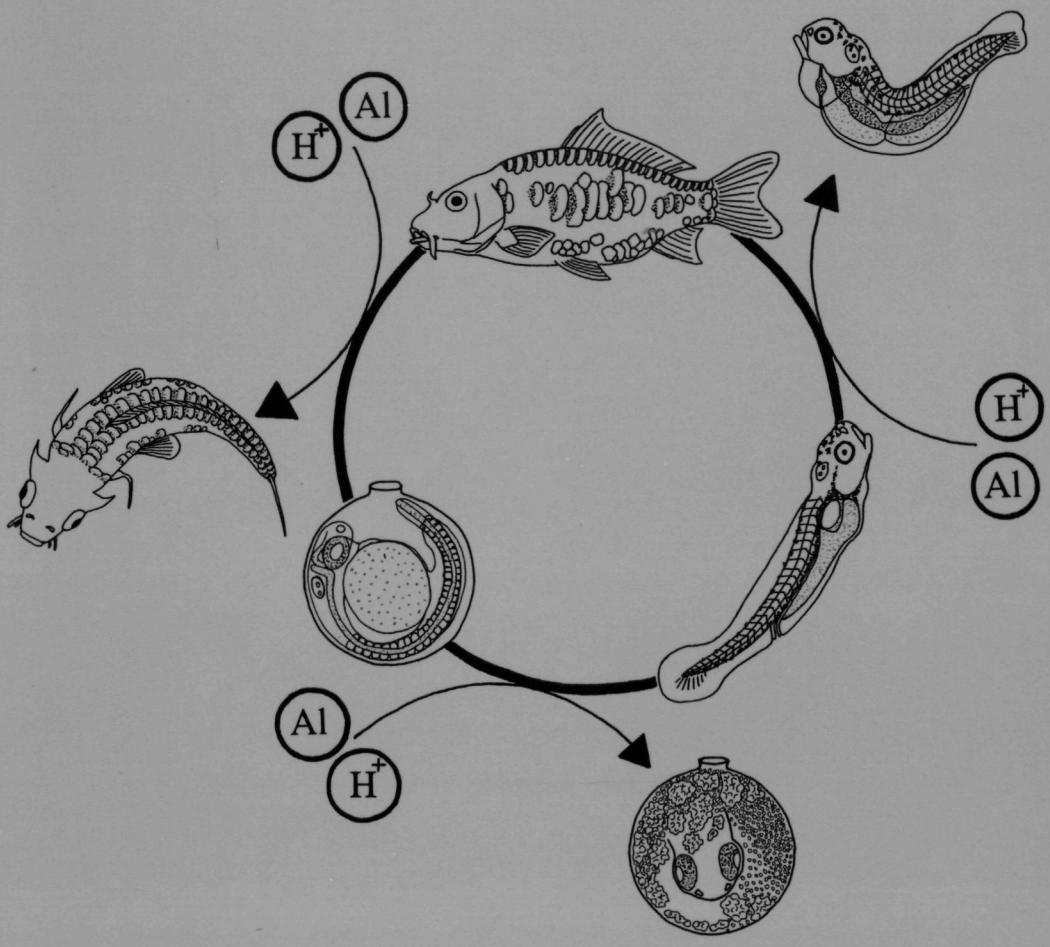
ALUMINIUM AND WATER ACIDITY

EFFECTS ON EARLY DEVELOPMENT AND JUVENILES OF THE CARP, *CYPRINUS CARPIO*



FERD OYEN

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EFFECTS ON EARLY DEVELOPMENT AND JUVENILES

OF THE CARP, *CYPRINUS CARPIO*

Een wetenschappelijke proeve op
het gebied van de natuurwetenschappen,
in het bijzonder de biologie

PROEFSCHRIFT

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FERDINAND GERARDUS FRANCISCUS OYEN
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Promotor: Prof. Dr. S.E. Wendelaar Bonga

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CHAPTER 1

General introduction and outline of the thesis

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General introduction

This study deals with the effects of water acidification and aluminium concentrations on embryos, larvae and juveniles of the common carp, *Cyprinus carpio*. Also, the protective effects of humic acids, natural substances which are known to form complexes with aluminium, have been investigated.

One of the consequences of a wasteful and energy consuming society is the emission of acidifying substances into the atmosphere. Emissions of sulphur and nitrogen oxides are caused by the burning of fossil fuels, while an intensive bio-industry is responsible for the release of large amounts of ammonia. After oxidation and nitrification, these emissions acidify the aquatic ecosystems. Acidification of fresh waters is defined as the decrease in acid-neutralizing capacity of the water (National Swedish Environment Protection Board, 1983; Leuven, 1988), and may result in a decrease of water pH. This buffering capacity is influenced by local geological and hydrological conditions. The drainage basins in mountainous areas of Scandinavia and North America are very vulnerable to acidification. They are characterised by relatively insoluble bedrock with little buffering capacity. Hence, the acid neutralizing capacities of waters in these regions are rapidly exhausted leading to a lowering of water pH. Therefore, it is no coincidence that most research on water acidification has been concentrated on this type of ecosystem, and on the salmonid fish that inhabit these waters.

Fish are vulnerable to low water pH. Exposure of salmonids to a sudden and severe drop in water pH may result in acute death. Failure of osmotic and ionic regulation, accompanied by blood acidosis, is considered the primary cause of this phenomenon (Fromm, 1980). Early life stages and smolts of salmonids are most susceptible to low pH (Daye and Garside, 1979; Kwain and Rose, 1985). Water acidity may inhibit hatching and increases egg and larval mortality (Carrick, 1979; Peterson *et al.*, 1980).

However, it has become clear that low water pH is not the only deleterious factor in acid water. A decrease in water pH is often accompanied by an elevation of the aluminium concentration. Aluminium has a relative atomic mass of 26.98. It is the most common metal and the third most abundant element (7-8 %) in the earth's crust. Aluminium compounds are found in almost all rocks and surface waters (Howells *et al.*, 1990). The release rate of aluminium from soils is dependent on the presence of mobile anions (Johnson and Cole, 1980), and on the exchange capacity with H^+ ions (Wollast *et al.*, 1984).

Aluminium concentrations in the water vary regionally. They reflect geological and hydrological characteristics, and the degree of soil weathering (Stumm and Morgan, 1981). Despite the abundance of aluminium, its concentration in most freshwaters is low as a consequence of the pH dependency of aluminium solubility. In the range of pH 5.5 to 8, aluminium solubility is very low, but at lower pH levels it rapidly increases (Smith and Hem, 1972). In acidified lakes in Scandinavia, aluminium concentrations of 3.7 to 29.6 μmol/l have been reported (Dickson, 1978; Overrein, 1980). In the Adirondacks in North America, 90 % of the lakes with a water pH of 5 or below have aluminium concentrations of 5.5 μmol/l or higher (Linthurst *et al.*, 1986). In waters with a pH lower than 5.5, dissolved aluminium can reach concentrations which are toxic to aquatic organisms. This implies that in most acid waters there are two factors that have a negative effect on the ecosystem: low pH and aluminium.

The toxicity of aluminium on aquatic life depends on the physical and chemical appearance of the metal in the water. When dissolved, aluminium can form organic and inorganic complexes which tend to polymerize (Driscoll and Schecher, 1988). The formation of various molecular species largely depends on pH, the presence of chelating substances, water temperature and time (Burrows, 1977; Leivestad *et al.*, 1987). Equilibria of Al^{3+} and its complexes are rapidly established (Tipping *et al.*, 1988). In the water, Al^{3+} forms soluble complexes with OH^-, F^-, SO_4^{2-} and organic ligands such as humic acids. Of these complexes, aluminium hydroxides prevail between pH 5 and 6. Different species of aluminium hydroxide exist, such as $AlOH^{2+}$, $Al(OH)_2^+$ and $Al(OH)_4^-$ (Driscoll and Schecher, 1988), while $Al(OH)_3$ has been hypothesized (Baes and Mesmer, 1976). Their relative dominance is pH dependent. The chemical speciation, complexation and changes in solubility of aluminium hydroxide are reflected in differences in toxicity at various pH levels. The presence of humic acids for example, and to a lesser extent of F^-, mitigate aluminium-hydroxide toxicity (Witters *et al.*, 1990). This complicates the study of the effects of aluminium on aquatic organisms.

Field and laboratory studies in North America and Scandinavia have shown that the high aluminium concentrations in waters of pH 4.8-6.0 have contributed significantly to the reduction of salmonid numbers in acid waters (Howells *et al.*, 1990). High aluminium levels can lead to excessive mucus production, resulting in gill clogging and reduction of gaseous exchange (Rosseland, 1980; Playle *et al.*, 1989). Aluminium may further cause the death of fish by disrupting branchial ion uptake. Although at pH levels < 4.8, aluminium may have some protective effect against water acidity, at higher pH levels it decreases plasma ion concentrations and reduces net influxes of Na^+ and Ca^{2+} (Muniz and Leivestad, 1980a,b; Rosseland and Skogheim, 1984; Verbost *et al.*, 1992). Aluminium may further reduce fish populations by recruitment failure. Not only low pH but also aluminium itself has a great impact on the early life stages. In particular emerging sac fry

and smolts of migrating salmonids are very susceptible (Howells *et al.*, 1990). However, at pH levels below 4.8 aluminium may protect embryos, as well as the adult fish, against the negative effects of water acidity. Remarkably, after hatching of the larvae this protective effect is reversed into a toxic one (Baker and Schofield, 1980). At pH > 4.8 (dependent on temperature and ionic strength of the water), aluminium increases egg mortality. Aluminium reduces larval growth and development (Cleveland *et al.*, 1986) and impairs mineralization of the skeleton.

With continuing acid deposition, also the water pH of more buffered aquatic ecosystems of Western Europe decreases. Like in Scandinavia and North America, this pH drop is often accompanied by an increase of aluminium levels. In Belgium, levels up to 30 μmol Al/l were encountered in humic moorland pools (Vangenechten and Vanderborght, 1980). In The Netherlands, the average aluminium concentration of moorland pools with a pH <4.5 was 27 μmol/l (Leuven, 1988). These ecosystems differ from those of North America and Scandinavia by containing more nutrients, and they may contain substantial amounts of organic substances. These include humic acids, which protect fish against aluminium toxicity (Witters *et al.*, 1990). The dominating fish species of these communities are mainly cyprinids. In contrast to salmonids, studies concerning the effects of aluminium on cyprinids are scarce. Their reproduction strategy is different from that of salmonids such as mating season (in late spring, avoiding snow melt events), a smaller egg size (less yolk) and a shorter incubation time.

In this thesis the common carp, *Cyprinus carpio,* was chosen as the test animal for acid and aluminium studies for the following reasons:

- It is considered to be a representative cyprinid, which implies that the results can contribute to the water quality criteria for the more nutrient-rich ecosystems in Western Europe.
- It is of great commercial importance to many countries in Eastern Europe and Asia.
- Its reproductive activity can be controlled efficiently, and fertilized eggs can be obtained throughout the year by hormonal therapy.

This study will focus on:

- The pH-dependency of aluminium toxicity to the early life stages of carp.
- The protective action of humic acids on the negative effects of low water pH and aluminium on the reproduction of carp.
- The mechanisms of action of low pH and aluminium on carp eggs.

This study may contribute to an understanding of the effects of water acidification and aluminium on freshwater fish populations of Western Europe. Furthermore, it could show to what extent the results of the studies on the early life stages of salmonids can be extrapolated to other fish families, such as the cyprinids.

Outline of the thesis

First, the influence of low water pH was determined on mortality, deformation rate, hatching time and rate of development of eggs and larvae (Chapter 2). As reported earlier, one of the primary effects of low pH on adult fish is disturbance of ion regulation. To investigate whether this also applies to the early life stages, we describe in Chapter 3 the effects of water pH on the uptake of Ca^{2+}, Na^{+} and Mg^{2+}, as well as the effects of humic acids on the impact of low pH.

In Chapter 4 we report on the effects of inorganic aluminium hydroxides on mortality, deformation rate and hatching time of eggs and larvae, and on the effect of complexation of aluminium by humic acids on the toxicity of this metal. We also discussed the pH dependency of the toxicity of inorganic aluminium to eggs by evaluating different models of aluminium interaction with gills.

Chapter 5 deals with the question whether embryonic deformation through water acidity and aluminium exposure is caused by, or resulted from, hatching time delay. Possible indirect effects of aluminium on deformation and hatching time, such as reduction of trunk movements and coagulation of the perivitelline fluid, were taken into consideration.

In Chapter 6 the effects of inorganic aluminium on larval ion uptake of Ca^{2+}, Na^{+} and Mg^{2+} are reported. We tested whether these aluminium effects are pH- and dose-dependent. Furthermore, we studied the adsorption of aluminium to embryo, chorion and larvae and whether humic acids could reduce aluminium-induced disturbances of ion uptake by the larvae.

Ion uptake in fish is dependent on the chloride cells in the integument. Since ion uptake in larvae was disturbed by inorganic aluminium, we investigated the effect of aluminium on these cells (Chapter 7).

Sensitivity of eggs to environmental stressors is partly determined by their quality, and thus by parental condition. Gamete production needs energy and is further dependent on the size of the fish. For this reason, we studied growth of juvenile carp during long-term exposure to different water acidity levels (Chapter 8). An effort was made to quantify the energy needed to compensate for negative effects of low water pH.

In Chapter 9 we report on the effects of low pH and inorganic aluminium on several physiological (haematocrit, plasma osmolarity) and morphological (number and size of gill mucous cells, number of opercular chloride cells) parameters of juvenile fish that are related to osmoregulation.

Finally, in Chapter 10 the major results are summarized and discussed. The responses to acid and aluminium exposure of carp are compared with those of other species, in particular salmonids. Recommendations on water quality criteria for carp are made.

References

Baes, C.F. and R.E. Mesmer, 1976. The hydrolysis of cations. J. Wiley, New York.

Baker, J.P. and C.L. Schofield, 1980. Aluminium toxicity to fish as related to acid precipitation and Adirondack surface water quality. Ecol. Imp. Acid Precip. Proc. Int. Conf., Sandefjord, Norway, March 11-14: 292-293.

Burrows, W.D., 1977. Aquatic aluminium: chemistry, toxicology and environmental prevalence. CRC Crit. Rev. Environ. Control 7: 167-216.

Carrick, T.R., 1979. The effect of acid water on the hatching of salmonid eggs. J. Exp. Biol. 14: 165-172.

Cleveland, L., Little, E.E., Hamilton, S.J., Buckler, D.R. and J.B. Hunn, 1986. Interactive toxicity of aluminium and acidity to early life stages of the brook trout. Trans Amer. Fish. Soc. 115: 610-620.

Daye, P.G. and E.T. Garside, 1979. Development and survival of embryos and alevons of the Atlantic salmon, *Salmo salar* L., continuously exposed to acidic levels of pH, from fertilization. Can. J. Zool. 57: 1713-1718.

Dickson, W., 1978. Some effects of acidification of Swedish lakes. Verein. Int. Verh. Limnol. 20: 851-856.

Driscoll, C.T and W.D. Schecher, 1988. Aluminium in the environment: 59-120 in: Metal ions in biological systems. Ed. H. Sigel, publ. Dekker, New York and Basel.

Fromm, P.O., 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Env. Biol. Fish. 5, 79-93.

Howells, G., Dalziel, T.R.K., Reader, J.P. and J.F. Solbe, 1990. EIFAC water quality criteria for european freshwater fish: report on aluminium. Chem. Ecol. 4: 117-173.

Johnson, D.W and D.W. Cole, 1980. Anion mobility in soils: Relevance to nutrient transport from forest ecosystems. Env. Int. 3: 79-90.

Kwain, W. and G.A Rose, 1985. Growth of brook trout *Salvelinus fontinalis* subject to sudden reductions of pH during their early life history. Trans. Am. Fish. Soc. 114. 564-570.

Leivestad, H.G., Hendry, G., Muniz, J.P. and E. Snekvik, 1976. Effects of acid precipitation on freswater organisms. Imp. Acid Precip. Forest and Freshwat. Ecosyst. Norway: 87-111. SNSF Project FR 6/76.

Leivestad, H., Jensen, E., Kjartansson, H. and L. Xingfu, 1987. Aqueous speciation of aluminium and toxic effects on Atlantic salmon. Ann. Soc. Roy. Zool. Belg. 117, (suppl. 1): 387-398.

Leuven, R.S.E.W., 1988. Impact of acidification on aquatic ecosystems in The Netherlands. Ph. D. thesis, University of Nijmegen, Toernooiveld 1, Nijmegen, The Netherlands: 181 pp.

Linthurst, R.A. *et al.*, 1986. Regional chemical characteristics of lakes in North America Part II: Eastern United States Water Air and Soil Poll. 31: 577-591.

Muniz, I.P. and H. Leivestad, 1980a. Acidification - effects on freshwater fish; in: Ecological Impact of Acid Precipitation, ed. D. Drablos and A. Tollan, Sandefjord, Norway, SNSF: 84-92

Muniz, I.P. and H. Leivestad, 1980b. Toxic effects of aluminium on the brown trout, *Salmo trutta* L. In: Ecological Impact of Acid Precipitation, ed. D. Drablos and A. Tollan, Sandefjord, Norway, SNSF: 320-321.

National Swedish Environment Protection Board, 1983. Ecological effects of acid deposition. Report and background papers of the expert meeting, I. Stockholm conference on the acidification of the environment. Report PM 1636: 340 pp.

Nelson, J.A., 1982. Physiological observations on developing rainbow trout, *Salmo gairdneri* (Richardson), exposed to low pH and varied calcium ion concentrations. J. Fish Biol. 20: 359-372.

Overrein, L.N., 1980. Acid precipitation - impact on the natural environment. In: Energy for our world 3, Proc. 11th. World Energy Conf., Munich: 570-583.

Peterson, P.H., Daye, P G. and J.L. Metcalfe, 1980. The effects of low pH on hatching of Atlantic salmon eggs. Proc. Int. Conf. Imp. Acid Precip. SNSF Project: p. 328.

Playle, R.S., Goss, G.G and C.M. Wood, 1989. Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminium exposures in soft water of two aluminium concentrations. Can. J. Zool. 67: 314-324.

Rosseland, B.O., 1980. Physiological responses to acid water in fish: 2, Effects of acid water on metabolism and gill ventilation in brown trout, *Salmo trutta* (L.), and brook trout, *Salvelinus fontinalis* (Mitchell). In: Ecological impact of acid precipitation. Ed. D. Drablos and A. Tollan, Sandefjord, Norway, SNSF: 348-349.

Rosseland, B.O. and O.K. Skogheim, 1984. A comparative study on salmonid fish species in acid aluminium-rich water. II. Physiological stress and mortality of one- and two-year old fish. Rept. Inst. Freshw. Res. Drottningholm 61: 186-194.

Skogheim, O.K., Rosseland, B.O. and I. Sevaldrud, 1984. Deaths of spawners of Atlantic salmon in River Ogna, S.W. Norway caused by acidified aluminium rich water. Rept. Inst. Fresw. Res. Drottningholm 61: 194-202.

Smith, R.W. and J.D. Hem, 1972. Effect of aging on aluminium hydroxide complexes in dilute aqueous solutions. Geological Survey Water-supply Paper 1827-D.

Stumm, W and J.J. Morgan, 1981. Aquatic Chemistry, J. Wiley & Sons, New York.

Tipping, E., Backes, C.A., and M.A. Hurley, 1988. The complexation of protons, aluminium and calcium by aquatic humic substances: a model incorporating binding-site heterogeneity and macroionic effects. Wat Res. 22: 597-611.

Vangenechten, J.D. and O.L.J. Vanderborght, 1980. Acidification of Belgian moorland pools by acid water sulphur-rich water; pp. 246-247 in: Ecological Impact of Acid Precipitation, eds. D. Drablos and A. Tollan, Sandefjord, Norway, SNSF.

Verbost, P.M., Lafeber, F.P.J.G., Spanings, T.A.T., Aarden, E.A. and S.E. Wendelaar Bonga, 1992. Inhibition of Ca^{2+} uptake in freshwater carp, *Cyprinus carpio*, during short-term exposure to aluminium. J. Exp. Zool. 262: 247-254.

Witters, H.E., Van Puymbroeck, S., Vangenechten, J.H.D. and O.L.J. Vanderborght, 1990. The effect of humic substances on the toxicity of aluminium to adult rainbow trout, *Oncorhynchus mykass* (Walbaum). J. Fish Biol. 37: 43-53.

Wollast, R., Chou, L., Debbaut, V., Wollast, A. and J. Zobrist, 1984. The influence of acid rains on the geochemistry of aluminium. Proc. Acid deposition and sulphur cycle. Brussels, 1984. 277-286.

CHAPTER 2

Effects of acid water on the embryonic development of the common carp.

F.G.F. Oyen, L.E.C.M.M. Camps, and S.E. Wendelaar Bonga

Effects of acid water on the embryonic development of the common carp.

Summary

Sensitivity of eggs and sac fry of the common carp (*Cyprinus carpio*) to pH levels in the range of 4.5 to 7.5 was assessed up till 117 h after fertilization. Studied parameters were rate of embryonic development, time and duration of hatching, and spinal cord deformation. In the pH range between 4.75 and 5.2 egg mortality was highest and a delay in the rate of embryonic development and hatching was observed in the surviving embryos. A strong increase of spinal cord deformation of sac fry occurred in the lower pH ranges (pH 4.75 - 5.5). Common carp appeared to be more sensitive to low water pH than many other species of fish.

Introduction

Numerous investigations have shown that acidification of poorly buffered aquatic systems leads to diminished fish populations (Fromm, 1980). This effect appears mainly due to recruitment failure (Leivestad et al., 1976). The dominant fish species in weakly buffered waters are mainly salmonids, and consequently studies on the effects of water acidification have primarily been focused on these fishes. For example, various studies on different trout species showed that eggs and larvae are more vulnerable to acid stress than adults (Daye and Garside, 1979; Rombough, 1982; Kwain and Rose, 1985). For trout, highest mortality occurred in the embryonic stages before retina pigmentation (Trojnar, 1977). In surviving embryos prolongation of the embryonic development occurs (Rask, 1984), delaying pre-hatching movements and time of hatching (Peterson and Martin-Robichaud, 1985), and increasing the risk of infection.

In western Europe the effects of acidification are also becoming more and more noticeable in moderately buffered waters (Leuven, 1988). These waters are mainly dominated by cyprinids. Effects of acidification on the reproductive success of this group are largely unknown. We therefore studied the effect of acid stress on the embryonic development of one of the most important cyprinids, the common carp (*Cyprinus carpio*), using the following parameters: rate of embryonic development, embryonic and larval death rate, hatching dynamics and spinal cord deformation.

Material and methods

Fertilization and incubation of eggs.

Carp gametes were obtained after artificial induction of ovulation and spermiation. To this end carp varying in weight between 1 and 1.5 kg were intramuscularly injected with a carp pituitary powder suspension (cps) in a 0.9 % NaCl solution. Females were first injected with 0.6 mg cps/kg fish, while males received 2.0 mg cps/kg fish. Twelve hours later, the females obtained an additional dose of 6.0 mg cps/kg fish. Twenty-four hours after the first injection, gametes of males and females were collected, mixed, and fertilization induced by addition of water. Immediately thereafter eggs were placed in the egg incubator. To avoid damage to the egg membrane, the routine chemical removal of the sticky layer of the eggs was omitted.

