{3+}, $Al(OH)^{2+}$) are more toxic than the monovalent hydroxide ($Al(OH)_2^+$). At pH 4.8 the former make up 84% of the total soluble Al, but this declines to 58% at pH 5.2 (Fig. 1A). The lower toxicity of pH 4.4 versus 4.8 could then, in turn, be explained if the divalent cation is more toxic than the trivalent cation.

However, it is important to emphasize that the two toxic mechanisms are probably not mutually exclusive. Under some circumstances, both may be equally active in producing gill damage whereas under others, one may predominate over the other. Factors determining the predominance of one over the other could include the pH or Ca^{2+} concentration of the external environment or simply the duration of exposure if there are major adaptive changes occurring at the surfaces of the gills. The observation by Wood et al. (1988) that respiratory distress becomes greater and ionic disturbance less when Ca^{2+} is elevated at constant pH and Al (pH 4.8/333 μg/L) lends support to the notion that Al can operate by more than one toxic mechanism and that its action is very much dependent on exposure conditions.

Protective Effect of Ca^{2+}

The range of water Ca^{2+} employed in the present study (0.025–0.4 mequiv/L) may not seem large but, in fact, it represents the full range found in waters sensitive to acidification (i.e. those with low calcium bicarbonate concentrations and therefore low acid neutralizing capacity, Harvey et al. 1981). Furthermore, this range is sufficient to provide a major protective effect. Based on present results, Ca^{2+} protects largely by reducing ion loss, thereby reducing mortality (Table 2; Fig. 3). Furthermore, Ca^{2+} is similarly antagonistic to low pH alone (Fig. 3, 6) and to Al, in combination with low pH (Fig. 3, 5).

The protective effect of Ca^{2+} is now well established in the toxicology literature as reducing the toxicity not only of H^+ and Al but of a number of other aquatic contaminants as well (e.g. Brown 1968; Alabaster and Lloyd 1980). The protection is thought to arise from weak ionic interactions between Ca^{2+} and surface ligands (membrane integral and peripheral proteins, mucopolysaccharides, anionic residues in intercellular cements). These interactions act to stabilize apical membranes and increase the tightness of intercellular tight junctions (reviewed by McDonald 1983b), thereby reducing permeability of the membrane and increasing its resistance to attack by surface-active toxicants. Therefore, much of the effect of Al and H^+ may be related to competitive interactions with Ca^{2+} for gill anionic sites. Furthermore, the result of increasing water Ca^{2+} may be to delay or reduce the binding of Al at these sites. Instead, high water Ca^{2+} may promote polymerization or precipitation of Al on the surface, since neither process would require prior binding of Al to gill anions. The end result may not necessarily be a change in the gill Al content so much as a difference in the form and reactivity of the Al present. Thus, one could explain the observation of greater respiratory and lower ion disturbance at high Ca^{2+} (Wood et al. 1988) if the result of this process was the formation of a thicker surface coat with a greater diffusion distance. The precise nature of Al and Ca^{2+} interactions at the gill surface is presently under investigation in our laboratories.

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