Two egg incubators were used to expose eggs and larvae to water of different pH. Both incubators contained 3 identical recirculation systems enabling us to vary the pH in each of the 6 systems separately. Each system consisted of 5 incubation units, containing a petridish suitable for rearing of about 200 eggs. The carp eggs adhered to the glass of the petridishes, thus facilitating observation during the experiments. The flow of water in every incubation unit was adjustable; and a sieve which covered the outlet prevented the escape of hatched larvae. The incoming water was sterilized with ultra violet light. No chemical disinfectants were used. In every system, the sterilization unit was surrounded by a thermal bath for temperature control (25 $\pm$ 0.2°C). Nine hours after fertilization, all non-fertilized eggs were removed. Nijmegen tap water (main ion concentration of the water in mmol/l: Ca, 0.8; Na, 1.9; K, 0.05; Cl, 3.1; Mg, 0.2) was used. The main ion concentrations of this water are within the normal range found in natural waters containing carp populations. The pH in the control system remained at 7.5. Experimental media were adjusted to pH 4.5, 4.75, 5.0, 5.08, 5.15, 5.35, 5.5 or 5.9 by adding diluted sulfuric acid (0.01 M). To maintain pH levels, a pH stat system (fluctuation 0.02 pH-units) was used. The CO_2, formed during the acidification of the test media was allowed to escape before the eggs were placed into the incubation units.

Rate of embryonic and larval development.

An embryonic development table of carp eggs was made under control conditions (pH 7.5, 25°C). Eggs were periodically examined under a dissection microscope (10-60x magnification) and the progressive embryonic stages described. During the first 20 h after fertilization, eggs were examined every 15 min; between 20 and 65 h every 20 min and between 65 and 125 h every 12 h. Effects of pH on eggs and larvae (n = 15) were

established by determination of the embryonic developmental stages every 12 hours. This was continued until 84 h after fertilization.

Determination of death rate, hatching dynamics and deformation.

During several experiments, eggs were exposed to water of pH 7.5 (control) and to pH 4.5, 4.75, 5.0, 5.08, 5.15, 5.35, 5.5 or 5.9. Each incubator contained one control group and two experimental groups. Every group consisted of 5 petridishes containing about 200 eggs each. Dead eggs and larvae were counted and removed every 12 h to prevent fungus growth, starting at 9 h and ending 117 h after fertilization. Eggs were considered dead when parts of the content turned opaque and white. At stages characterised by heart beat, its absence was used as criterium.

Hatching dynamics were studied in experimental groups submitted to media of pH 4.75, 5.0, 5.15, 5.35, or controls at pH 7.5. Every hour the percentage of hatched larvae was established for each petridish, starting at 50 h until 106 h after fertilization. Hatching was defined as rupture of the egg membranes by the tail. Fully as well as partially hatched larvae were counted. The hatching period was defined as the time span between hatching of the first and the last larvae. After hatching, some larvae appeared to have a deformed (curved) spinal cord.

Deformed larvae of the groups incubated at pH 4.75, 5.0, 5.5, 5.9, or 7.5 were counted immediately after hatching.

Data were analyzed for statistical significance with the Student t-test. Significance was accepted at the 5 % level.

Results

Effect of pH on the rate of embryonic and larval development.

Embryonic development of carp eggs was classified into the most important developmental stages at pH 7.5 (Table 1). These stages were used to compare the rate of egg and larval development in controls with those in acidified water (experimental group). The results are presented in Fig. 1. Compared to controls no difference in developmental rate of any of the experimental groups was observed during the first 15 stages. From stage 15 onward development was delayed at low pH. At pH 4.5 development stopped completely about 21 h after fertilization (stage 16). Embryos exposed to pH levels between 5.0 and 5.5 hatched at earlier developmental stages.

Table 1: Appearance of embryonic stages under control conditions (pH 7.5, temp. 25 $\pm$ 0.2 °C) for common carp, arranged in chronological sequence. Eggs were examined every 15 min during 0-20 h after fertilization; every 20 min during 20-65 h and every 12 h after 65 h.

Stage	Main developmental feature	Time after fertilization (h)
1	fertilization	0
2	2-cell stage	1.00
3	4-cell stage	1.15
4	8-cell stage	1.40
5	16-cell stage	2.00
6	32-cell stage	2.30
7	early morula	3.00
8	late morula	5.00
9	early gastrula (30 % invagination)	6.30
10	late gastrula (blastopore)	9.00
11	neurulation	12.00
12	chordation	14.00
13	4 somite pairs	15.00
14	16-18 somite pairs	17.00
15	26-28 somite pairs	20.00
16	ventriculation	21.20
17	otic capsule visible, start of embryonic movements	22.40
18	brain ventricles present	25.40
19	heart activity	26.40
20	pigmentation of the eye	30.00
21	blood circulation, otholiths	33.00
22	appearance of pectoral fins	34.00
23	melanophores appear on yolksac	35.00
24	melanophores appear on side in line	40.00
25	few melanophores appear in head region (20)	43.00
26	many melanophores appear in head region	45.20
27	angle between head and body axis is 70-80 degree; no blood vessel in pectoral fin	53.00
28	angle between head and body axis is approximately 30 degree; pectoral fin can move and contains vena subclavia	56.00
29	angle between head and body axis is approximately 19 degree; development of operculum and gill filaments; operculum covers the first three gill arches	60.00
30	operculum covers five gill arches; cloaca breaks through to external environment	65.00
31	swim bladder filled with air	77.00

Effect of pH on mortality, hatching dynamics and deformation.

Mean cumulative mortality percentages of eggs and larvae exposed to acid water after fertilization are shown in Fig. 2. Malfunction of the pH stat systems regulating the pH at pH 5.08 and 5.35 resulted into abortion of these experimental groups after 33 and 69 hrs respectively. Cumulative mortality increased progressively with decreasing pH. Death rate

after 21 h of exposure was already increased at pH 5.5 ($P \leq 0.05$). The highest mortality occurred during the first 45 h after fertilization. At pH 4.5 death rate reached 100 % 57 hours after fertilization. Fig. 3 presents the relationship between the mean cumulative mortality percentage at every sampling time and the different water pH levels. An exceptional increase in mortality at pH 5.0 was observed when compared to the group at pH 5.15. The 96LC50 value is pH 5.04. In Fig. 3 (insert) the death rate of the different pH groups per 12 h is shown. Mortality increased progressively with decreasing pH; the most sensitive stage appeared to be the first day after fertilization. Afterwards the death rates dropped almost to control levels, but increased again during and after hatching at pH 5.0 and 4.75. No eggs survived at pH 4.5, at which the death rate remained high after day one following fertilization.

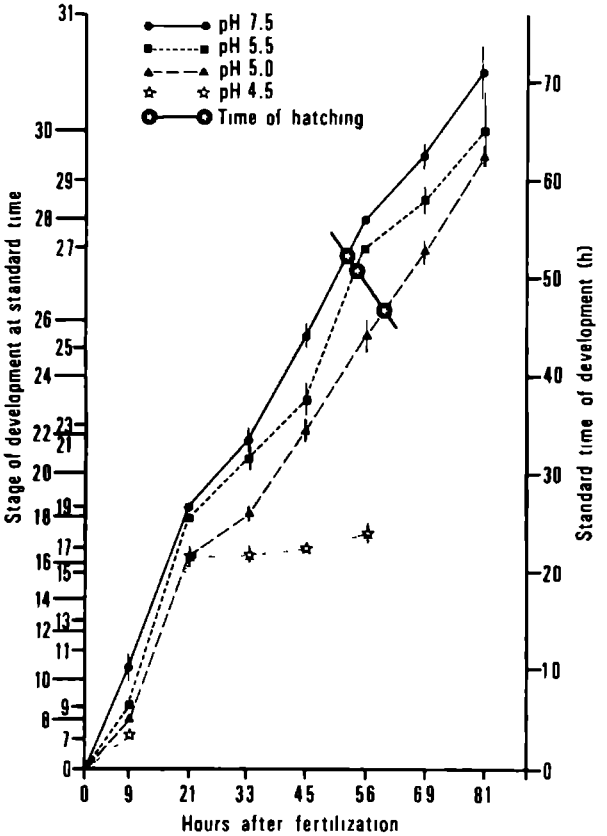


Fig. 1: The effects of acidification on the rate of development. Every point is the mean of 15 individual values; bars indicate minimum and maximum stages. The horizontal axis shows time in hours after fertilization, the vertical axis the serial numbers of the sequential stages, spatially arranged according the corresponding times of the standard development at 25°C.

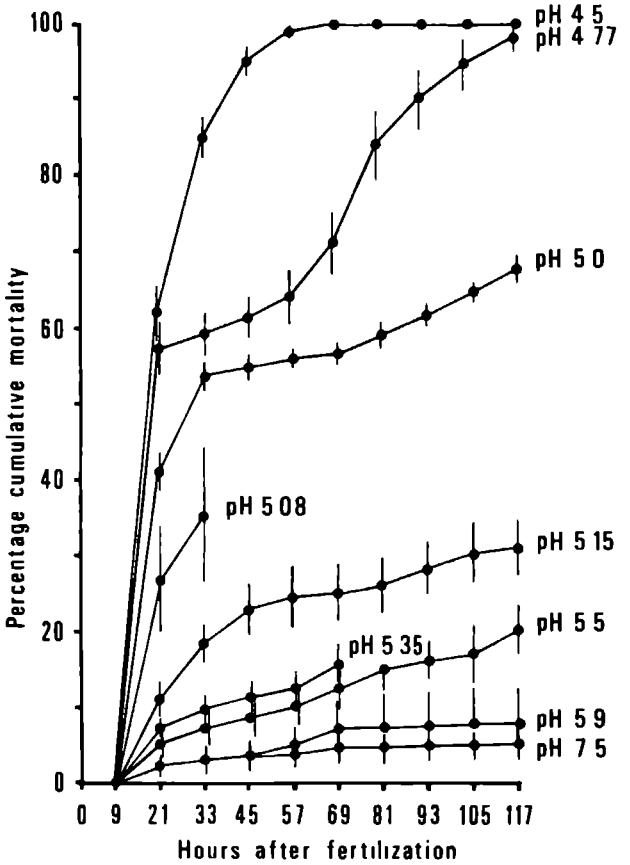


Fig. 2: Cumulative mortality percentages (means ± SD; n=5) as function of time after fertilization, shown for every experimental group.

Fig. 4 shows the relationship between the mean cumulative hatching percentages and hours after fertilization at pH 7.5, 5.35, 5.15 and 5.0. With decreasing pH, hatching was progressively delayed. After 58 h only 54 % of the eggs at pH 5.15 had hatched, while the hatching percentage at pH 7.5 was already 95 % ($P \leq 0.02$). According to Fig. 4 (insert), the delay of the 50 % hatching point was most prominent between pH 5.2 and 4.75. The 50 % hatching point at pH 4.75 was delayed with 45 % when compared to the controls. The delay in hatching time was much more pronounced during the later period of the hatching process than at earlier hatching periods. Apart from hatching time, duration of hatching also appeared to be affected by acid water. As shown in Fig. 5 the duration of the hatching increased with decreasing water pH below pH 5.35. At pH 5.15, this increase was significant ($P \leq 0.02$) when compared to pH 7.5.

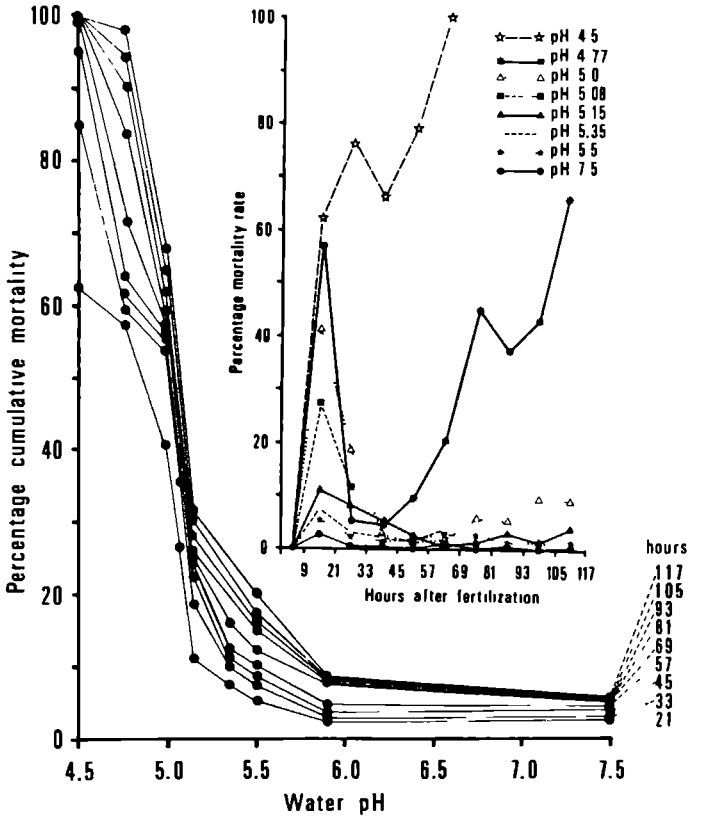


Fig. 3: Relationship between cumulative mortality (means; n=5) and pH of experimental groups at each sampling time. Inset: mortality rate/12 h (calculated values) related to time after fertilization, for each experimental group.

In Fig. 6 the percentage of deformed larvae is related to water pH. Between pH 5.5 and 4.75 a very strong increase in the number of hatched larvae with deformed spinal cord was apparent. This increase was already significant ($P \leq 0.001$) at pH 5.5. At pH 4.75, up to 65 % of the larvae were deformed.

Discussion

Water acidification below pH 5.2 appeared lethal to carp larvae. Already at pH 5.5 effects became apparent like delayed rate of embryonic development, increased mortality percentage, delayed hatching time, prolonged hatching process, and elevated deformation percentage. Negative effects of such relatively moderate levels of acidification on teleost fish are, in our opinion, unexpected. Contradictory observations have been reported for

salmonid species. For example Kwain and Rose (1985) observed delayed organogenesis of *Salvelinus fontinalis* embryos, only after hatching at pH 5.0. Furthermore, Daye and Garside (1979) did not find any influence of low water pH on the rate of development of Atlantic salmon (*Salmo salar*) at pH 6.8 - 3.7. Trojnar (1977) even found a more rapid development at pH levels lower than pH 5.0 for brook trout (*Salvelinus fontinalis*). Despite differences in experimental conditions, these data suggest that eggs of salmonids are less sensitive to acid water than those of common carp.

One might expect that the observed delay in hatching time parallelled the developmental delay, leading to hatching at the same stage in all the experimental pH groups. However, this was only partly the case. Delay in hatching time was less pronounced than the developmental delay. Larvae submitted to acid water did hatch at an earlier developmental stage than control larvae. This phenomenon has not been reported before. It would implicate that newly hatched carp larvae exposed to low pH are more sensitive to environmental stressors because of incomplete development.

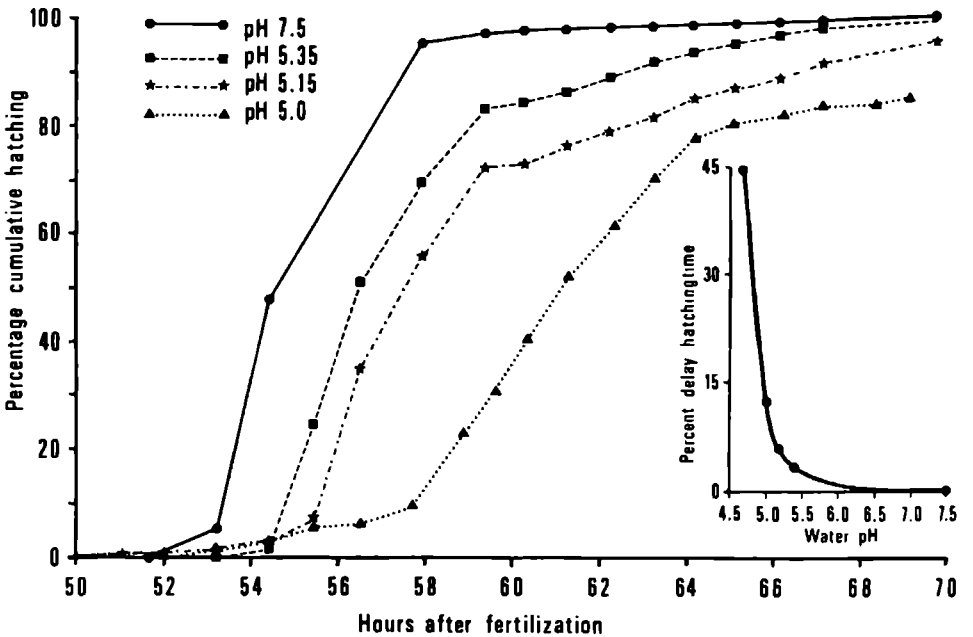
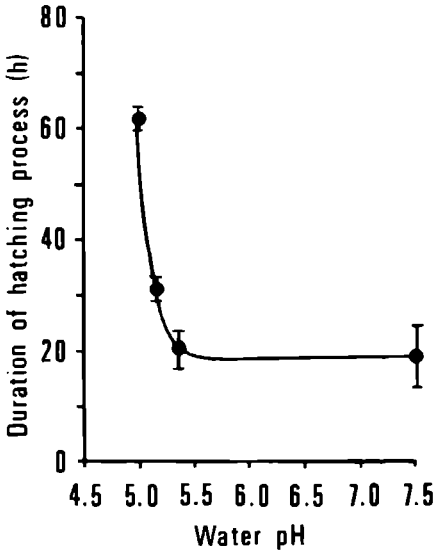


Fig. 4: Relationship between relative cumulative hatching percentage (means; n=5) and time after fertilization, at different pH values. Inset: 50 % hatching time delay relative to control (calculated values) versus water pH.



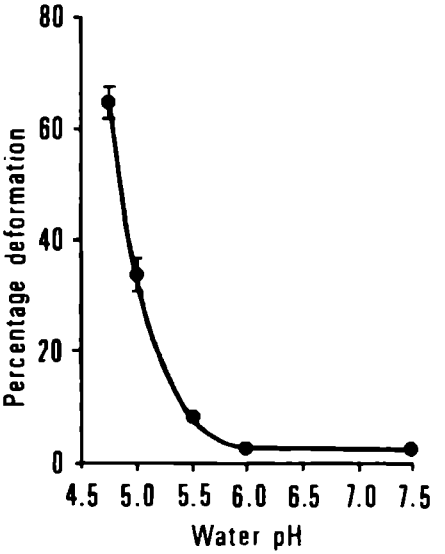


Fig. 5: Duration time of the hatching process (Means ± SD; n=5), related to water pH.
Fig. 6: Percentage spinal cord deformation (means ± SD; n=5) versus water pH.

We observed that the mortality increased progressively with increasing water acidity. The critical level was between pH 5.15 and 4.75. A very acid-sensitive period appeared to be the first stages after fertilization. This phenomenon has also been reported for other species of fish such as *Stizostedion vitreum*, *Catostomus commersoni*, *Coregonus clupaeiformis* and *Notropis cornutus* (Holtze and Hutchinson, 1989). In Table 2 critical pH levels for eggs and larvae of different species of fish are listed. Common carp seem to be more sensitive to acid water than other species of fish.

We found that both hatching time and hatching period of carp eggs were influenced by pH. Similarly, Rask (1983) found that eggs of *Perca fluviatilis* reared at pH 4.0 showed a delay in hatching time relative to that of the control. He also found a pH-dependent elongation of the hatching period. Delay in hatching time as a result of acid water was further found for *Pimephalus promelas* (Mount, 1973), *Salmo trutta fario* (Brown & Lynam, 1982) and *Salvelinus fontinalis* (Swarts et al., 1978). For *Salmo salar*, Peterson et al. (1980) found no delay in the hatching time upon exposure of the eggs to low pH immediately after fertilization. However, when eggs were exposed after the eye pigmentation stage, considerable hatching delay was observed. This was also the case for *Stizostedion vitreum* (Holze & Hutchinson, 1989) and *Salvelinus fontinalis* (Kwain & Rose, 1985). Carrick (1977) however, found no effect of acid water on the hatching time of eggs from *Salmo salar*, *Salmo trutta* and *Salmo trutta fario*. Nor did Menendez (1976) find such an effect for *Salvelinus fontinalis*. Trojnar (1977) found a reduced hatching time

Table 2: Lethal pH levels for eggs and larvae of different teleost species.

Fish species	Lethal pH	Reference
Alosa aestivalis	96LC50: 6.3	Klauda et al., 1987
Notropis cornutus	96LC50: 5.4	Holtze & Hutchinson, 1989
Cyprinus carpio	96LC50: 5.04	this manuscript
Stizostedion vitreum	96LC50: 4.9	Holtze & Hutchinson, 1989
Catostomus commersoni	96LC50: 4.7	Holtze & Hutchinson, 1989
Coregonus clupaeiformis	96LC50: 4.6	Holtze & Hutchinson, 1989
Jordanella floridae	5.0 - 6.0	Graig & Baksi, 1977
Morone saxilatis	5.5	Mehrle et al., 1984
Rutilus rutilus	4.7 - 5.6	Johansson & Kihlstrom, 1976
Cyprinus carpio	4.8 - 5.4	this manuscript
Micropterus dolomieu	5.1	Kane & Rabeni, 1987
Pimephalis promelas	4.5 - 5.2	Mount, 1973
Salmo gairdneri	4.75	Kwain, 1975
Salvelinus alpinus	4.6 - 5.0	Jagoe et al., 1984
Salvelinus fontinalis	4.5 - 5.0	Kwain & rose, 1985
Salmo salar	4.0 - 5.0	Peterson et al., 1980 Daye & Garside, 1979 Carrick, 1979
Salmo trutta	4.5	Carrick, 1979
Salmo trutta fario	4.5	Carrick, 1979
Perca fluviatilis	4.0 - 4.6	Rask, 1983; Leuven et al., 1987; Johansson & Millsbrink, 1976
Esox lucius	4.2 - 4.3	Johansson & Kihlstrom, 1975; Leuven et al., 1987

for eggs of *Salvelinus fontinalis* upon exposure to acid water. Thus, delay of hatching time as observed in carp has frequently been found for eggs of other fish species. This implicates that the eggs in acid water are longer vulnerable to predation than eggs in neutral water. Delay in hatching time has been attributed to the decrease in the activity of secreted chorionase by acid exposed larvae (Peterson et al., 1989). This enzyme has its optimum at pH 8.5 (Hagenmaier, 1974). Rask (1983) and Peterson et al. (1980) found that the pH of the perivitelline fluid drops within a few hours to nearly that of the incubation medium. Since the hatching enzyme is secreted into the perivitelline fluid, this pH drop may cause its inactivation. Peterson and Martin-Robichaud (1983) suggested that decreased trunk movements of embryos reared at low pH contributes to delayed hatching

through less efficient chorionase distribution, causing delayed rupture of the chorion. In our opinion pH dependent deformation of the larvae could result in weaker trunk movements which are less effective in rupturing the egg membrane. Furthermore, the perivitelline fluid became less fluid at very low pH levels, thereby impairing embryonic movements. These factors, in combination with the pH-dependent delay in rate of development, may very well be the main causes of hatching time delay of carp eggs.

We did not observe any difference in larval deformation percentage in the range from pH 7.5 to 5.9. Between pH 5.5 and 4.75 however, the percentage of deformation increased strongly (up to 65 %). Larval deformation at low pH was also found for *Pimephalus promelas* (Mount, 1973) and *Perca fluviatilis* (Runn et al., 1977), whereas for *Salmo salar* no relation between larval deformation and water pH was found (Daye and Garside, 1979; Peterson et al., 1989). Runn et al. (1977) have suggested that hatching delay coupled with the smaller inner egg volume (as a result of low pH) may cause larval deformation. We found that embryos were already deformed before the hatching started. This means that the deformation is not a result of the delay in hatching time. Also the degree of deformation appears stronger after increased delay of hatching. The deformed embryo may not be able to rupture the chorion at the right time, preventing the larvae from stretching and forcing its further growth within the egg shell.

We found that the critical pH level for carp eggs ranged between 4.75 and 5.2, which is about 1 pH unit higher than the critical value for juveniles of about 30 g (personal observations). Although extrapolation from the laboratory to the field situation requires caution, it appears probable that carp populations in nature are already seriously threatened at pH 5.2. In addition, at this pH aluminium exerts maximum toxicity (pH 5.2 - 5.4; Baker, 1982). Since acidification is often accompanied by elevated levels of aluminium in natural water bodies (Driscoll, 1980), the lowest pH limit where successful reproduction can be expected in nature may even be higher than pH 4.75 - 5.2. This is currently under investigation.

References

Baker, J.P. 1982. Effects on fish of metal associated with acidification. in: Johnson, R.E. (Ed.). Acid rain/Fisheries. Proc. Int. Symp. Acid Precip. Fish. Imp., American Fisheries Society, Bethesda, Maryland: 165-175.

Brown, D.J.A. and S. Lynam. 1981. The effect of sodium and calcium concentrations on the hatching of eggs and the survival of the yolk sac fry of brown trout, *Salmo trutta* L. at low pH. J. Fish Biol. 19: 205-211.

Carrick, T.R. 1979. The effect of acid water on the hatching of salmonid eggs. J. Fish Biol. 14: 165-172.

Crai, G.R. and F.W. Baksi. 1977. The effects of depressed pH of flagfish reproduction, growth and survival. Water. Res. 11: 621-626.

Daye, P.G., and E.T. Garside. 1979. Development and survival of embryos and alevins of the Atlantic salmon, *Salmo salar* L., continuously exposed to acidic levels of pH, from fertilization. Can. J. Zool. 57: 1713-1718.

Daye, P.G., and E.T. Garside. 1975. Lethal levels of pH for brook trout, *Salvelinus fontinalis* (Mitchell). Can. J. Zool. 639-641.

Driscoll, C.T. 1980. Chemical characterization of some dilute acidified lakes and streams in the Acirondack Region of New York State. Ph. D. thesis, Cornell Univ., Ithaca, New York.

Fromm, P.O. 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Env. Biol. Fish. 5: 79-93.

Hagenmaier, H.E. 1974. The hatching process of fish embryos-IV. The enzymological properties of a highly purified enzyme (chorionase) from the hatching fluid of the rainbow trout, *Salmo gairdneri* Rich. Comp. Biochem. Physiol. 49B: 313-324.

Holtze, K.E. and N.J. Hutchinson, 1989. Lethality of low pH and Al to early life stages of six species inhabiting Precambrian Shield waters in Ontario. Can. J. Fish. Aquat. Sci. 46: 1188-1202.

Jagoe, C.H., T.A. Haines, and F.W. Kircheis. 1984. Effects of reduced pH on three life stages of sunapee char *Salvelinus alpinus*. Bull. Environ. Contam. Toxicol. (1984) 33: 430-438.

Johansson, N. and J.E. Kihlstrom. 1975. Pikes shown to be affected by low pH values during first weeks after hatching. Environ. Res. 9: 12-17.

Johansson, N. and G. Milbrink. 1976. Some effects of acidified water on the early development of roach, *Rutilus rutilus* L. and perch, *Perca fluviatilis* L. Water Res. Bull.12, 39-48.

Kane, D.A. and C.F. Rabeni. 1987. Effects of aluminium and pH on the early life stages of smallmouth bass (*Micropterus dolomieu*). Water Res. 21: 633-640.

Klauda, R.J., R.E. Palmer, and M.J. Lenkevich. 1987. Sensitivity of early life stages of blueback herring to moderate acidity and aluminium in soft freshwater. Estuaries 10: 44-53.

Kwain, W. 1975. Effects of temperature on development and survival of rainbow trout, *Salmo gairdneri*, in acid waters. J. Fish. Res. Bd Can. 32: 493-497.

Kwain, W. and G.A. Rose. 1985. Growth of brook trout *Salvelinus fontinalis* subject to sudden reductions of pH during their early life history. Trans. Am. Fish. Soc. 114: 564-570.

Leivestad, H., G. Hendrey, J.P. Muniz, and E. Snekvik. 1976. Effects of acid precipitation on freshwater organisms. Imp. Acid Precip. Forest and Freshwat. Ecosyst. Norway: 87-111. SNSF Project FR 6/76.

Leuven, R.S.E.W. 1988. Impact of acidification on aquatic ecosystems in the Netherlands. Ph D. thesis, Catholic University Nijmegen, The Netherlands.

Leuven, R.S.E.W., S.E. Wendelaar Bonga, F.G.F. Oyen and W. Hagemeijer. 1987. Effects of acid stress on the distribution and reproductive means of freshwater fish in Dutch waters. Ann. Soc. R. Zool. Belg./Ann. K. Belg. Ver. Dierkd. 117: 231-242.

McDonald, D.G. 1983. The effects of H^+ upon the gills of freshwater fish. Can. J. Zool. 61: 691-703.

Mehrle, P.M., D. Buckler, S. Finger, and L. Ludke. 1984. Impact of contaminants on striped bass. U.S. Fish and Wildlife Service, Columbia National Fisheries Research Laboratory, Interim Report. Columbia, MO.

Menendez, R. 1976. Chronic effects of reduced pH on brook trout (*Salvelinus fontinalis*). J. Fish Res. Bd Can. 33: 118-123.

Mount, D.I. 1973. Chronic effect of low pH on fathead minnow survival, growth and reproduction. Water Res. 7: 987-993.

Peterson, R.H., P.G. Daye, and J.L. Metcalfe. 1980. The effects of low pH on hatching of Atlantic salmon eggs. Proc. Int. Conf. Imp. Acid Precip. 328. SNSF Project.

Rask, M. 1983. The effect of low pH on perch, *Perca fluviatilis* L. I. Effects of low pH on the development of eggs of perch. Ann. Zool. Fennici 20: 73-76.

Rask, M. 1984. The effect of low pH on perch, *Perca fluviatilis* L. I. Effects of low pH on the development of eggs of perch. Ann. Zool. Fennici 21: 9-13.

Rombough, P.J. 1982. Effects of low pH on eyed embryos and alevins of Pacific salmon. Can. J. Fish. Aquat. Sci. 40: 1575-1582.

Runn, P., N. Johansson, and G. Milbrink. 1977. Some effects of low pH on the hatchability of eggs of perch, *Perca fluviatilis* L. Zoon 5: 115-125.

Srivastava, R.S. and P. Dwivedi. 1979. Studies on the effects of acidic medium on the fry of *Cyprinus carpio*. Acta Hydrochem. Hydrob. 7: 421-430.

Swarts, F.A., W.A. Dunson and J.E. Wright. 1978. Genetic and environmental factors involved in increased resistance of brook trout to sulphuric acid conditions and mine acid polluted waters. Trans. Am. Fish. Soc. 107: 651-677.

Troynar, J.R. 1977. Egg hatchability and tolerance of brook trout (*Salvelinus fontinalis*) fry at low pH. J. Fish. Res. Bd Can. 34: 575- 579.

Wendelaar Bonga, S.E. and L.H.T. Dederen. 1986. Effects of acidified water on fish. Endeavour 10: 198-202.

CHAPTER 3

Effects of acid water and humic acids on mortality, development and body ion concentrations of sac fry of the common carp

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Abstract

The effects of seven different water acidity levels, ranging from pH 7.5 to pH 4.5, were studied on the eggs and larvae of *Cyprinus carpio*. In addition, the effects of humic acids at these pH levels were examined. The 96-h death rate, the rate of body deformation, as well as the body dry weight and the total body concentrations of Ca, Na and Mg were determined in developing embryos and larvae.

At pH 7.5, whole body Ca, Na and Mg concentrations increased with respectively 500 %, 62 % and 27 % in the time between hatching and yolk-sac absorption, while the dry body weight decreased with 32 % during the same period. At pH 5.2 and lower, the mortality and deformation rate increased. Effects on the dry body weight of the larvae were not observed. Total body Ca and Na decreased at lower pH levels, while Mg increased. The presence of humic acids did not affect the above parameters.

Introduction

When fish are exposed to acid water, the efflux of ions increases, while the active uptake of ions from the surrounding water decreases (McDonald, 1983; Battram, 1988; Playle *et al.*, 1989). The resulting disturbance of ion homeostasis is one of the major causes of death of fish in acid water (McDonald, 1983; Battram, 1988).

Water acidity may also affect fish populations in a more serious way, namely by means of inducing recruitment failure (Rask, 1984). This may occur at pH levels much higher than those inducing fish kills. It is caused by the high sensitivity of developing eggs and larvae as a result of the high surface-volume ratio and the high intensity of the metabolic processes of these early life stages, including mineralisation of the skeleton (Zeitoun *et al.*, 1976; Talbot *et al.*, 1982). In salmonids ion regulation of eggs and larvae may also be affected by low pH (Peterson and Martin-Robichaud, 1986; Wood *et al.*, 1990).

In this chapter we investigated the effects of water acidity on ion homeostasis of eggs and larvae of the common carp. As parameters for the effects of low water pH we used mortality, skeletal deformation and the accumulation of Ca, Mg and Na.

We have studied the eggs and larvae in acid water in the presence and in the absence of humic acids. Humic acids are characterised by a strong ability to interact with metal ions, including Ca^{2+}, and are known to protect fish against toxic metals such as aluminium. Most studies on the effects of humic acids relate to observations on aluminium-containing

water. It is well known that humic acids bind aluminium and in this way reduce the toxicity of aluminium-containing water of low pH (Witters *et al.*, 1990). Studies on the effects of humic acids on the impact of acid water on fish are not available. Humic acids could modify the effect of low water pH on fish by binding Ca^{2+} ions. Water Ca^{2+} ions are protective against water acidity (Brown and Lynam, 1981; Nelson, 1982; Brown, 1982). Although the binding constants of these organic acids for Ca^{2+} are low, in particular at low pH (Schnitzer, 1969), humic acids could negatively affect the availability of Ca^{2+} in water. It is possible therefore that the presence of organic acids increases the impact of water acidity on eggs and larvae.

Material and methods

Fertilization and incubation of eggs.

Eggs and sperm of carp were obtained as described in Chapter 2. The incubator systems were the same as described in Chapter 2.

Exposure conditions

Exposure of the eggs and larvae to the experimental conditions took place immediately after fertilization until 168 h afterwards. One group of eggs was exposed to water of the following pH: 7.5, 5.9, 5.4, 5.2, 5.0, 4.8 and 4.5. Another group of eggs was simultaneously exposed to water of the same pH levels containing humic acids (Fluka AG, CH-9470 Buchs). This exposure experiment was repeated 3 times.

The pH levels of the experimental media were obtained by adding dilute sulfuric acid (0.01 M). A pH stat system (fluctuation 0.02 pH units) was used to maintain a proper pH. The CO_2 formed during the acidification of the media was allowed to escape before the eggs were placed into the incubation units.

Humic acids were first dissolved in 0.1 M potassium hydroxide, and then added to the water at a concentration of 33 mg/l. The concentration of humic acid was checked every 24 h by monitoring the light absorption at 450 nm, and adjusted when necessary. The light absorption by humic acids was corrected for the influence of low pH.

The medium used in the experiments was prepared by adding salts to demineralized water to avoid possible effects of aluminium present in the tap water. This water contained in mmol/l: NaCl, 3.8; KCl, 0.06; $CaCl_2$, 0.8; $MgSO_4$, 0.2 and $NaHCO_3$: 0.335. These ion concentrations are within the normal range found in natural waters containing carp populations.

Parameters and analyses

The observed parameters were 96-h mortality percentage, deformation percentage, body weight (dry weight basis), and total body concentrations of Ca, Mg and Na.

Mortality was checked every 24 hours until the end of the experiment. Dead eggs and larvae were removed to prevent fungal growth. Eggs were considered dead when parts of the yolk content or the embryo itself turned opaque and white. For stages characterised by heart beat, its absence was used as a criterium.

The criterium for body deformation was a curved spinal cord. This phenomenon was often accompanied by other anomalies. The percentage deformation was determined after total hatching. When hatching did not occur, four groups of 25 larvae were prepared out of the egg by removing the chorion mechanically. After giving the larvae the opportunity to stretch, the deformation percentage could be established.

To determine their dry weight four times 25 embryos or larvae were collected at 48 h, 72 h (around hatching time), 116 h and 168 h after fertilization. They were dried at 70°C for 24 h. At 48 h after fertilization, and at later sampling-times in case hatching was delayed, the embryos were removed from the eggs.

Total body Ca, Mg and Na concentrations of embryos and larvae were calculated on a dry weight basis. Samples were destructed for 24 h at 70°C in 65 % NHO_3 and after dilution analyzed with an Inductively Coupled Plasma Atomic Emission Spectrometer (Instrumentation Laboratory, plasma 200).

Statistics and mathematics

The significance of effects of pH was tested against the pH 7.5 control group by means of the Mann-Whitney-U test. ANOVA was used for testing possible effects of humic acids in water of different pH against controls from water of the same pH without humic acids.

Results

Temporal changes

Fig. 1 shows the changes in body ion concentrations (dry weight basis) of Ca, Na and Mg, and of the dry weight per embryo or larvae. Between 48 and 72 hours after fertilization, around hatching time, only minor changes occurred. In the period after hatching (72-168 h after fertilization) the Ca and Na concentrations increased by 500 and 62 % respectively. The body concentrations of Mg appeared to increase slightly when compared to those of Ca and Na. During the same period, the dry body weight decreased

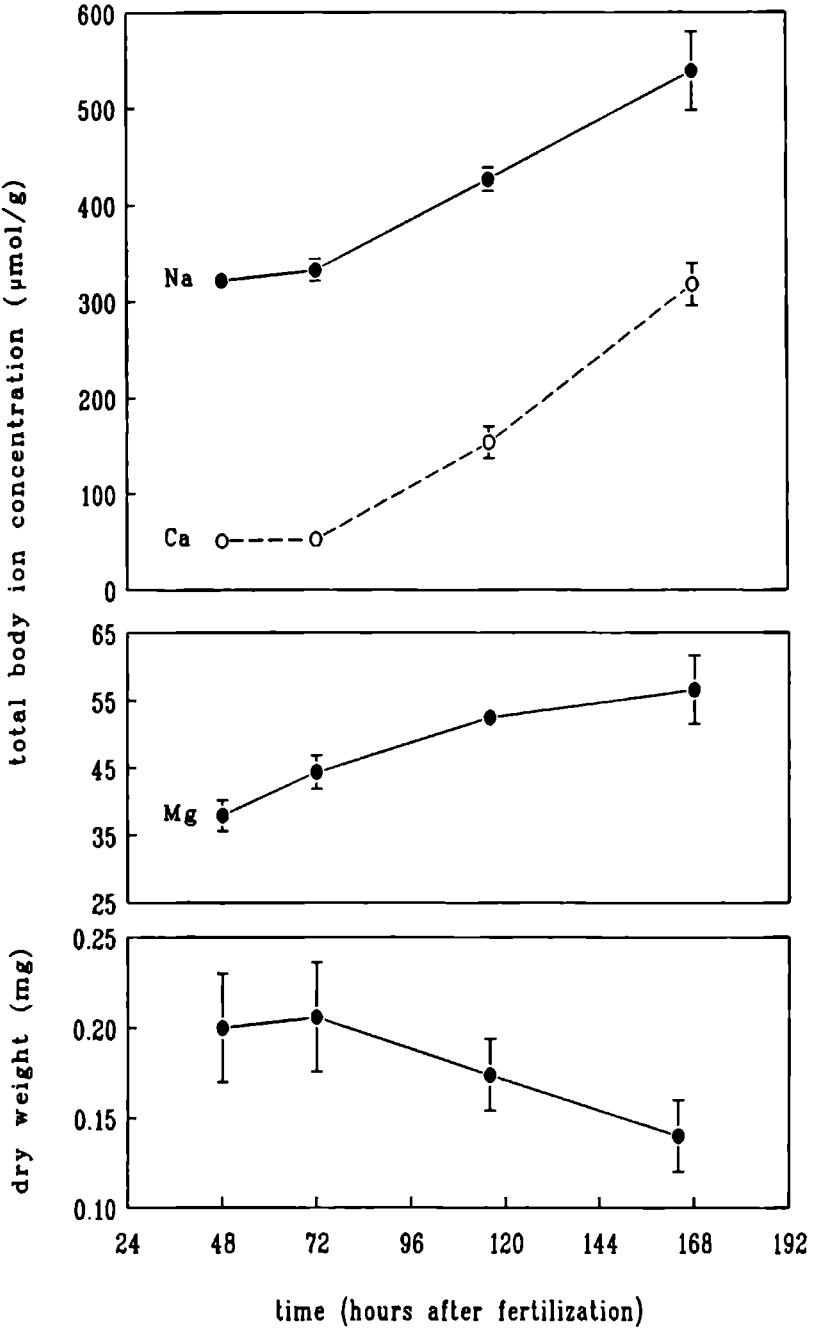

Fig. 1: Temporal changes of total body concentrations of Ca, Na and Mg (µmol/g dry weight) and dry body weight (mg) of eggs and larvae (mean values ± SD; n = 16).

with 32 %. The increase of the Ca and Na concentrations could not be explained by a loss of body weight, and must therefore be attributed to an active uptake from the environment. In contrast, the concentration of Mg increased only 27 %, which can fully be explained by the loss of weight of the larvae during development. On an individual basis, there was even a loss of Mg^{2+} to the environment (14 % at 168 h).

Influence of pH and humic acids

For each parameter studied, the pH dependency with and without humic acids is shown in Figures 2-7. Total body concentrations of Ca, Na and Mg as well as dry body weight were determined at 168 h after fertilization. The data for pH 4.5 are lacking because of the high death rate.

Effects on 96-h mortality.

As shown in Fig. 2, 96-h mortality increased strongly at pH 5.2 and lower. At pH 4.5., 94 % of all eggs died. Addition of humic acids did not influence the mortality of the eggs and larvae.

Effects on deformation %.

The deformation % of the larvae also showed a strong increase at pH levels lower than 5.4 (Fig. 3). Again, humic acids had no effect.

Effects on dry body weight at 168 h.

Neither low pH nor humic acids had any influence on the dry body weight at 168 h after fertilization (Fig. 4).

Effects on total body Ca.

Concentrations of total body Ca at 168 h after fertilization significantly ($P < 0.005$) dropped with about 25 % at pH levels of 5.2 and lower (Fig. 5). Uptake of Ca^{2+} was not affected by the presence of humic acids.

Effects on total body Na.

Exposure of eggs to pH 4.8 resulted in a total body Na concentration of the larvae which was only 67 % of that of larvae reared at pH 7.5. Fig. 6 clearly shows a positive relationship between total body Na concentrations and water pH. Humic acids had no effect.

Effects on total body Mg.

Mg increased when eggs and larvae were exposed to low pH (Fig. 7), but this effect was only apparent at the lowest pH level studied.

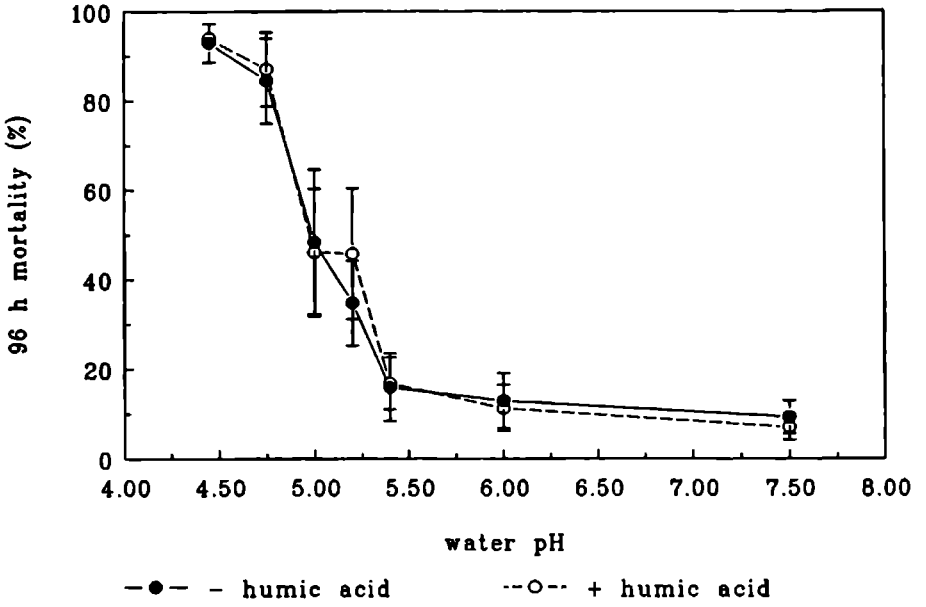

Fig. 2: Relationship between 96-h mortality (%) and pH in the presence or absence of humic acids (mean values ± SD; n = 12).

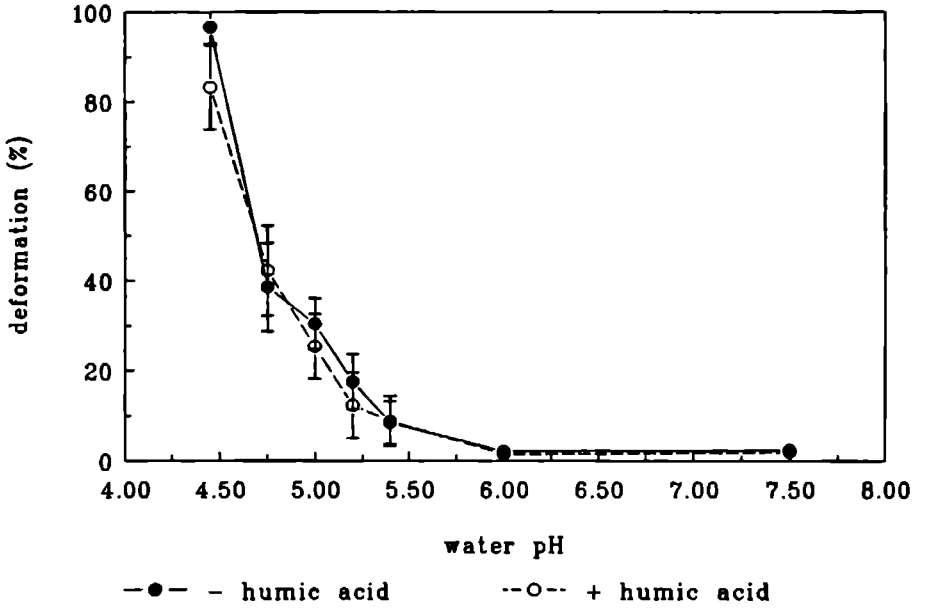

Fig. 3: Relationship between spinal cord deformation (%) and water acidity in the presence or absence of humic acids (mean values ± SD; n = 12).

Temporal changes under control conditions (pH 7.5)

At control conditions, loss of dry body weight (32 %) of carp sac fry occurred only at the time between hatching and the end of yolk sac absorption. This is normal for sac fry and is caused by the conversion of yolk into body tissues. Natochin *et al.* (1976) reported a similar drop for the larvae of the Russian sturgeon (*Acipenser guldenstadti*). A 20 % reduction of dry body weight in the sac fry period was described for rainbow trout (*Oncorhynchus mykiss*; Zeitoun *et al.*, 1976). Wood *et al.* (1990a) observed in brook trout (*Salvelinus fontinalis*) that the wet body weight declined with 20 % and the dry weight with 60 % in the time period of 70-111 days after fertilization.

During early development, a rapid mineralization of the body takes place as a result of an intensive uptake of ions from the water (Zeitoun *et al.*, 1976; Talbot *et al.*, 1982). However, when calculating the uptake in terms of mineral concentration per unit of dry body weight, one has to take into account the mass losses of the organism during development. Organic matter is reduced during yolk conversion, which leads to a relative increase of mineral content per unit of dry body weight.

Uptake of Ca^{2+} out of the water by embryos was negligible when surrounded by the chorion. This is in line with Zeitoun *et al.* (1976), who also found that Ca^{2+} uptake before hatching was almost zero. In contrast, during the yolk sac stage we observed a 500 % increase of the total body Ca concentration (on dry weight basis), which could not be explained by the loss of dry body weight. Gain of Ca in the period after hatching was also reported by Natochin *et al.* (1976) for larvae of the Russian sturgeon; by Gunn and Noakes (1987) for sac fry of lake trout (*Salvelinus namaycush*) (57 %); by Zeitoun *et al.* (1976) for sac fry of rainbow trout (230 % on individual basis) and by Wood *et al.* (1990a) for fry of brook trout (165 % on wet weight basis). We therefore conclude that, like other fish species, sac fry of carp use the water and not the yolk as their principal source for Ca^{2+} uptake. In accordance with adult fish, most of this Ca^{2+} is needed for the calcification of the skeleton.

Prior to hatching no net gain of Na was registered. This is in line with the results of Eddy and Talbot (1982), who found that the Na balance of the eggs of Atlantic salmon, *Salmo salar* is only slightly positive until hatching. In our work, uptake of Na^{+} by carp larvae did occur after hatching, but was less pronounced than Ca^{2+} uptake. The Na gain on dry weight basis was only 60 %, slightly more than could be explained by the decrease in body weight. In the Russian sturgeon, Na levels declined during the first 2 days after fertilization, remaining constant until hatching, and increased again during the yolk sac stage after hatching. This increase surpassed that of Ca (Natochin *et al.*, 1976).

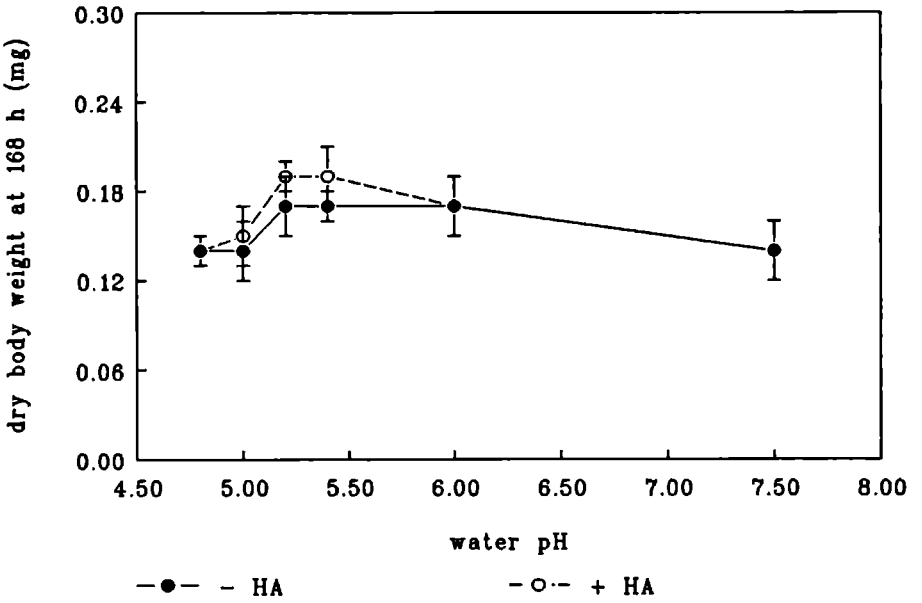

Fig. 4: Dry body weight at 168 h after fertilization (mg) related to water pH in the presence or absence of humic acids (mean values ± SD; n = 12).

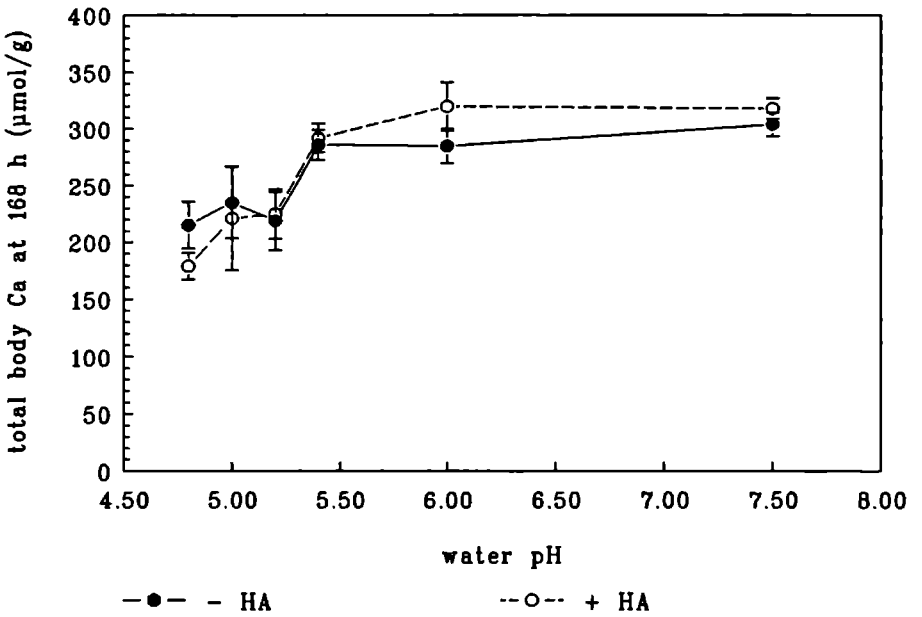

Fig. 5: Relationship between total body Ca concentration (µmol/g dry weight) and water pH in the presence or absence of humic acids (mean values ± SD; n = 12).

A similar development-dependent pattern of Na levels was described by Zeitoun *et al.* (1976), who found a Na gain of 200 % in sac larvae of rainbow trout. Reader *et al.* (1988) reported a gain of Na in sac fry of brown trout (*Salmo trutta*) and Wood *et al.* (1990a) a gain of 132 % for brook trout larvae in the period between 70 and 111 days after fertilization. No gain was found by Gunn and Noakes (1987) for lake trout. In general, one can conclude that no net Na^+ uptake from the water in the pre-hatching stages occurs. After hatching, the water significantly contributes to the Na requirements of the larvae in the yolk absorbing stage. The active uptake of ions like Ca^{2+} and Na^+ at this stage could be facilitated by the chloride cells in the integument, which are already present at hatching (Chapter 7).

During 48-168 h after fertilization, total body Mg concentrations increased only by 27 %. Between 48 and 72 h this increase was also noticeable per animal, but after hatching (72 h) Mg was lost to the environment.

In the pre-hatching period we concluded that the embryo obtained Mg^{2+} from the ambient water through the chorion. This is in line with earlier observations in our laboratory. An increase of 93 % of the Mg content of carp eggs in the period from 6 h after fertilization to just prior to hatching was found by Van der Velden *et al.* (1991). They concluded that the amount of Mg in the yolk sac was not sufficient for development, and that at the time before hatching, Mg^{2+} uptake from the external environment was required. Their results on carp are in conflict with the general opinion that Mg is available in excess in the yolk. Perhaps, the gain in the Mg concentration of the eggs can partly be attributed to the perivitelline fluid, instead of to the embryo itself.

In most cases described in the literature, Mg levels in the pre-hatching stage are constant, whereas after hatching a decline is measured (Zeitoun *et al.*, 1976; Natochin *et al.*, 1976; Gunn and Noakes, 1987; Reader *et al.*, 1988; Wood *et al.*, 1990a). These authors generally considered the total body Mg concentration as a marker for the development stage, because this concentration is influenced by development-dependent parameters as body water content and loss of organic matter. They further suggested that during larval stages Mg is stored in excess in the yolk, and uptake from the surrounding water may occur only via the food.

Effects of low water pH

Low water pH causes mortality and induces a high rate of body deformation in carp embryos and larvae. This is in agreement with our earlier results (Chapter 2) and marks carp as relatively sensitive to water acidification.

We observed no effects of low pH on the dry body weight at the end of the experiment. Our observations on carp larvae are in line with those of others. For example,

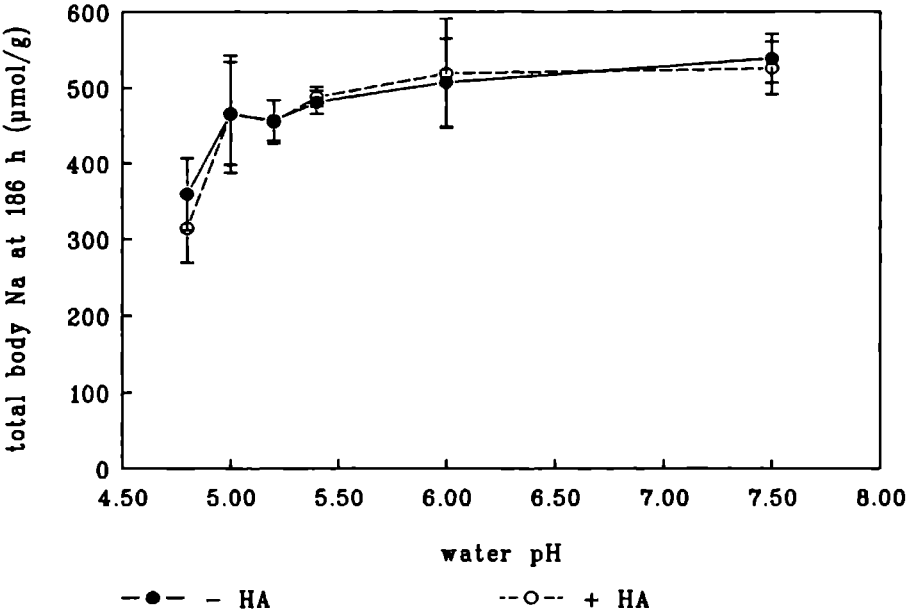

Fig. 6: Relationship between the total body Na concentration (µmol/g dry weight) and water pH in the presence or absence of humic acids (mean values ± SD; n = 12).

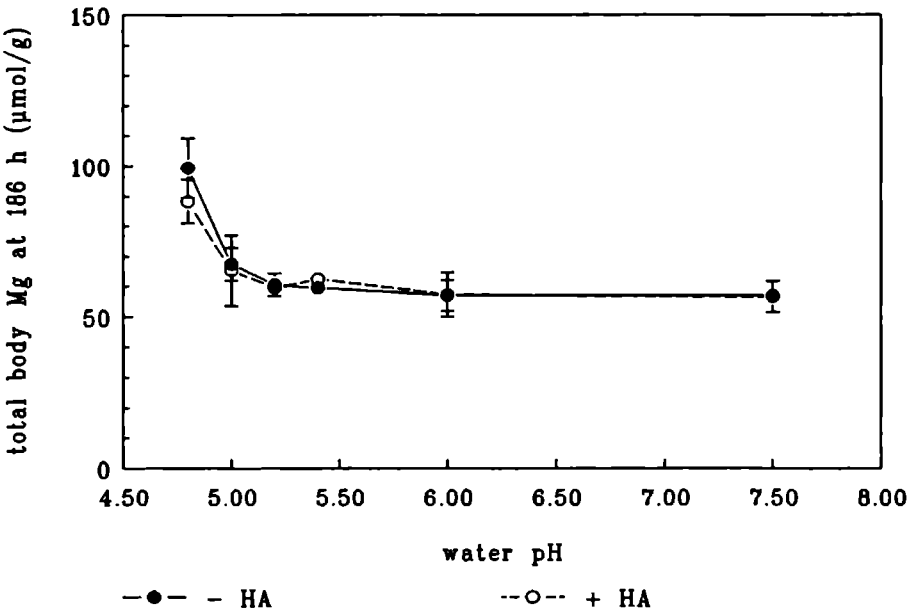

Fig. 7: Relationship between total body Mg concentration (µmol/g dry weight) and water pH in the presence or absence of humic acids (mean values ± SD; n = 12).

exposure of sac fry of brown trout to pH 4.5 did not result in any reduction of dry body weight (Reader *et al.*, 1988). Similarly, Gunn and Noakes (1987) found no differences in body weight between lake trout embryos raised at pH 5.1 or at pH 6.0. In addition, Nelson (1982) found that alevins of rainbow trout exposed to pH 4.3-4.8 were shorter in length but did not differ in weight from those reared at pH 7.1. Also, when brook trout fry were exposed to low pH levels after fertilization, no effect of pH on dry body weight could be observed (Wood *et al.*, 1990a). Moreover, when exposure started a few days after hatching, neither an effect on dry weight nor on wet weight could be observed (Wood and McDonald, 1990b).

The total body Ca levels of larval carp at the end of yolk absorption were reduced as a result of acid exposure. The same phenomenon was reported by Wood *et al.* (1990a) for brook trout when exposed to low pH after fertilization, or even after hatching. Similar results were reported by Peterson *et al.* (1982) and Peterson and Martin-Robichaud (1986). Reader *et al.* (1988) mentioned that the net Ca^{2+} uptake at pH 4.5-4.8 was lower than at pH 5.4 and 6.5 for sac fry of brown trout. No relation between water pH and total body Ca concentrations of eggs and embryos of lake trout was observed by Gunn and Noakes (1987). However, only a relatively moderate acidity level (pH 5.1) was studied by the latter.

In our experiments, Na^+ uptake of carp larvae was reduced after exposure to low water pH. Post hatch exposure of brook trout to pH 4.4 resulted in a 50 % reduction of whole body Na concentrations compared to larvae reared at pH 6.5 (Wood *et al.*, 1990a,b), and similar effects occurred when exposure started after fertilization. Reductions of Na were also observed for brown trout by Peterson *et al.* (1982), Peterson and Martin-Robichaud (1986) and Reader *et al.* (1988). Contrastingly, Gunn and Noakes (1987) observed no effects of pH on lake trout embryos, but as mentioned before, only moderate levels of water acidity were studied. Even before hatching, negative Na balances were reported. Net Na losses were found by Vinogradow and Komev (1985) for perch eggs, and by Eddy and Talbot (1985) for eggs of Atlantic salmon. In the latter study it was shown that this effect was caused by inhibition of the Na^+ influx at low pH. The Na^+ efflux was unaffected.

In contrast to the reduction of the total body Ca and Na concentrations in our experiments, the Mg levels on a dry weight basis increased at low pH. Wood *et al.* (1990a) found a small increase of total body Mg levels on a wet weight basis in larvae of brook trout when exposed to low pH up from fertilization. This effect disappeared when exposure occurred only after hatching. They mentioned that the Mg level in yolk was much higher than that in the embryo, and that with increasing development (= yolk conversion), the Mg concentration of the larvae became lower. Thus, the relatively high Mg level at the end of yolk sac absorption of acid-exposed sac fry observed in their

experiments might be attributed to a delay in development. Such a delay probably did not occur when exposure started after hatching. This approach can not be applied to our observations, since our Mg measurements were based on dry weight. Earlier observations indicated that development of carp eggs and larvae were delayed in acid water (Chapter 2), which would predict a reduced Mg concentration on dry weight basis. The relative increase in the Mg concentrations in acid water might reflect a greater loss of organic matter of the embryo during development, and thus a less efficient conversion of yolk into larval tissues than under control conditions. Loss of dry body weight during development however, appeared to be insensitive to pH. This points to increased Mg^{2+} uptake from the water as a result of acid exposure. Wood *et al.* (1990a) found the Mg content of brook trout larvae dropped at higher water Ca^{2+} levels. An interaction between Mg^{2+} and Ca^{2+} was also reported by Van der Velden *et al.* (1991), who found that uptake of Ca^{2+} by carp eggs was stimulated by decreasing water Mg^{2+} levels. They concluded that Ca^{2+} and Mg^{2+} compete for uptake sites in developing eggs. The reduced Ca^{2+} uptake and increased Mg^{2+} uptake we found during early development in carp supports this contention.

The differences of ion concentrations in larvae as a result of acid exposure could be attributed to ion regulatory disturbances as well as to retarded development. It is difficult to make a choice between both alternatives. Accumulation of Ca^{2+} and Na^{+} occurred mainly after hatching while the increase in the Mg concentration occurred mainly in the egg stage. The delay of hatching of carp eggs in acid water (Chapter 2), could therefore result in a shorter accumulation period for Ca^{2+} and Na^{+}. In contrast, the period of Mg^{2+} uptake from the water was prolonged in acid water and this might explain the higher Mg levels. High deformation rates could also contribute to lower Ca and Na contents at low pH. Reduced mobility might influence the refreshment of the water layer at the body surface where ion uptake occurs, and this may lead to a lower ion gradient between the larvae and the surrounding water.

Effects of humic acids at low water pH.

The presence of humic acids did not influence the impact of water pH on any of the studied parameters. The few reports on the effects of humic acids on the impact of low water pH are contradictory. Witters *et al.* (1990) observed an initial negative effect of humic acids at pH 4.7 on plasma Cl^{-} concentrations of rainbow trout. Contrastingly, Hargeby and Petersen (1988) mentioned a protective effect of humic acids: it reduced the osmotic disturbance in the amphipod *Gammarus pulex* at low pH. No general conclusion can therefore be drawn with respect to the effects of humic acids in acid water.

References

Battram, 1988. The effects of aluminium and low pH on chloride fluxes in the brown trout, *Salmo trutta* L. J. Fish Biol. 32: 937-947.

Brown, D.J.A., 1982. Influence of calcium on the survival of eggs and fry of brown trout (Salmo trutta) at pH 4.5. Bull. Environm. Contam. Toxicol. 28: 664-668.

Brown, D.J.A. and S. Lynam, 1981. The effect of sodium and calcium concentrations on the hatching of eggs and the survival of the yolk sac fry of brown trout, *Salmo trutta* L. at low pH. J. Fish Biol. 19: 205-211.

Eddy, F.B. and C. Talbot, 1985. Sodium balance in eggs and dechorionated embryos of the atlantic salmon, *Salmo salar* L., exposed to zinc, aluminium and acid waters. Comp. Biochem. Physiol. 81C: 259-266.

Gunn, J.M. and D.L.G. Noakes, 1987. Latent effects of pulse exposure to aluminium and low pH on size, ionic composition, and feeding efficiency of lake trout (*Salvelinus namaycush*) alevins. Can. J. Fish. Aquat. Sci. 44: 1418-1424.

Hargeby, A. and R.C. Petersen, 1988. Effects of low pH and humus on the survivorship, growth and feeding of *Gammarus pulex* (L.)(Amphipoda). Freshwat. Biol. 19: 235-247.

McDonald, D.G., 1983. The effect of H^+ upon the gills of freshwater fish. Can. J. Zool. 61: 691-703.

Natochin, Yu.V., V.I. Lukianenko, E.A. Lavrova and G.F. Metallov, 1976. Electrolyte composition of embryo and larva of the Russian sturgeon in the process of development. Comp. Biochem. Physiol. 55A: 57-59.

Nelson, J.A., 1982. Physiological observations on developing rainbow trout, *Salmo gairdneri* (Richardson), exposed to low pH and varied calcium ion concentrations. J. Fish Biol. 20: 359-372.

Peterson, R.H., Daye, P.G. and J.L. Metcalfe, 1980. The effects of low pH on hatching of Atlantic salmon eggs. Proc. Int. Conf. Imp. Acid Precip.: 328. SNSF Project.

Peterson, R.H., Daye, P.G., Lacroix, G.L. and E.T Garside, 1982. Reproduction in fish experiencing acid and metal stress, p. 177-196. in R.E. Johnson [ed.] Acid Rain/Fisheries Am. Fish. Soc. Bethesda, M.D.

Peterson, R.H., and D.J. Martin-Robichaud. 1986. Growth and major inorganic cation budgets of Atlantic salmon alevins at three ambient acidities. Trans. Am. Fish. Soc. 115: 220-226.

Playle, R.S., Goss, G.G. and C.M. Wood, 1989. Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminium exposures in soft water of two calcium concentrations. Can. J. Zool. 67: 314-324.

Rask, M., 1984. The effect of low pH on perch, *Perca fluviatilis* L. II. The effect of acid stress on different developmental stages of perch. Ann. Zool. Fennici 21: 9-13.

Reader, J.P., Dalziel, T.R.K. and R. Morris, 1988. Growth, mineral uptake and skeletal calcium deposition in brown trout, *Salmo trutta* L., yolk-sac fry exposed to aluminium and manganese in soft acidic water. J. Fish Biol. 32: 607-624.

Schnitzer, M., 1969. Reactions between fulvic acid, a soil humic component, and inorganic constituents. Soil Scio. Soc. Amer. Proc. 33, 75-81.

Talbot, C., Eddy, F.B. and J. Johnston, 1982. Osmoregulation in salmon and sea trout alevins. J. Exp. Biol. 101: 61-70.

Velden vd, J.A., Spanings, F.A.T., Flik, G. and S.E. Wendelaar Bonga, 1991. Early stages of carp (*Cyprinus carpio* L.) depend on ambient magnesium for their development. J. Exp. Biol. 158: 431-438.

Vinogradov, G.A. and V.T. Komev, 1985. Ion regulation in the perch, *Perca fluviatilis*, in connection with the problem of acidification of water bodies. Scripta Technica, Inc. Originally published in Vopr. Ikhtiol. 1: 135-144 (1985).

Witters, H.E., Van Puymbroeck, S., Vangenechten, J.H.D. and O.L.J. Vanderborght, 1990. The effect of humic substances on the toxicity of aluminium to adult rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Biol. 37: 43-53.

Wood, C.M., McDonald, D.G., Ingersoll, C.G., Mount, D.R., Johansson, O.E., Landsberger, S. and H.L. Bergman, 1990a. Effects of water acidity, calcium, and aluminium on whole body ions of brook trout (*Salvelinus fontinalis*) continuously exposed from fertilization to swim-up: A study by instrumental neutron activation analysis. Can. J. Fish. Aquat. Sci. 47: 1593-1603.

Wood, C.M., McDonald, D.G., Ingersoll, C.G., Mount, D.R., Johansson, O.E., Landsberger, S. and H.L. Bergman, 1990b. Whole body ions of brook trout (*Salvelinus fontinalis*) alevins: Responses of yolk-sac and swim-up stages to water acidity, calcium, and aluminium, and recovery effects. Can. J. Fish. Aquat. Sci. 47: 1604-1615.

Zeitoun, I.H., D.E. Ullrey, W.G. Bergen, and W.T. Magee, 1976. Mineral metabolism during the ontogenesis of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Bd Can. 33: 2587-2591.

CHAPTER 4

Effects of acid water and humic acids on the toxicity of aluminium to eggs and sac fry of the common carp.

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Effects of acid water and humic acids on the toxicity of aluminium to eggs and sac fry of the common carp.

Summary

The pH dependency of the acute toxicity of inorganic aluminium (Al) to eggs and larvae of the common carp (*Cyprinus carpio*) was tested in the pH range of 7.5-4.8. Studied parameters were mortality, time of hatching and percentage of spinal cord deformation. Above pH 5.9, inorganic Al (7.4 μmol/l) did not exert any toxic effects within 168 h; between pH 5.9 and 5.1 it increased mortality and deformation ratios, and delayed hatching time. At pH 5.1 - 4.8, inorganic Al levels $<$ 13.5 μmol/l showed a mitigating effect on acid stress, which disappeared with time and with increasing Al levels: then the toxic effects of inorganic Al reappeared. Complexation of inorganic Al with humic acids prevented toxic Al effects as well as the transient mitigating effect of Al on acid stress. Our results support the Al-speciation model as well as the free ion binding theory with H^+ competition for binding sites and the formation of ligand-hydroxy-Al complexes, as proposed by Neville and Campbell (1988).

Introduction

The deleterious effects of water acidification on fish in natural waters are not only caused by the low water pH, but also by the high Al levels that usually go with it. Like low water pH, elevated Al levels are toxic to the adult brown trout (*Salmo trutta*) as well as to their early life stages (Brown, 1983). Data on fish other than salmonids are scarce. This also holds thrue for cyprinids. In Chapters 1 and 2, we have shown that carp eggs and larvae are very sensitive to low water pH. Data on the effects of elevated Al levels in the environment on the reproductive success of carp are absent. We have therefore investigated the toxicity of Al in acid water to the embryonic and larval development of the common carp.

Al in natural water may occur in different chemical forms, of which Al hydroxide is the most predominant one between pH 5 and 6 (Lazerte, 1984; Burrows, 1977). Inorganic monomeric Al hydroxide forms different species, depending on water pH (Smith and Hem, 1972). Since these species have different properties, the toxicity of Al was studied at various pH levels. According to Morris and Krueger (1984), complexation of inorganic monomeric Al hydroxide with organic matter such as humic acids reduces Al toxicity to adult trout. Data on the effects of humic acids on Al toxicity to eggs and embryos are scarce. Therefore, the protective effect of humic acids on the toxicity of monomeric Al hydroxide was also studied. For both studies we used the following parameters: embryonic and larval mortality, hatching

time and spinal cord deformation. Several theories on the interaction of Al with fish gills have been presented (Helliwell *et al.*, 1983; Leivestad *et al.*, 1987; Neville and Campbell, 1988; Exley *et al.*, 1991). An attempt was made to explain our results on the basis of these theories.

Materials and methods

Fertilization and incubation of eggs.

Collection of eggs and sperm from carp, fertilization techniques, and incubation procedures were identical to those used in Chapter 2). The medium used for the experiments was prepared by adding salts to demineralized water in the following concentrations: NaCl, 3.8; KCl, 0.06; $CaCl_2$, 0.8; $MgSO_4$, 0.2 and $NaHCO_3$: 0.335 mmol/l. This resulted in ion concentrations within the normal range found in natural waters containing carp populations. The pH of the experimental media was adjusted by adding diluted sulfuric acid (0.01 M). To maintain pH levels, a pH stat system (fluctuation 0.02 pH units) was used. The CO_2 formed during the acidification of the test media was allowed to escape before the eggs were placed into the incubation units. Al was added as $Al_2(SO_4)_3$. Every 12 hour, the monomeric Al concentrations of the media were measured by the colorimetric determination with pyrocatechol violet (Wilson and Sergeant, 1963), and adjusted when necessary. Humic acids (Fluka AG, CH-9470 Buchs) were first dissolved in a basic solution of NaOH, and then added in the amounts required.

Effect of inorganic monomeric Al on mortality, hatching dynamics and deformation.

Eggs and larvae were exposed to 32 combinations of pH levels (pH range 7.5 - 4.8) and Al concentrations (Al range 0 - 13.5 μmol/l) after fertilization.

Dead eggs and larvae were counted every 12 h up until 260 h after fertilization, depending on the mortality rate. Eggs were considered dead when parts of the content turned opaque and white. For heart beat stages stopping of the beat was used as criterium.

Hatching was evaluated by calculating the percentage of the hatched larvae for each Petri dish every 3 h, starting at 48 h and ending at 96 h after fertilization. Further monitoring was done every 8 h, until all eggs had either hatched or were dead. Hatching and the median hatching time were defined as in Chapter 2.

After hatching, a proportion of the total larvae appeared to have a deformed (curved) spinal cord. Deformed larvae of the experimental groups were counted immediately after completion of hatching.

Effect of organically complexed monomeric Al hydroxide on mortality, hatching time and deformation.

This experiment was essentially the same as the previous one, except that a concentration of 33 mg/l humic acid (well within the natural range of waters in Western Europe (Tipping, personal communication) had been added to every experimental group. At more acidic pH and higher Al concentrations, humic acids were seen to to precipitate. Under these conditions, the level of humic acids was corrected every 12 hours. This was done by measuring the extinction of the media at 450 nm, and when necessary, adding humic acids until the desired concentration was reached. The influence of water pH on the adsorption of humic acids at 450 nm was taken into account.

Calculations and statistics.

The relationships of mortality, hatching time and deformation with pH and with Al were plotted as three-dimensional surfaces. To this end, the mean raw data, the mean pH values and monomeric Al levels were modelled by the computer program SURFER version 3.00 (Golden Software Inc., 1987). Experiments done at Al levels above 7.7 μmol Al/l (pH 4.8) were not included because of the low Al solubility at pH 6.0 - 5.5.

To compare our observations with the Al-speciation model (Helliwell *et al.*, 1983; Leivestad *et al.*, 1987), the ion activity fractions of the different Al species at the studied pH values were calculated for the experimental conditions, using the method described by Butler (1964). The equilibrium constants used were from Smith and Hem (1968).

Statistical analysis was performed by testing the effects of Al on the studied parameters against their own pH control values, applying analysis of variance for mortality, hatching time and deformation, and the Mann-Whitney-U test for the mortality rate.

Results

Effects of low water pH, inorganic Al and inorganic Al plus humic acids on 50 h mortality of eggs.

Fig. 1 shows the combined effects of pH and inorganic aluminium hydroxide on the 50 h mortality of the eggs.

a) Effects of low water pH.

Reduction of water pH to values lower than 5.3 resulted in elevated mortality percentages.

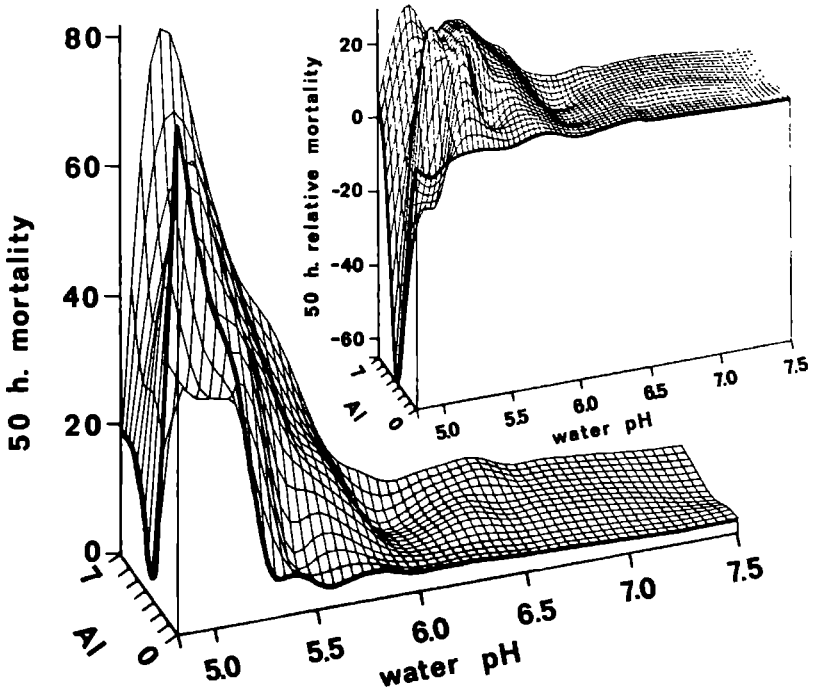

Fig. 1: Three-dimensional surface plot of the 50 h-mortality (%) of eggs during exposure to different water pH levels and different inorganic Al concentrations (μmol/l). Inset: three-dimensional surface plot of the 50 h Al-dependent (mortality pH + Al minus mortality pH) mortality (%) under these conditions.

b) Effects of low water pH and inorganic Al.

At pH 7.5 - 6.0, no effect of inorganic Al on the eggs was observed (Fig. 1). Between pH 6.0 - 5.1 addition of inorganic Al increased mortality significantly when compared to the mortality induced by exposure to pH alone. Exposure to low inorganic aluminium levels in the pH range of 5.1 - 4.8, partially counteracted the negative effects of low pH. This protective effect disappeared at higher Al levels; Al even increased mortality. This phenomenon is visualised in Fig. 2, where the mortality of experimental groups exposed to different inorganic Al levels at pH 4.8 is plotted against time after fertilization. Up from 72 hours after fertilization the protective effect of inorganic Al disappeared and eventually all the larvae died, even those exposed to the lowest Al concentration. Two periods showed high mortality rates: the first 30 hours and the period after 70 hours. To study this in more detail, mortality rates per 12 h of living eggs and larvae were determined. Results are presented in Fig. 3. During the first 30 hours, low Al levels suppressed significantly ($P < 0.001$) the pH-induced mortality rate. Between 30 and 70 h the eggs appeared relatively resistant. Up from 70 hours (the normal hatching time) the mortality rate rose markedly at pH 4.8, with and

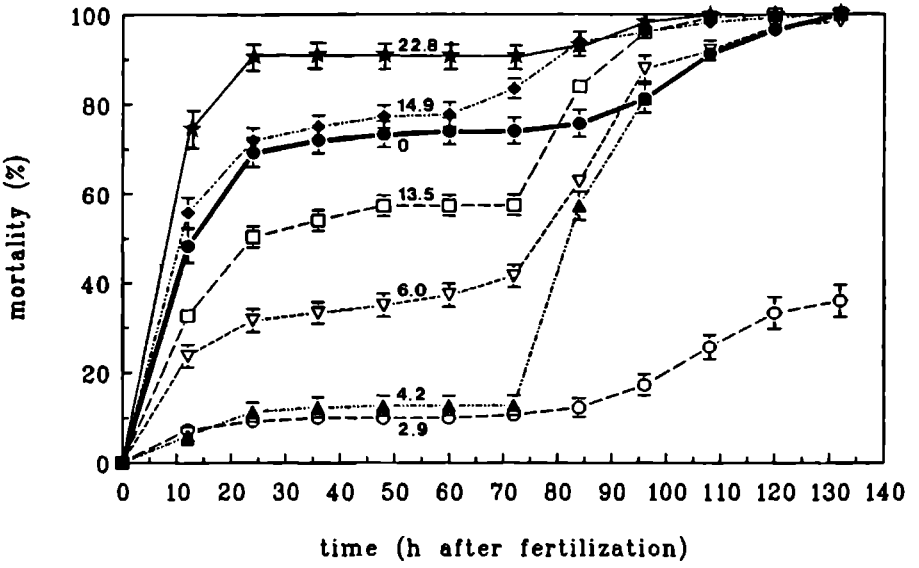

Fig. 2: The effect of different inorganic Al concentrations (indicated in the graph in µmol/l) at pH 4.8 on mortality of eggs and sac fry (mean values ± SE; n = 5).

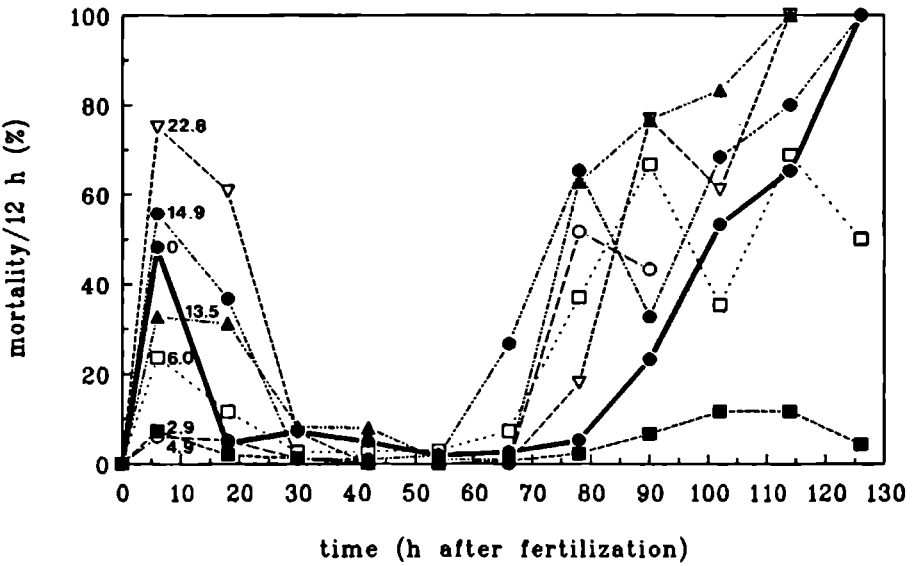

Fig. 3: Mortality rates (mortality percentage of surviving/12 h) of eggs and sac fry at different times during exposure to different inorganic Al concentrations (indicated in the graph in µmol/l) at pH 4,8 (mean values; n = 5).

without Al. The mortality rates of the Al-exposed groups were however higher.

c) Effects of low water pH, inorganic Al and humic acids.

Fig. 4 shows the effects of humic acids (33 mg/l), with or without Al at varying pH. Al did not significantly elevate mortality in the pH range studied. The protective effect of inorganic Al hydroxide on pH toxicity between pH 5.1 and 4.8 (Fig. 1) disappeared in the presence of humic acids. Under these conditions, Al did no longer contribute to the mortality at low pH (Fig. 4 inset).

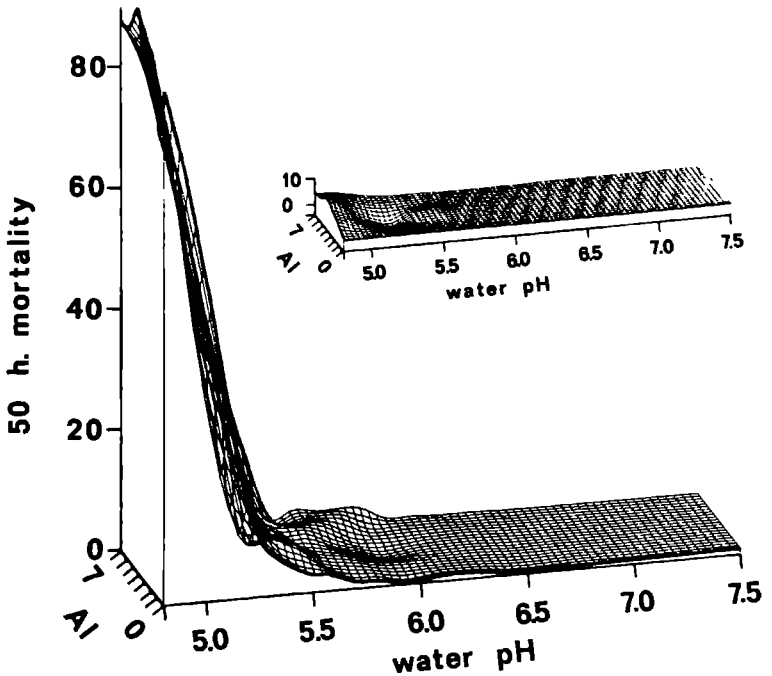

Fig. 4: Three-dimensional surface plot of the 50 h-mortality (%) of eggs, during exposure to different water pH levels and different Al concentrations (μmol/l), in the presence of humic acids (33 mg/l). Inset: three-dimensional surface plot of the 50 h Al-dependent mortality (%) under these conditions (i.e. the difference in mortality between the groups of eggs exposed to these conditions in the presence and absence of Al).

Effects of low water pH, inorganic Al and inorganic Al plus humic acids on hatching time.

The combined effects of pH and Al on hatching time, expressed as the time point when 50 % of the total number of hatched larvae had hatched (the 50 % hatching point), are shown in Fig. 5.

a) Effects of low water pH.

Hatching time delay became apparent at pH levels lower than 5.3.

b) Effects of low water pH and inorganic Al

The presence of Al had no influence on the 50 % hatching time, in the pH range from 7.5 - 5.9. Between pH 5.9 and 5.1, Al strongly delayed hatching time. Contrastingly, at pH 5.1 - 4.8, hatching time delay was less than at the same pH without Al, at least at the lower Al concentrations indicating that Al had a protective effect at this pH. This effect disappeared at higher Al levels. In Fig. 6, time of hatching at pH 5.2 in the presence of different inorganic Al concentrations is shown in more detail. At pH 5.2 without Al, the 50 % hatching point was reached after 68 h. At the same pH with 4.3 μmol Al/l, the 50 % hatching pointwas only reached after 161 h, which represents a hatching time delay of 138 %. Without Al, 95 % of the eggs that eggs that hatched showed hatching within a period of 13 h. In the

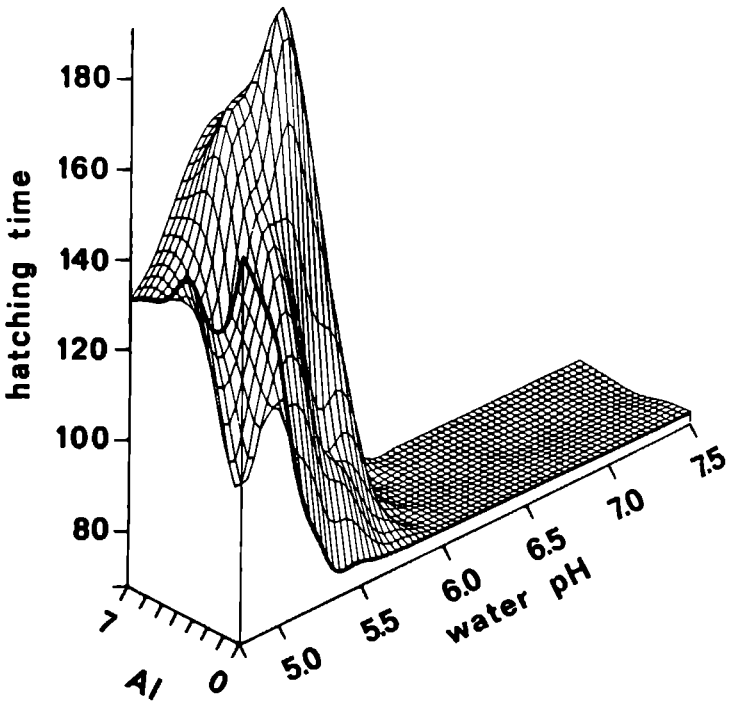

Fig. 5: Three-dimensional surface plot of the 50 % hatching times during exposure to different water pH levels and different inorganic Al concentrations (μmol/l).

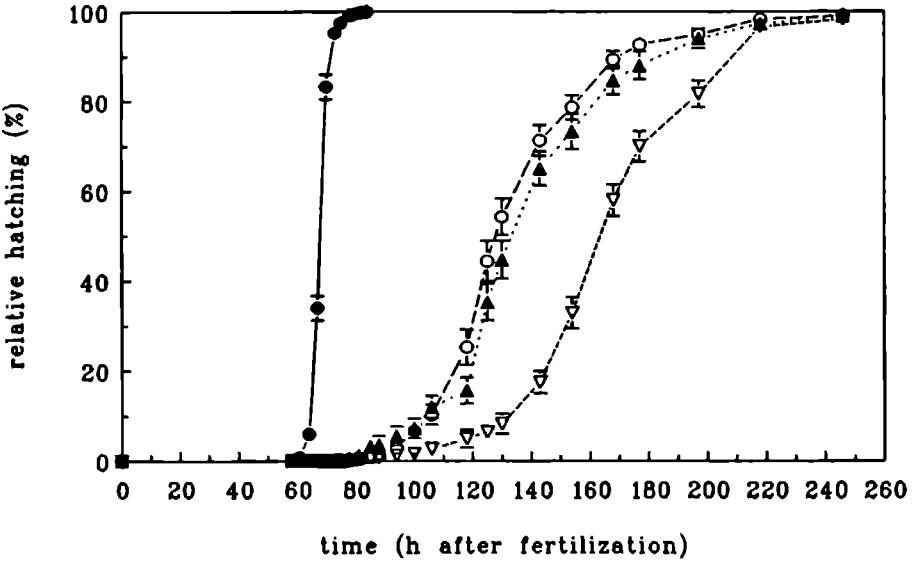

Fig. 6: Relative hatching percentages (% of total hatch) at different time-intervals of embryos exposed to different inorganic Al levels at pH 5.2 (mean values ± SE; • = 0 µmol Al/l; o = 2.0 µmol Al/l; ▲ = 2.2 µmol Al/l; ▽ = 4.3 µmol Al/l).

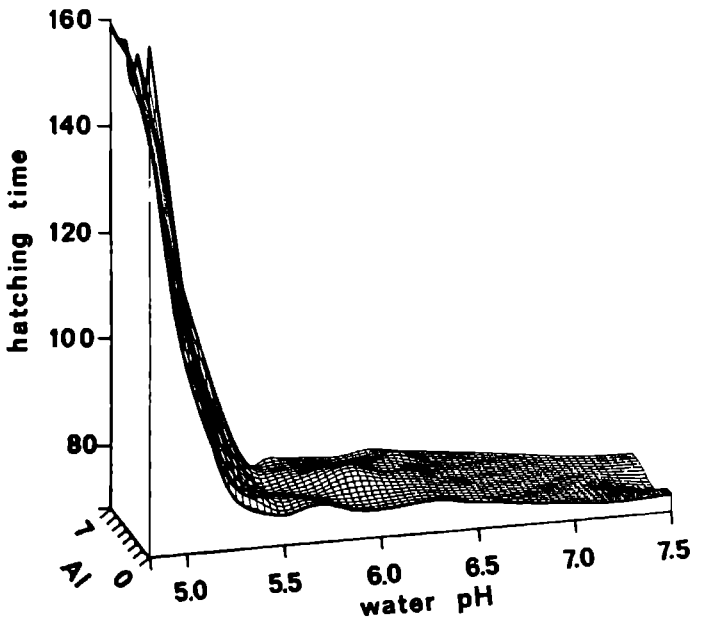

Fig. 7: Three-dimensional surface plot of 50 % hatching times during exposure to different water pH levels and different Al concentrations (µmol/l), in the presence of humic acids (33 mg/l).

presence of Al this period was 136 h, indicating that Al caused a substantial individual variation in the time of hatching. Some eggs even hatched at 250 h after fertilization, prolonging their embryonic stage with 182 hours.

c) Effects of low water pH, inorganic Al and humic acids.

In the presence of both Al and humic acids (33 mg/l) in the water, any effect of Al disappeared: hatching time was no longer different from that of eggs incubated in acid water without Al (fig. 7) in the whole pH range tested (pH 7.5 - 4.8). The protective effect of low inorganic Al concentrations also disappeared in the presence of humic acids.

Effects of low water pH, inorganic Al and inorganic Al plus humic acids on deformation.

The deformation percentage of the larvae as a result of the presence of inorganic Al at different water pH is shown in Fig. 8. The deformation percentages at pH 4.8 were not presented in this figure because the numbers of hatched larvae were too small for establishing reliable deformation percentages.

a) Effects of low water pH.

In neutral or slightly acid water (pH 7.5 - 5.9), no effects of acidification or aluminium were noticeable. At lower pH levels, the deformation percentage increased slowly, and at pH levels lower than 5.1 very dramatically.

b) Effects of low water pH and inorganic Al.

Addition of Al resulted in a pronounced increase of the deformation percentage, beginning already at pH 5.9 and at relatively low aluminium concentrations (1.3 μmol/l). For example, at pH 5.5 without Al, the deformation percentage of the hatched larvae is 8.5 %. Addition of only 1.7 μmol Al/l resulted in an elevation of this deformation percentage to 98.6 %. A protective effect of low Al levels, as observed for mortality and hatching time delay in water of pH 5.0 - 4.8, could not be observed with respect to the deformation percentage since data at pH 4.8 were lacking. However, when the degree of deformation was taken into consideration instead of the percentage of deformed larvae, a protective effect of Al became noticeable. Microscopic analysis showed that larvae from water of pH 4.8 with Al were deformed to a much lower extent than larvae from water of the same pH without Al.

c) Effects of low water pH, inorganic Al and humic acids.

The effect of humic acids on the Al-induced deformation percentage of the larvae is shown in Fig. 9. At none of the studied pH levels, any effect of Al on the deformation percentage could be observed when humic acids were present. The detoxifying effect of humic acids on inorganic monomeric Al toxicity, as found for mortality and hatching time, was also noticeable for the deformation percentage.

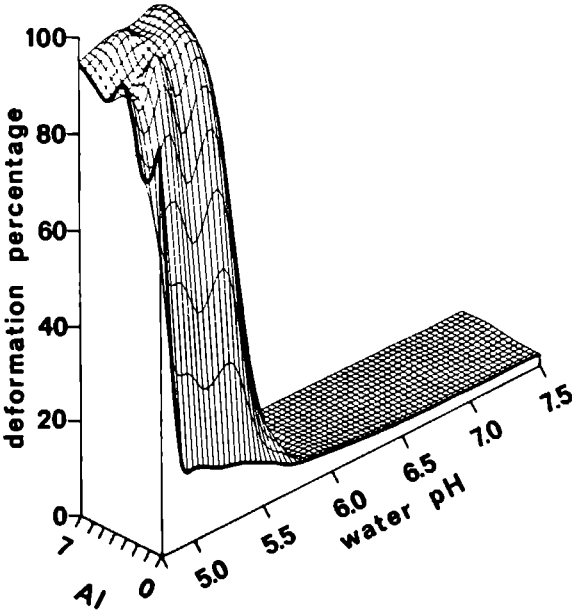

Fig. 8: Three-dimensional surface plot of larval deformation percentages (%) induced by different water pH levels and different inorganic Al concentrations (μmol/l).

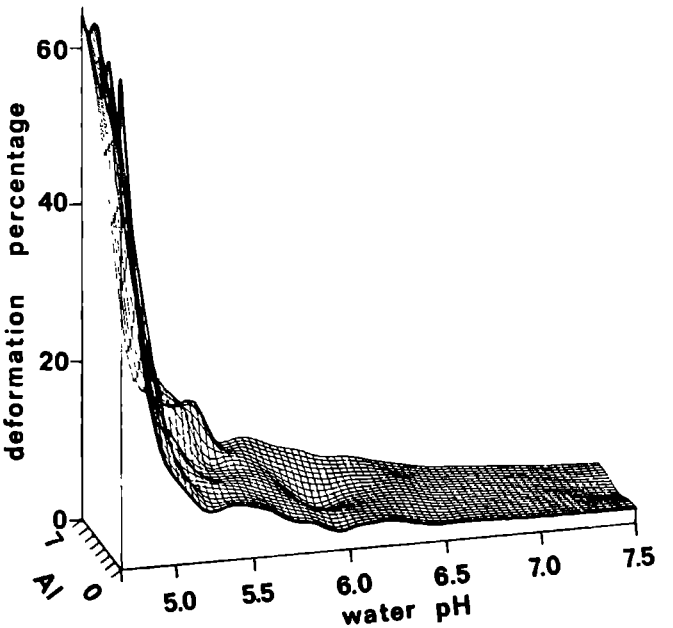

Fig. 9: Three-dimensional surface plot of larval deformation percentages (%) induced by different water pH levels and different Al concentrations (μmol/l), in the presence of humic acids (33 mg/l).

Effects of low water pH, inorganic Al, and inorganic Al plus humic acids on 50 h mortality.

a) Effects of low water pH.

Our results showed that water acidification at pH levels lower than 5.3 increased mortality of eggs and larvae. This confirms our earlier results (Chapter 2).

b) Effects of low water pH and inorganic Al.

No increased mortality was observed for inorganic Al concentrations up to 7.4 μmol inorganic Al/l in the pH range of 7.5 - 5.9. Inorganic Al showed strongest toxicity in the pH range of 5.9 - 5.1. Between pH 5.1 and 4.8, low Al levels had a mitigating effect on pH toxicity, at least before hatching. This protective effect disappeared when the Al levels rose: at higher concentrations Al became toxic. The sensitivity of carp eggs for low pH and Al changed during embryonic development. The eggs appeared to be most sensitive during the first 24 h after fertilization. Then followed a relatively insensitive period which lasted until the time when unexposed eggs normally hatch. The period around hatching represented a second sensitive period.

Al did not influence mortality from pH 7.5 to pH 5.9. When comparing the results of different authors working in this field, some differences become evident. Cleveland *et al.* (1986) found that exposure of eyed eggs of brook trout (*Salvelinus fontinalis*) to 11 μmol Al/l at pH 7.2 had no effect on mortality. Hunn *et al.* (1987) found no effect of 11 μmol Al/l at pH 7.2 on eyed eggs of the same species. Exposure of hatched larvae of smallmouth bass (*Micropterus dolomieu*) to 8 μmol Al/l and 36 umol Al/l at pH 7.2 or pH 6.1 did not influence mortality (Kane and Rabeni, 1987). According to Klauda *et al.* (1987), exposure of yolk-sac larvae of blueback herring (*Alosa aestivalis*) to levels up to 15.5 μmol Al/l at pH 7.8 and 6.5 did neither result in elevated mortality. The LC50 value of eggs of rainbow trout (*Oncorhynchus mykiss*) at pH 7 was 141 μmol Al/l (Thomsen *et al.*, 1988). This is very high and indicates a low toxicity of Al at this pH. In contrast, Buckler *et al.* (1987) found increased mortalities when eleven day old larvae of East coast striped bass (*Morone saxilatus*) were exposed to 14.8 μmol Al/l at pH 7.5. Thus, our findings for the pH 7.5 - 5.9 range are in line with literature, with exception of the last study mentioned.

Inorganic Al showed strongest toxicity in the pH range 5.9 - pH 5.1. Most authors reported similar results. Klauda *et al.* (1987) observed elevated mortality of yolk sac larvae exposed to concentrations up to 15.5 μmol Al/l at pH 5.7 or 5.0. The effect was most pronounced at pH 5.0. Thomsen *et al.* (1988) reported similar results for eggs of rainbow trout. Exposure of smallmouth bass larvae to 7.4 μmol Al/l at pH 5.7 - 5.1 also increased mortality, with an LC50 value after 96 h at pH 5.1 of 4.8 μmol Al/l (Kane and Rabeni, 1987). Baker and Schofield (1980) found that Al levels up to 18.5 μmol Al/l increased the mortality of brook

trout fry in the pH range of 5.5 - 5.0. They further demonstrated that Al levels of 7.4 μmol/l and lower were toxic between pH 5.7 and pH 5.2 for eggs of the white sucker (*Catostomus commersoni*; Baker and Schofield, 1982). Al was toxic for larvae of this species in the pH range 5.6 - 4.2, with most pronounced effects between pH 5.4 - 5.2. Larvae of brown trout reacted in the same way as larvae of the white sucker (Baker and Schofield, 1982). Contrastingly, eyed eggs of brook trout incubated in water of pH 5.5 with 11.1 μmol Al/l did not show increased mortality, and neither did larvae of this species (Cleveland *et al.*, 1986). Under the same conditions, Hunn *et al.* (1986) found no effect of Al on eggs of brown trout. Rosseland and Skogheim (1982) did not observe toxic effects of 17.1 μmol Al/l on eyed eggs of atlantic salmon (*Salmo salar*).

Between pH 5.1 and 4.8 Al exerted toxic as well as protective effects, depending on the Al concentration level and the developmental stage. Our results are in line with those of Baker and Schofield (1980), who found that 18.5 μmol Al/l reduced mortality of brook trout eggs in the pH range 5.0 - 4.2. For sac fry, Al was toxic between pH 5.0 - 4.2, and thus the mitigating effect of Al on acid stress was only transient. Hunn *et al.* (1986), observed that 11.1 μmol Al/l at pH 4.5 protected against acid stress suffered by eyed eggs of brook trout. Cleveland *et al.* (1986) did not find an effect of Al under the same conditions. The latter authors however noticed a toxic effect of Al at this pH on brook trout larvae. A developmental stage-dependent change from a protective into a toxic effect of Al was also found by Baker and Schofield (1982). Acid stress on eggs of the white sucker was reduced by Al concentrations lower than 7.4 μmol/l in water with a pH between 5.0 and 4.2. Around hatching time this mitigating effect was reversed into a toxic one: larvae showed an elevated mortality at the same concentration of Al in the pH range 5.0 - 4.8. These authors found similar results for brook trout. The detoxifying effect of Al at low pH on pre-hatching stages, combined with a toxic effect on the stages after hatching appears a general phenomenon. However, the observed concentration-dependent reversal of the effect of Al has not been mentioned in the literature.

Our finding that the sensitivity of embryos to low water pH and Al is related to the developmental stage has been mentioned before. Literature on the effects of Al during the first sensitive period, i.e. 0-30 h after fertilization, is scarce since most authors exposed eggs only up from the eye point stage. However, Holtze and Hutchinson (1989) found an early sensitive period for water pH immediately after fertilization for eggs of *Stizostedion vitreum*, *Catostomus commersoni*, *Coregonus clupaeiformis* and *Notropis cornutus*. A similar sensitive period was identified by Thomsen *et al.* (1988) for eggs of rainbow trout.

It is of interest to consider some morphological and physiological changes in the egg, during the first 24 h, which could have some relevance for the increased sensitivity of the eggs for acidity and Al during this period. According to Kudu (1982), polymerisation of the crystalline material from cortical granules, needed to create the perivitelline space, is Ca

dependent. Since H^+ and Al^{3+} can interfere with Ca (Reid *et al.*, 1991), these ions could influence the formation of the perivitelline space. Gajduzek and Rubcov (1983) studied the changes of the egg membranes of carp. During the first 24 h after fertilization, when the egg was swelling, the thickness of the outer membrane diminished, and the apertures and channels in this membrane disappeared. Meanwhile, the thickness of the inner membrane (zona radiata) also became reduced. The diameter of the channels increased and lamellae of the zona radiata fused, thus closing the channels. During the first time after fertilization, when the channels were still open, H^+ and Al^{3+} could possibly enter the egg, together with the incoming water. According to Zotin (1966), water exchange between the perivitelline fluid and the surrounding bulk water is diminished shortly after fertilization. Potts and Rudy (1969) also stated that the permeability to water and ions of the vitelline membrane during the colloid release phase is very high, but strongly reduced during and after the hardening of the chorion.

According to Peterson *et al.* (1981), embryos in the early cleaving stages are most sensitive to low pH. Epidermal cells, necessary for the development of tissues with complex functions in older embryos, may die faster than they can be replaced. Without an intact integument, survival is impossible. The same authors mentioned that older embryos are perhaps the most tolerant of all lifestages of fish. They have a relative thick tissue mass which could result in an extra ion gradient, like the perivitelline fluid and the chorion. This might explain our observation of a relative insensitive period between the hardening of the chorion and hatching.

A second sensitive period around hatching, as observed in our experiments, was also found by Thomsen *et al.* (1988). The sensitivity may be related to the swelling of the eggs that occurs around hatching. The outer egg membrane disappears or becomes very thin, exposing the channels of the zona radiata to the surrounding water. The lamellae in the channels of this inner egg membrane become disrupted because of stretching, thus reopening the channels. In this situation, H^+ and Al^{3+} could enter the egg again. Another explanation of this second sensitive period might be that just before hatching the embryo is experiencing an oxygen debt (Kamler, 1976). Because the movements of the deformed embryos are less pronounced than those of normal embryos, the circulation of the perivitelline fluid may be reduced and this could impair the oxygen supply to the embryo. In this way, the oxygen deficiency of the embryo would become even higher.

c) Effects of low water pH, inorganic Al, and humic acids.

Our results show that humic acids reduce Al toxicity very effectively. Al forms complexes with carboxyl groups of humic acids (Mak and Langford, 1982) and, for instance, binds 2.5 and 50 times stronger than Fe and Mn, respectively (Khan, 1969; Snitzer, 1981). Trivalent ions like Al^{3+} bind more effectively than divalent and monovalent ions to fulvic acids, which are chemically related to humic acids (Snitzer and Kerndorff, 1980).

Effects of low water pH, inorganic Al and inorganic Al plus humic acids on hatching time.

The effects of water pH and inorganic monomeric Al on hatching delay and on mortality ran parallel. Between pH 5.9-7.5, Al had no influence on hatching time; between pH 5.1 and 5.9 exposure to Al resulted in a very strong delay of hatching time. Below pH 5.1 low Al levels reduced the delay caused by low water pH.

a) Effects of low water pH.

The observation that exposure of carp eggs to a water pH lower than 5.5 results in delay of hatching confirms earlier results (Chapter 2).

b) Effects of low water pH and inorganic Al.

A pronounced delay of hatching time as we observed for low Al between pH 5.1 and 5.9 has not been published before. An explanation could be that our carp eggs were exposed to Al immediately after fertilization, while other authors started exposure after hardening of the chorion. Hunn *et al.* (1986) found that eyed eggs of brook trout showed no delay of hatching when exposed to 11.1 μmol Al/l at pH 4.5 and 5.5. An effect no influence on mortality was neither found. According to Cleveland *et al.* (1986), eyed eggs of the same species experienced a higher mortality rate under these conditions. Delayed hatching was not observed, although the occurrence of partially hatched larvae increased. Delayed hatching as a result of low water pH only has been reported for *Perca fluviatilis* (Rask, 1983), *Pimephalus promelas* (Mount, 1973), *Salmo trutta fario* (Brown and Lynam, 1981), *Salvelinus fontinalis* (Swarts *et al.*, 1978; Kwain and Rose, 1985), *Salmo salar* (Peterson *et al.*, 1980), and *Stizostedion vitreum* (Holze and Hutchinson, 1989).

c) Effects of low water pH, inorganic Al and humic acids.

Our observations show that the presence of humic acids in the water prevents toxic effects of Al on hatching. To our knowledge this phenomenon has not been observed before.

Effects of low water pH, inorganic Al and inorganic Al plus humic acids on deformation.

The incidence of deformation of larvae, already high at low water pH, increased further in the presence of Al. Similar to the mortality rate and the hatching time delay, the effect of Al on the deformation rate was dependent on water pH. The effect was most pronounced at pH levels lower than 5.9, while at higher pH values no effects were observed.

a) Effects of low water pH.

Increasing effects of pH values lower than 5.2 on the deformation rate were already reported (Chapter 2).

b) Effects of low water pH and inorganic Al.

Reports on the effects of inorganic Al on the deformation rate of hatched larvae are scarce. For common carp, exposure to low Al levels at pH 5.4 already resulted in deformation ratios

of almost 100%. In contrast, Cleveland *et al.* (1986) found no effect of 11.1 μmol Al/l on eyed eggs of brook trout at pH 4.5 and 5.5. They found no effect on mortality and hatching. Kane and Rabeni (1987) noticed a very small increase of deformation of hatched smallmouth bass larvae as result of exposure to Al and at low pH. Elevated deformation ratios as result of acid stress were also recorded for *Pimephalus promelas* (Mount, 1973) and *Perca fluviatilis* (Runn *et al.*, 1977).

c) Effects of low water pH, inorganic Al and humic acids.

The increase of the deformation rate induced by Al at low pH disappeared completely when humic acids were added. As far as we know this has not been reported before.

4) Interpretation of the results with current models of Al toxicity.

We will interpret our observations on the basis of two models proposed for explaining Al-toxicity in fish gills: The Al-speciation model and the free ion binding model.

The Al-speciation model postulates that different products of Al hydrolysis dominate at different pH and that these $Al(OH)_x$ forms compete for binding sites on living tissue, each expressing a specific toxicity. The main (simplified) reactions are (Smith and Hem, 1972):

$$Al^{3+} + H_2O \rightleftarrows Al(OH)^{2+} + H^+$$

$$Al(OH)^{2+} + H_2O \rightleftarrows Al(OH)_2^+ + H^+$$

$$Al(OH)_2^+ + 2H_2O \rightleftarrows Al(OH)_4^- + 2H^+$$

Changes in water pH leading to shifts from one species of Al to another will also modify Al binding to a living tissue. The various positively charged Al species could theoretically bind with different affinities to for instance negatively charged glyco-proteins in mucus. Most pronounced negative effects of Al were observed when $Al(OH)_2^+$ was the dominant form in solution. When $Al(OH)_2^+$ is present, Al starts to polymerize, and it is this initial Al-polymerization which is thought to be toxic (Lydersen *et al.*, 1990). To test whether this model could explain our observations, 50 h mortality rates of carp eggs exposed to 0 and 2.8 μmol Al/l as well as the Al-induced mortality ((mortality pH + Al)-(mortality pH)) were plotted against pH (Fig 11). Al toxicity was compared with the expected corresponding activity fractions of the $Al(OH)_x$ forms in our experimental conditions. In the pH range 7.5 - 5.9, when Al is not toxic, $Al(OH)_4^-$ is the dominant aluminium form. At $5.9 < pH > 5.1$, when Al induces high mortality, the $Al(OH)_2^+$ form dominates. Between pH 5.1 and 4.8, the situation is complex: Al was protective at low, but toxic at high concentrations. At this pH the most dominating Al form is Al^{3+}. Apparently, at this pH and at a low total Al concentration, the concentration of $Al(OH)_2^+$ was not high enough to induce toxic effects.

The mitigating effect of low concentrations of Al on acid stress at these pH levels could be the result of competition of Al^{3+} with H^+ for binding sites. The toxicity of $Al(OH)_2^+$ became noticeable when total aluminium levels were higher. Thus, so far our results like those of others point to $Al(OH)_2^+$ as the most toxic form. However, the situation might be more complicated because the water pH experienced by the embryo might differ from the bulk water pH. Kugel and Peterson (1989) found for Atlantic salmon that the perivitelline fluid (pvf) had a small buffering capacity around water pH 5.3, and it is obvious that this buffer capacity influences the Al speciation experienced by the embryo. In the lower part of fig. 11, the Al speciation in the pvf of Atlantic salmon is visualised as function of the pH of the pvf if carp eggs show a similar difference between water pH and the pH of the pvf as eggs of Atlantic salmon. If this indeed applies to carp eggs, then exposure of the embryo in the eggs to $Al(OH)_2^+$ as the dominant Al form occurs in a broader pH range than the pH range where the toxic action of Al is expressed, and this makes the link between $Al(OH)_2^+$ and Al toxicity less exclusive.

One aspect that has been connected with Al toxicity to fish gills is Al-precipitation. The pH at the gill boundary layer is higher than that of the surrounding water as a result of metabolic processes (Exley *et al.*, 1991). Al could therefore precipitate on the gills because of reduced Al solubility in this layer. However, such a situation is unlikely for eggs. According to Peterson (1980), the pH of the perivitelline fluid of eggs of Atlantic salmon matched that of the surrounding water at pH 5.5. As mentioned above, at water pH > 5.3, the pH of the pvf was lower than the pH of the water. At water of pH < 5.3, the pH of the pvf was higher than that of the surrounding water, but never higher than pH 5.3 (Kugel and Peterson, 1989). From their data can be concluded that, if carp eggs behave similarly, the pH within the pvf never reach the values that will strongly reduce Al solubility. Thus, Al-precipitation is unlikely to occur in the pH range where Al appeared toxic in our experiments.

The second approach to explain Al toxicity is that of the free-ion binding model. It assumes that the toxicity is attributed to Al^{3+} binding at the surface of the exposed tissue. Some important assumptions are implicit in this model (Neville and Campbell, 1988):

1) The interaction of Al with the egg is represented in terms of $(X)_n$-Al-(L_n)-egg complexes ($X = H_2O$ or OH; L = ligand).
2) The toxic response is proportional to the concentration of the (X_n)-Al-L-egg complexes.
3) An equilibrium is rapidly established among Al species in solution, and between the solution and the egg surface.

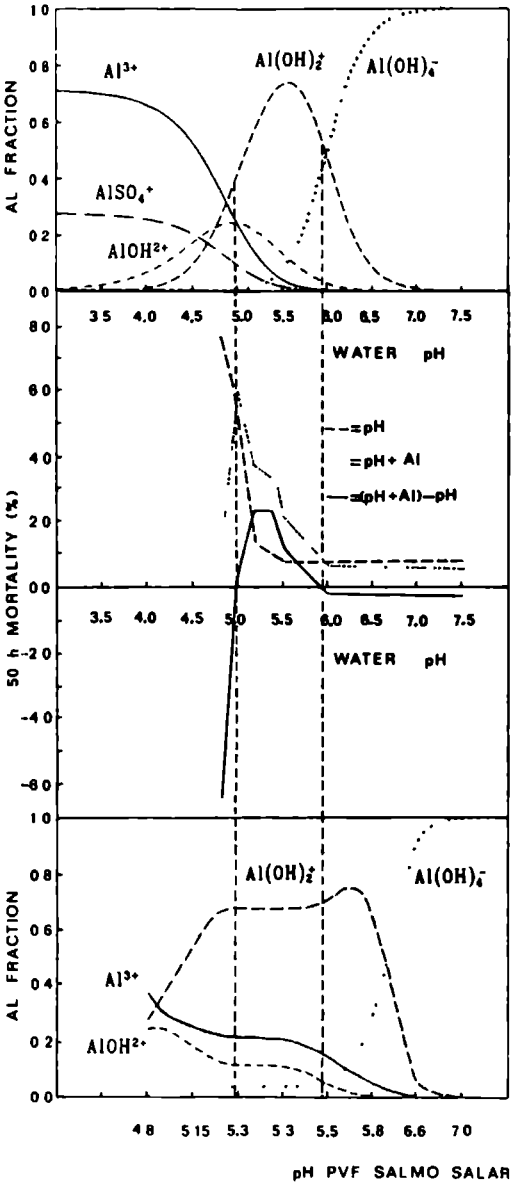

Fig. 10: Centre: 50 h mortality rates (%) of eggs exposed to different water pH levels with or without 2.8 µmol/l inorganic Al (mean values). In the upper graph the pH-dependent activities of the different Al forms in the surrounding water are plotted; in the lower part the corresponding activities of the different Al forms in the perivitelline fluid (and thus experienced by the embryo in the egg) are plotted. This curve is based on the assumption that the perivitelline fluid of these eggs has the same buffering capacity as that of eggs of *Salmo salar* (according to pH data of Kugel and Peterson, 1989).

According to Martin (1986), the only accessible oxidation state for Al in biological systems is Al^{3+}. It associates with oxygen donor ligands (L) like carboxylate and phosphate groups. Neville and Campbell (1988) studied Al effects on fish gills at different pH levels, and described the toxic action as a function of Al^{3+} complexation including competition with H^+ for the binding sites on the gills. This free ion model is described by the following reactions:

$$Al^{3+} + \text{L-gill} \rightleftarrows \text{Al-L-gill}$$
$$H^+ + \text{L-gill} \rightleftarrows \text{H-L-gill}$$
$$2H^+ + \text{L-gill} \rightleftarrows H_2\text{-L-gill}$$

On the basis of assumed pK_x values for gills, they calculated the [Al-L-gill] at different pH levels. They concluded that there was no interaction between Al^{3+} and the gills at pH > 6.5. A maximum Al-L-gill complexation was reached at bulkwater pH 5.0, which decreased at lower pH due to competition with H^+ ions. The pH of the maximum [Al-L-gill] can shift to lower or higher pH, depending on the strength of the competition by H^+. We found maximum mortality at pH 5.2. This value is very close to the maximum [Al-L-egg] predicted by this model. The model further predicts a diminished Al toxicity at pH levels lower than pH 5.0, and this is also in line with our observations. However, according to Neville & Campbell (1988) not only Al-L-egg effects but also H-L-egg effects have to be taken into account. At pH 5.1 - 4.8, we observed a mitigating effect of Al on H^+ toxicity on the eggs. The small amount of Al adhered to the egg shell or the perivitelline fluid might protect the embryo, possibly by lowering the [H_x-L-egg] by competition of Al^{3+} for these binding sites. The Al^{3+} absorbed to the chorion could prevent the entering of H^+ ions into the egg shell because of the strong positive charge of Al^{3+}. A similar explanation has been presented for the transient protection effect of Al found on the gills in this pH range (Neville and Campbell, 1988). Perhaps the disappearance of this mitigating effect of Al on the pH toxicity after hatching is due to the disappearance of the protective chorion. Thus, the free ion model can explain our observations of Al toxicity on egg mortality.

Neville and Campbell (1988) found two peaks of Al toxicity for fish gills: one in the pH range from 4.5 - 4.0 and one in the pH range from 6.5 - 5.5. The first peak was associated with osmoregulatory disturbances. For this peak, that could be predicted by the free ion model, Neville & campbell found similar pH-related toxicity curves for mature fish as found in our experiments with eggs. The peak situated in the pH-range 6.5 - 5.5 was associated with asphyxiation and could not be explained by the free ion binding model. This was a reason for Neville and Campbell's (1988) postulation of mixed ligand hydroxy-Al complexes:

$$Al^{3+} + OH^- + \text{L-gill} \rightleftarrows \text{HO-Al-L-gill}$$

With this addition, the model could explain both Al toxicity peaks. One explanation of the incorporation of mixed ligand hydroxy-Al species in this model was a shift from the highest concentration point of the [Al-L-gill] complex from pH 5.0 to pH 4.7. Although this could explain the first Al-toxicity peak in the studies of Neville & Campbell, it does less satisfactorily explain our observations on carp eggs because we found an Al-toxicity peak at pH 5.9 - 5.1. The extended model further predicted a bell-shaped curve for [HO-Al-L-gill] complexes with highest concentration levels between pH 6.5 and 5.5, explaining the Al-toxicity peak found by Neville and Campbell (1988) in this pH range. If this second Al toxicity peak is the result of the pH shift at the gill boundary layer, a possibility mentioned before, this extended model could also explain our results. Then, the observed Al toxicity to carp eggs (pH 5.9 - 5.1) matches the low-pH Al toxicity peak for the fish gills which disturbs osmoregulation (pH 4.5 - 4.0). In addition, the high-pH Al toxicity peak which disturbs oxygen uptake in fish gills can not express itself when eggs are concerned, since, as discussed above, the pH shift needed for Al precipitation does not occur. Although the model of free ion binding can explain our results without the assumption of HO-AL-egg complexes, with the assumption of these complexes our results on carp eggs fit a more widely applicable model for Al toxicity.

Humic acids showed a very pronounced detoxifying effect on Al, probably by reducung the Al bioavailability. An attempt was made to integrate this action of humic acids into the proposed models.

a) Al-speciation model.

Next to Al^{3+}, humic acids also bind to divalent and monovalent ions, in this case to $Al(OH)^{2+}$ and $Al(OH)_2^{+}$, although in general these complexes are less stable (Kawaguchi and Kazutake, 1959). This might result in complexes that are not able to interact with the egg anymore. One might speculate that HA protects better against Al^{3+} than against $Al(OH)_x$ at higher pH levels. On the other hand, competition with H^+ ions is less at these higher pH levels. When humic acids remove Al^{3+} from the solution, toxic $Al(OH)_x$ will be transformed into Al^{3+} in accordance to the equillibrium reactions. Binding of humic acid to Al^{3+} ions (Mak and Langford, 1982) explains why the protective effect of Al^{3+} at low pH disappeared when humic acids were added.

b) Binding of Al^{3+} to ligands on the eggs with competition of H^+.

Introduction of humic acids (HA) into this model leads to the following possibilities:

$$H^+ + \text{L-egg} \rightleftarrows \text{H-L-egg} \quad (1)$$

$$2H^+ + \text{L-egg} \rightleftarrows H_2\text{-L-egg} \quad (2)$$

$$HA + xH^+ \rightleftarrows HA(H)_x \quad (3)$$

$$Al^{3+} + \text{L-egg} \rightleftarrows \text{Al-L-egg} \quad (4)$$

$$HA + Al^{3+} + \text{L-egg} \rightleftarrows \text{HA-Al} + \text{L-egg} \rightleftarrows \text{HA-Al-L-egg} \rightleftarrows HA + \text{AL-L-egg} \quad (5)$$

Since humic acids have a very high affinity for Al^{3+} (5), the free concentration of this ion will become very small. Thus, the formation of Al-L-egg (4) will be slowed down. It might even be possible that Al will be removed from the Al-L-egg complex by humic acids (5). Another factor contributing to the decrease of Al toxicity in the presence of HA is that the HA-Al complex most likely is not toxic. The detoxification capacity of humic acids is predicted to be less at very low pH because of competition of Al^{3+} with H^+ ions (3); possibly because the tested concentration of humic acids was very high in relation to the Al levels, such an effect was not observed in our experiments.

c) Binding of Al^{3+} to egg ligands with H+ competition, with the addition of mixed ligand hydroxy-Al complexes.

The binding strengths of HO-Al to HA or to L-egg, which are not known at the moment, will determine whether HA is able to remove HO-Al from the HO-Al-L-egg complex or that L-egg can remove HO-Al from the HA-HO-Al complex. If the complex HO-Al-L-egg is created as a transformation of Al-L-egg, then formation of this complex will be very slow when HA is added, since this leads to low Al-L-egg concentrations (5).

Conclusions

- Toxicity of inorganic monomeric hydroxide did result in:
  1) Elevated mortality.
  2) Delayed hatching and elongation of the hatching process, making eggs more vulnerable to predation.
  3) Elevated deformation percentages. Deformed larvae can not catch their food or escape from predators. In the natural situation this leads to elevated secondary mortality levels.

- Acute toxicity of inorganic monomeric Al hydroxide is pH dependent:
  pH 7.5 - 5.9: - No effect on mortality, hatching time or deformation rate.
  pH 5.9 - 5.1: - Increasing mortality, directly related to Al levels.
  - Very strong hatching time delay, positively related to Al levels; elongation of the hatching process.
  - Deformation rate is increasing markedly with the Al concentration; carp is very sensitive in this aspect when compared to other species.

pH 5.1 - 4.8: - At low levels, Al protects eggs against acid stress but becomes toxic during and after hatching. This protective effect also disappears when Al levels are higher, and Al promotes mortality. Regarding hatching time, low levels of Al protect against low pH resulting in a less pronounced delay than from low pH alone. This protective effect disappears at higher Al concentrations.

- Although not reflected in the percentage of deformed larvae, the protective effect of low aluminium concentrations against acid stress expresses itself via a lowered degree of deformation.

- When extrapolating the results to the field situation, one may conclude that the presence of inorganic monomeric hydroxide in combination with low pH elevates the pH level critical for survival of the population from pH 5.2 - 5.0 (Chapter 2) to pH 5.9 - 5.5.

- When monomeric Al hydroxide is complexed with humic acids, it has no effect on mortality, hatching time and deformation in the pH range of 7.5 - 4.8. Humic acids have a very pronounced detoxifying effect on the toxicity of Al hydroxide. They also delete the transient protective effect of inorganic Al on acid stress at low pH.

- The effects of Al on carp eggs, as observed during our experiments, are compatible with most of the current theories (Al speciation model, free ion binding model).

References

Bache, B.W., 1986. Aluminium mobilization in soils and waters. J. Geol. Soc. (Lond.) 143: 699-706.

Baker, J.P., 1982. Effects on fish of metal associated with acidification. In: Acid rain/fisheries, R.E. Johnson [ed.], Proc. Int. Symp. acid Precip Fish Imp , American Fisheries Society, Bethesda, MD, 165-175.

Baker, J.P., 1984. Fishes, pp 5-75 to 5-139. In: A A. Altshuller and R.A. Linthurst [ed]: The acid deposition phenomenon and its effects: Critical Assessment Review Papers, Vol II., U.S. Environmental Protection Agency, Washington, DC.

Baker, J.P. and C.L. Schofield, 1982. Aluminium toxicity to fish in acidic waters. Water Air and Soil Poll. 18. 289-309.

Baker, J.P. and C.L. Schofield, 1980. Aluminium toxicity to fish as related to acid precipitation and Adirondack surface water quality. Ecol. Imp. Acid Precip. Proc. Int. Conf., Sandefjord, Norway, March 11-14, 1980, pp. 292-293.

Birchall. J.D., C. Exley, J.S. Chappell and M.J. Phillips, 1989. Acute toxicity of aluminium to fish eliminated in silicon-rich acid waters. Nature 339, 146-148.

Brown, D.J.A., 1983. Effect of calcium and aluminium concentrations on the survival of brown trout (*Salmo trutta*) at low pH. Bull. Environ. Contam. Toxicol. 30, 582-587.

Brown, D.J.A. and S. Lynam, 1981. The effect of sodium and calcium concentrations on the hatching of eggs and the survival of the yolk sac fry of brown trout, *Salmo trutta* L. at low pH. J. Fish Biol. 19, 205-211.

Buckler, D.R., P.M. Mehrle, L. Cleveland and F.J. Dwyer, 1987. Influence of pH on the toxicity of aluminium and other inorganic contaminants to East coast striped bass. Water Air and Soil Poll. 35, 97-106.

Burrows, W.D., 1977. Aquatic aluminium: chemistry, toxicology, and environmental prevalence. CRC Crit. Rev. Environ. Control 7, 167-216.

Butler, J.N., 1964. Ionic equilibrium. A mathematical approach. Addisson-Wesley Publishing Company; Inc., Reading, Massachussets.

Cleveland, L., E.E. Little, S.J. Hamilton, D.R. Buckler and J.B. Hunn, 1986. Interactive toxicity of aluminium and acidity to early life stages of brook trout. Trans. Am. Fish. Soc. 115, 610-620.

Cronan, C.S., and C.L. Schofield, 1979. Aluminium leaching response to acid precipitation: Effects on high-elevation watersheds in the northeast. Science 204: 304-306.

Daye, P.G. and E.T. Garside, 1979. Development and survival of embryos and alevins of the Atlantic salmon, *Salmo salar* L., continuously exposed to acidic levels of pH, from fertilization. Can. J. Zool. 57, 1713-1718.

Driscoll, C.D., J.P. Baker, J.J. Bisgoni, Jr., and C.L. Schofield, 1980. Effects of aluminium speciation on fish in dilute acidified waters. Nature 284, 161-164.

Exley, C., J.S. Chappell and J.D. Birchall, 1991. A mechanism for acute aluminium toxicity in fish. J. Theor. Biol. 151, 417-428.

Fromm, P.O., 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Env. Biol. Fish. 5, 79-93.

Gajduzek, J. and V. Rubcov, 1983. The microstructure of egg membranes in carp, *Cyprinus carpio*. Folia Zool. 32, 271-279.

Helliwell, S., G.E. Batley, T.M. Florence and B.G. Lumsden, 1983. Speciation and toxicity of aluminium in a model fresh water. Environ. Technol. Lett. 4: 141-144.

Holtze, K.E. and N.J. Hutchinson, 1989. Lethality of low pH and Al to early life stages of six species inhabiting Precambrium Shield waters in Ontario. Can. J. Fish. Aquat. Sci. 46, 1188-1202.

Hunn, J.B., L. Cleveland and E.E. Little, 1987. Influence of pH and aluminium on developing brook trout in a low calcium water. Environm. Poll. 43, 63-73.

Jensen, K.W. and E. Snekvik, 1972. Low pH levels wipe out salmon and trout populations in southern Norway. Ambio 1, 223-225.

Kane, D.A. and C.F. Rabeni, 1987. Effects of aluminium and pH on the early life stages of smallmouth bass (*Micropterus dolomieu*). Water Res. 21, 633-640.

Kamler, E., 1976. Variability of respiration and body composition during early developmental stages of carp. Pol. Arch. Hydrobiol. 23/3, 431-485.

Kawaguchi, K. and K. Kazutake, 1959. On the complex formation between soil humus and polyvalent cations. Soil Plant Food 5, 54-63.

Khan, S.U., 1969. Soil Sci. Soc. Am. Proc. 33, 851.

Kossakowski, K., 1988. Larval development of Carp, *Cyprinus carpio* L., in acidic water. J. Fish Biol. 32, 17-26.

Kudo, S., 1982. Ultrastructural modifications of fertilization envelopes in the carp egg. Annot. zool. Japon. 55, 224-235.

Kugel, B. and R.H. Peterson, 1989. Perivitelline fluid pH of rainbow trout (*Oncorhynchus mykiss*) eggs in relation to ambient pH. Can. J. Fish. Aquat. Sci. 46, 2070-2073.

Klauda, R.J., R.E. Palmer and M.J. Lenkevich, 1987. Sensitivity of early life stages of blueback herring to moderate acidity and aluminium in soft freshwater. Estuaries 10, 44-53.

Kwain, W. and G.A. Rose, 1985. Growth of brook trout *Salvelinus fontinalis* subject to sudden reductions of pH during their early life history. Trans Am. Fish. Soc , 114-570.

LaZerte, B., 1984. Forms of aqueous aluminium in acidified catchments of southern Ontario: A methodological analysis. Can. J. Fish. Aquat. Sci. 41, 766-776.

Leivestad, H., E. Jensen, H. Kjartansson and L. Xingfu, 1987. Aqueous speciation of aluminium and toxic effects on Atlantic salmon. Annls. Soc. R. Zool. Bel. 177, suppl. 1, 387-398.

Leuven, R.S.E.W., 198 . Impact of acidification on aquatic ecosystems in the Netherlands. Ph. D. thesis, Catholic University Nijmegen, The Netherlands.

Lydersen, E., A.B.S. Poleo, I.P. Muniz, B Salbu and H.E. Bjornstad, 1990. The effects of naturally occurring high and low molecular weight inorganic and organic species on the yolk-sac larvae of Atlantic salmon (*Salmo salar* L.) exposed to acidic aluminium-rich lake water. Aquat. Tox. 18, 219-230.

Mak, M.K.S. and C.H. Langford, 1982. A kinetic study of the interaction of hydrous aluminium oxide colloid with a well-characterized soil fulvic acid. Can J. Chem, 60: 2023-2028.

Martin. R.B., 1986. The chemistry of aluminium as related to biology and medicine. Clin. Chem. 32, 1797-1860.

Mount, D.I., 1973. Chronic effect of low pH on fathead minnow survival, growth and reproduction. Water Res. 7, 987-993.

Morris, T.K. and G.L. Krueger, 1984. Ecological effects of aluminium complexes on freshwater fish and their environments. In: Bhumralkar, C.N. [ed.]. Meteorological aspects of acid rain. Butterworth Publishers, Boston, 231-239.

Muniz, I.P. and H. Leivestad, 1980. Toxic effects of aluminium on the brown trout, *Salmo trutta* L.. Proc. Int. Conf. Ecol. Impact Acid Precip., SNSF project, 320-321.

Neville, C.M. and P.G.C. Campbell, 1988. Possible mechanisms of aluminium toxicity in a dilute, acidic environment to fingerlings and older life stages of salmonids. Water Air Soil Pollut. 42, 311-321.

Peterson, R.H., P.G Daye and J L Metcalfe, 1980. The effects of low pH on hatching of Atlantic salmon eggs. Proc. Int Conf Ecol. Imp. Acid Precip., SNSF Project, p. 328.

Peterson. R.H., P.G. Daye and J.L. Metcalfe. 1981. Inhibition of Atlantic salmon (*Salmo salar*) hatching at low pH. Can. J. Fish Aquat. Sci. 37, 770-774.

Playle, R.C. and C.M. Wood, 1990. Is precipitation of aluminium fast enough to explain deposition on fish gills? Can. J. Fish. Aquat. Sci. 47, 1558-1561.

Potts, W.T.W. and P.P. Rudy, Jr. 1969. Water balance in the eggs of the Atlantic salmon, *Salmo salar*. J. Exp. Biol. 50. 223-237.

Rask, M., 1983. The effect of low pH on perch, *Perca fluviatilis* L. I. Effects of low pH on the development of eggs of perch. Ann. Zool. Fennici 20, 73-76.

Reid, S.D., McDonald, D.G. and R.R. Hem, 1991. Acclimation to sublethal aluminium: Modifications of metal-gill surface interactions of juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 48, 1996-2005.

Rombough, P.J., 1982. Effects of low pH on eyed embryos and alevins of Pacific salmon. Can. J. Fish. Aquat. Sci. 40, 1575-1582.

Rosseland, B.O. and O.K. Skogheim, 1982. Physiological stress and mortality of Atlantic salmon, *Salmo salar* L. in acid water with high levels of aluminium. Ices Council Meeting, Copenhagen (Denmark), 1982: 16 pp.

Rubtsov, V.V., 1982. The dependence of some morphophysiological characteristics of the eggs of the carp *Cyprinus carpio* L. on changes in the microstructure of the egg membrane. J. Ichthyol. 21, 43-51.

Runn, P., N. Johansson and G. Milbrink, 1977. Some effects of low pH on the hatchability of eggs of perch, *Perca fluviatilis* L., Zoon 5, 115-125.

Schnitzer, M. and H. Kerndorff, 1980. Reactions of fulvic acid with metal ions.

Schnitzer, M., 1969. Reactions between fulvic acid, a soil humic component and inorganic soil constituents. Soil Sci. Soc. Amer. Proc. 33, 75-81.

Skogheim, O.D. and B.O. Rosseland, 1984. A comparative study on salmonid fish species in acid aluminium-rich water I. Mortality of eggs and alevins. Rep. Inst. Freshwat. Res. 61, 177-185.

Smith, R. W. and J.D. Hem, 1972. Effect of aging on aluminium hydroxide complexes in dilute aqueous solutions. Geological Survey Water-supply Paper 1827-D, 51pp.

Swarts, F.A., W.A. Dunson and J.E. Wright, 1978. Genetic and environmental factors involved in increased resistance of brook trout to sulphuric acid conditions and mine polluted waters. Trans. Am. Fish. Soc. 107, 651-677.

Thomsen, A., B. Korsgaard and J. Joensen, 1988. Effect of aluminium and calcium ions on survival and physiology of rainbow trout *Salmo gairdneri* Richardson) eggs and larvae exposed to acid stress. Aquatic Toxicol., 291-300.

Wilkinson, K.J., P.G.C. Campbell and P. Couture, 1990. Effect of fluoride complexation on aluminium toxicity towards juvenile Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 47, 1446-1452.

Wilson, A.D. and G.A. Sergeant, 1963. The colorimetric determination of aluminium in mineralsby pyrocatechol violet. Analyst 88: 109-112.

Wollast, R., L. Chou, V. Debbaut, A. Wollast and J. Zobrist, 1984. The influence of acid rains on the geochemistry of aluminium. Proc. "Acid deposition and sulphur cycle" Brussels, June 1984, 277-286.

Zotin, A.I., 1966. The uptake and movement of water in embryos. Symp. Soc. Biol. 19, 365-384.

CHAPTER 5

Induction of spinal cord deformation and delay of hatching of early life stages of carp by acid water and aluminium: causative factors.

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Induction of spinal cord deformation and delay of hatching of early life stages of carp by acid water and aluminium: causative factors.

Abstract

Factors were examined that possibly induce elevated spinal cord deformation rates and delay of hatching in developing eggs of carp reared in acid water with or without aluminium (Al). Under both conditions, deformation was already induced before normal hatching time, suggesting that delayed hatching was not caused by the elevated deformation rates. Conversely, elevated deformation rates might result in delayed hatching.

Elevated Al concentrations at pH 5.4 effectively reduced the frequency of trunk movement. This inhibits mechanical rupture of the chorion, possibly leading to delayed hatching.

Light and electron microscopic studies of eggs exposed to low water pH revealed coagulation of the perivitelline fluid. This was more prominent in the presence of Al. Coagulation may cause a reduced motility of the embryo and, in addition, may hamper the dispersion of the hatching enzyme. It may further contribute to spinal cord deformation and delayed hatching.

Introduction

One of the major mechanisms underlying the negative effects of acid water is recruitment failure. In the studies on the effects of low pH and Al on early life stages of fish, most attention has been paid to mortality. Few data are available on sublethal effects on the early life stages, such as hatching delay and deformation of larvae. Such effects have been reported for eggs and larvae of perch, brook trout and Atlantic salmon when exposed to acid water (Rask, 1983; Runn *et al.*, 1977; Swarts *et al.*, 1978; Peterson *et al.*, 1980).

Studies in our laboratory have shown that low pH, with or without elevated Al levels, induced pronounced hatching delay and elevated deformation percentages of embryos of the common carp (Chapter 2). In particular the increased deformation rate of carp larvae was much higher than that recorded for salmonids (Swarts *et al.*, 1978; Peterson *et al.*, 1980). In these studies, several explanations for the hatching delay were proposed, such as retardation in development, inhibition of the hatching enzyme, or reduced trunk movements. The latter may result in a less efficient distribution of the hatching enzyme or in hampered mechanical rupture of the chorion. Our studies on the development of carp (Chapters 2,4) suggest two other possibilities. First, the hatching delay may be caused by the deformation of the embryo. Hatching delay and deformation both increased markedly in acid water, and both were further stimulated when Al was present in addition. Second, we presented evidence indicating that the proteins of the perivitelline fluid (pvf) may coagulate. The resulting

increased viscosity of the pvf may inhibit trunk movements. Both possibilities were studied in this investigation.

Materials and methods

Fertilization and incubation of eggs.

Carp gametes were obtained as described in Chapter 2, as were incubation methods. The ionic composition of the water and the techniques to maintain water pH levels and Al concentrations were identical to those described in Chapter 4.

Relationship between deformation and hatching time.

In the first experiment, four groups of approximately 1000 eggs each, kept at pH 5.4, were exposed to inorganic Al (added as aluminium hydroxide) at concentrations known to induce larval deformation and hatching delay (Chapter 4): 0 (controls), 1.85 and 3.7 μmol Al/l. One group kept at pH 5.4 was exposed to 3.7 μmol Al/l only during the first 20 h; the other groups during 10 days.

Numbers of hatched larvae were counted every 3 h, starting at 48 h and ending at 96 h after fertilization. Further monitoring was done every 8 h, until all eggs had either hatched or were dead. Hatching was defined as rupture of the egg membrane and the partial or total emergence of the larvae.

At 48, 72, 96,120, 168 and 240 h after fertilization, the numbers of deformed larvae were counted. Deformed larvae were defined as larvae with a deformed spinal cord. The first two time points were chosen to see whether deformation already occurred before normal hatching time. At the time points where hatching had not yet occurred, 100 embryos per experimental group were dissected out of the egg shell with fine needles in order to count the number of deformed larvae. The dissected larvae were examined 30 minutes later, when the normal larvae had stretched themselves and could be distinguished from the deformed larvae. After hatching, the deformation percentage of all hatched larvae was determined at the time points indicated above.

Effects of Al on frequency of trunk movements.

In a second experiment, we determined the effects of Al on body motility. For this purpose, eggs were exposed after fertilization to 0 (control), 1.4, 2.0, 3.0 and 5.2 μmol Al/l (mean measured values; see Chapter 4) at pH 5.4. The number of trunk movements was recorded at 52 $\pm$ 0.5 h after fertilization, just before the time that hatching could be expected

under normal conditions. Per experimental group, 30 embryos were observed for 1 min. under a dissection microscope.

Effects of low water pH and Al on the perivitelline fluid.

To examine the effects of low water pH and Al on the perivitelline fluid, eggs were reared at pH 4.8 and at pH 5.4 + 2.0 μmol Al/l, and compared with eggs reared at pH 7.5 and pH 5.4 without Al (controls), where no such effects do occur (Chapters 2 and 4). At 48 h, eggs were collected and fixed in a mixture of osmium tetroxide, glutaraldehyde and $K_2Cr_2O_7$ (Wendelaar-Bonga *et al.*, 1990), embedded in Spurr's resin, and sectioned for light or electron microscopic observation. Prior to light microscopic examination, the sections were stained with methylene blue. Intact living eggs and larvae were photographed at 48, 60, 84 and 144 h after fertilization, using a dissection microscope.

Statistical analysis.

The deformation rates of the first experiment were analyzed with the Student t-test for statistical significance at the time-point before normal hatching. Significance was accepted at $P < 0.005$.

In the second experiment, the same test was applied to test the significance of the differences in the trunk movement frequencies between embryos exposed to the different Al concentrations and controls.

Results

Relationship between deformation and hatching time.

Fig. 1 shows the relationship between deformation and hatching time delay. The percentage of spinal cord deformation of eggs reared in water of pH 5.4 without Al was similar to that found at pH 7.5. Exposure to Al at pH 5.4 increased the deformation percentages, and further resulted in a delay of hatching and a prolongation of the hatching process. The deformation rate changed with time. Almost all the deformation occurred before the time that 95 % of the larvae would have hatched under normal conditions, thus indicating that hatching delay could not be the cause of the increase in deformation percentage.

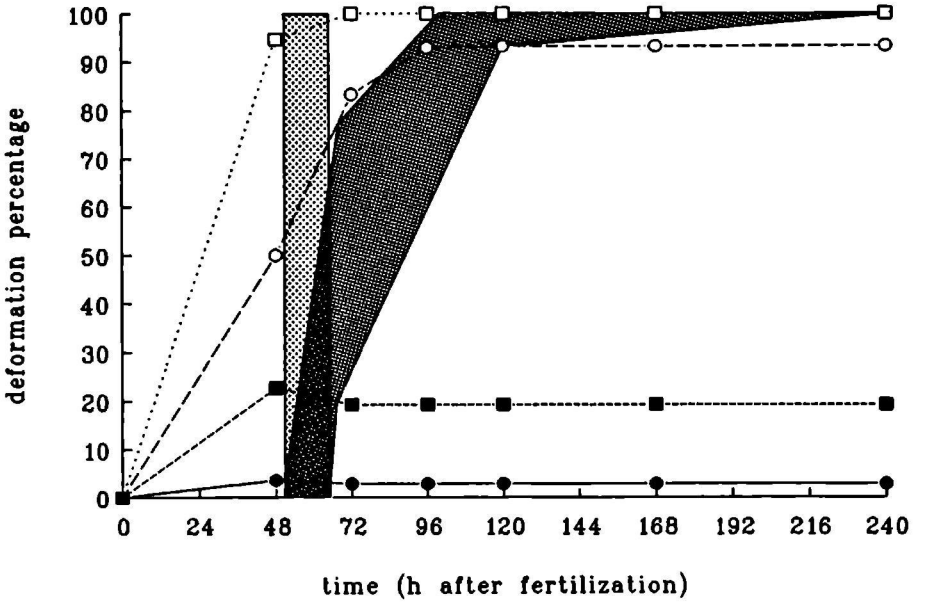


Fig. 1: Changes of Al induced spinal cord deformation rates (• = pH 5.4; □ = pH 5.4 + 20 h 3.7 μmol Al/l; o = pH 5.4 + 1.85 μmol Al/l; □ = pH 5.4 + 3.7 μmol Al/l) in time and their corresponding hatching periods at pH 5.4; light area represents 5-95 % hatching time interval at pH 5.4; dark area represents 5-95 % hatching time interval at pH 5.4 in the presence of the Al concentrations mentioned.

Effects of Al on frequency of trunk movements.

Fig. 2 presents the influence of different Al concentrations in the water on the frequency of trunk movements. It illustrates clearly that exposure to Al results in a dose- dependent reduction of the trunk movements. Even if the Al concentration was as low as 1.4 μmol/l, the activity of the embryos was reduced.

Effects of low water pH and Al on the perivitelline fluid.

In 24 or 48 h old encapsulated embryos reared at pH 7.5 and pH 5.4 development was normal, and no visible changes in the perivitelline fluid were observed in the living eggs (Fig. 3a). This was not the case when eggs were reared at pH 4.8 and pH 5.4 + 2 μmol Al/l. Large coagulating bodies, being initially transparent, but turning opaque in time, were present at 24 and 48 h after fertilization (Fig. 3b). During dissection of living eggs, these structures appeared to be rigid and gelatinous. After 60 h, the perivitelline space was almost completely filled with these opaque structures (Fig. 3c).

Light microscopic study of the pvf also learned that coagulation occurred in the perivitelline fluid of eggs reared at pH 4.8 (Fig. 4) and at pH 5.4 + 2 μmol Al/l.

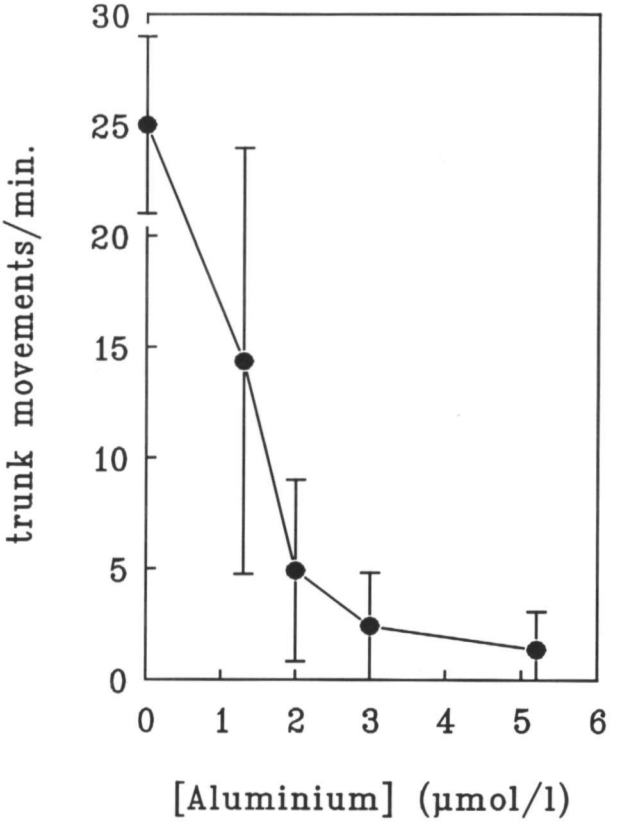


Fig. 2: Number of trunk movements per minute of 52-h encapsulated embryos reared at different Al concentrations at pH 5.4 (mean values ± SD; n = 30).

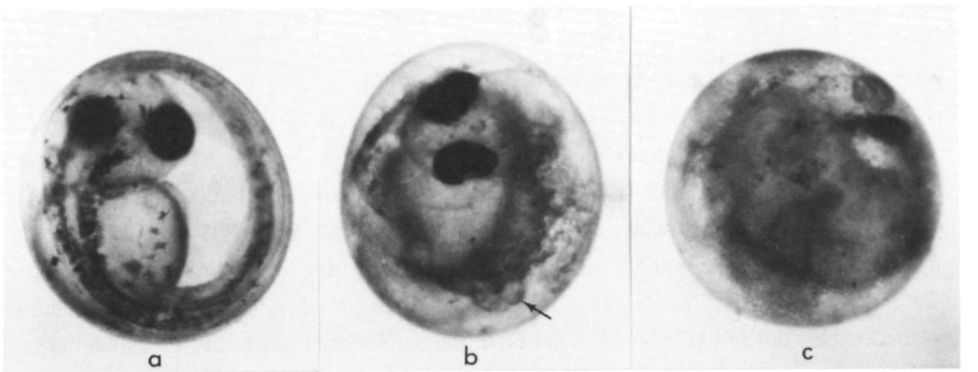


Fig. 3: Micrographs of 48-h carp eggs reared at pH 5.4 control (3a) and pH 5.4 + 2 µmol Al/l (3b), and of 60-h carp egg exposed to pH 5.4 + 2.0 µmol Al/l (3c); 15 x. Figs. 3b and 3c show the occurrence of perivitelline coagulations.

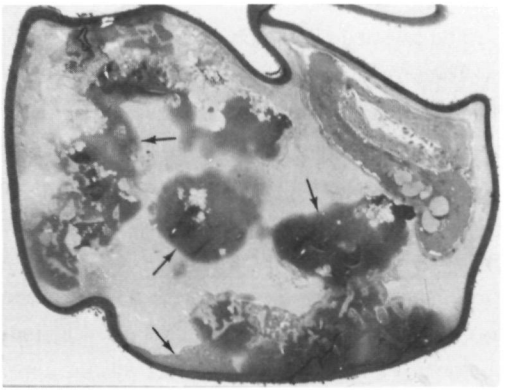


Fig. 4: Light microscopic picture of perivitelline space of 48-h carp egg exposed to pH 4.8, showing coagulations (arrows) in the pvf (40 x).

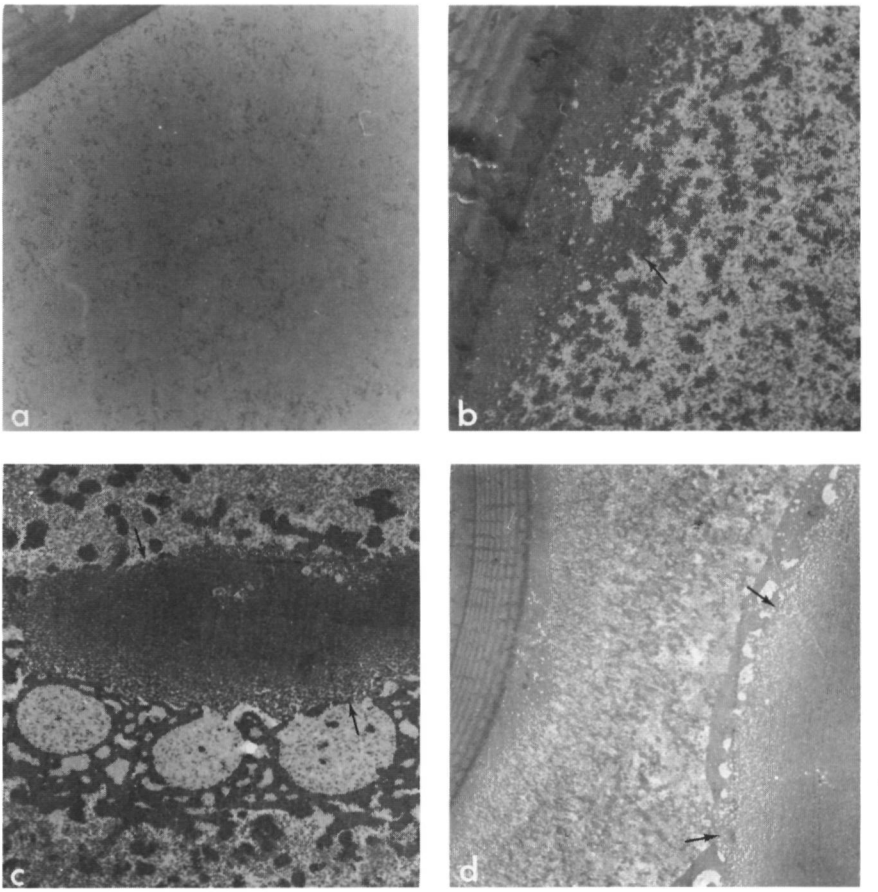


Fig. 5: Electron microscopic pictures of perivitelline space of 48-h carp eggs reared at pH 7.5 (5a, 1300 x) and at pH 4.8 (5b, 2000 x; 5c, 3500 x; 5d, 700 x). Arrows point to large coagulations.

A considerable part of the pvf appeared to be affected. These phenomena were not observed at neutral pH or at pH 5.4 without Al. The structures formed in the pvf at pH 4.8 were studied at the electron microscopic level and compared with controls at pH 7.5 (Fig. 5). Only few very small granulated structures were found at neutral pH (Fig. 5a). Exposure to pH 4.8 resulted in a strongly increased number of these granulated structures, that tended to coagulate at the egg membrane (Fig. 5b). Furthermore, large accumulations of these granules were visible, surrounded at the border by droplet-like structures (Fig. 5c,d).

Larvae which hatched from eggs with coagulations in the pvf showed very pronounced spinal cord deformations. The presence of Al at pH 5.4, and water of pH 4.8, induced a curved spinal cord and swelling of the coelome (Fig. 6 b). Another effect was the appearance of two-headed larvae (Fig. 6 c) in about 2 % of the deformed larvae. Fig. 6d shows a typical example of a larvae which did not hatch. Its body was curved into the shape of the egg.

Discussion

Spinal cord deformation.

We have demonstrated that body deformation can occur already before hatching, which indicates that deformation is not caused by delayed hatching. Conversely, it is possible that hatching delay is caused by reduced motility of the embryo, i.e., its unability to rupture the chorion mechanically. However, this does not exclude that hatching delay has an influence on deformation. Although hatching delay had no or little influence on the percentage of deformation, it apparently aggravated the degree of deformation: larvae showing severely delayed hatching were much more deformed than larvae showing only a slight hatching delay. The spinal cord of the severely deformed larvae followed the curvature of the egg shell. Runn *et al.* (1977), who exposed eggs of perch (*Perca fluviatilis*) at pH 4.5 and 4.0, also observed hatching delay and a dramatic increase in deformation rates (up to 100 %). In contrast to our results, they found that elevated deformation rates occurred only after the time of normal hatching. They considered the elevated deformation rates a result of the delay in hatching. When perch eggs were exposed to low pH conditions immediately after fertilization, a reduction of the inner egg volume was measured (Shephard, 1987; Runn *et al.*, 1977), and thus mechanical compression of the larvae might contribute to spinal cord deformation.

The perivitelline fluid.

We observed coagulation of the perivitelline colloidal material when carp eggs were reared at pH 4.8 and at pH 5.4 + 2 μmol Al/l. After the eggs enter the water, their cortical alveoli release a colloidal material into the perivitelline space. Eddy and Talbot (1983) described the

pvf as a relatively open network structure of colloidal polymers with gel-like properties. It is osmotically active, causing the water to enter the egg and form the perivitelline space (Peterson and Martin-Robichaud, 1982). According to Eddy (1974) the pvf consists for 58 % of water. This perivitelline colloid has a net negative charge at normal pH levels and can accumulate cations in concentrations in excess of the ambient water. Thus, it can maintain an equilibrium potential across the chorion. The pvf and the chorion are thought to influence the availability of ions for the embryo and also to prevent ion loss (Eddy and Talbot, 1985).

Effects of low pH and Al on the pvf have been described earlier. Kudo (1982) mentions that polymerisation of the crystalline material from the cortical alveoli to form the colloid is calcium dependent. Since low water pH and Al often interfere with calcium mediated processes (Verbost *et al.*, 1992), effects on the formation of the colloid material into the pvf might be possible. Indeed, Peterson and Martin-Robichaud (1982) observed a decreased water uptake and content of the pvf at acidity levels of pH 5.0-4.0, while the pvf formation in salmon eggs was almost completely inhibited in the presence of Al. They suggested that H^+ has a similar effect as Al, although at much higher concentrations. These authors proposed denaturation of the colloids in the perivitelline space by low pH and Al, likely reducing the osmotic activity of the colloids. No denaturation was observed in the pvf, although white areas of denaturated yolk were reported. Additionally, reduction of the egg diameters has been observed in Atlantic salmon eggs at low pH, indicating also a reduced osmotic activity of the pvf (Shephard, 1987). Eddy and Talbot (1983; 1985) reported that the Na^+ influx into eggs of Atlantic salmon was almost completely inhibited at pH 4.0 because of saturation of the perivitelline binding sites for cations by H^+ and Al. Some studies have been performed on the perivitelline potential, which reflects the ability of the pvf to accumulate cations (Peterson, 1984; Shephard, 1988). This potential was reduced or reversed from negative to positive when pH was lowered or Al was added, thus reducing the capacity of the pvf to accumulate cations.

In conclusion, it is clear that an interaction takes place between the pvf and H^+ and Al, and that these ions change the properties of the pvf. However, the formation of big coagulates as observed in our study has not been reported before. Possibly, the proteins coagulate when cations are gradually being replaced by H^+ and Al. The coagulated structures become visible only at 24 h after fertilization and not immediately after hatching. The effect of H^+ and Al appears to be similar but larger amounts of H^+ than Al are needed to obtain the same effect. A similar difference in effectiveness of H^+ and Al has been observed by Peterson and Martin-Robichaud (1982), who studied the inhibition of the formation of the pvf in salmon eggs by H^+ and by Al.

Coagulation could have different effects. The mobility of the embryos in the partially or fully coagulated fluid is probably reduced, and this may result in forced growth in a fixed body position. Coagulation may also reduce diffusion of catabolites out of the egg.

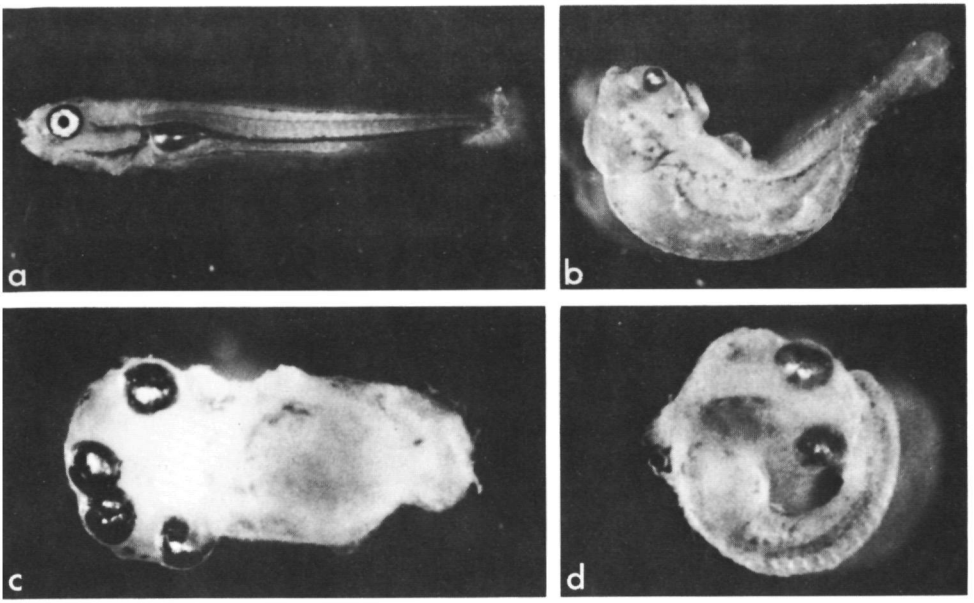
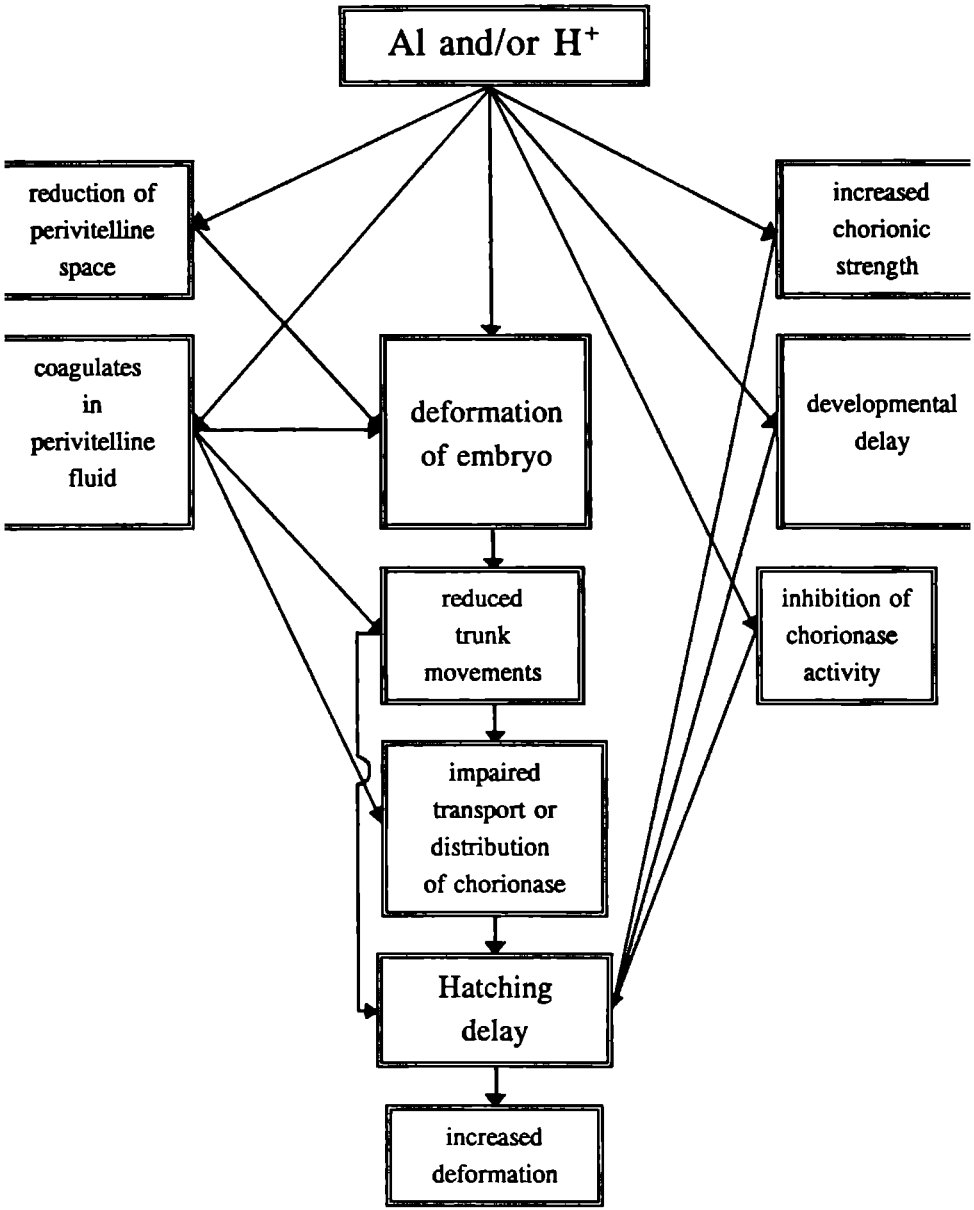


Fig. 6: 144-h larvae reared at pH 7.5 (6a, normal larvae, 8 x) and at pH 5.4 + 2 μmol Al/l (6b, deformed larvae, 9 x; 6c, two-headed larvae, 16 x; 6d, non-hatched larvae, 14 x).

Accumulation of these substances into the pvf might enhance deformation. Finally, oxygen diffusion via the chorion through the pvf to the embryo could be diminished. Low oxygen levels are known to induce skeletal anomalies (Bengtsson, 1979).

Delayed hatching.

The mechanisms of hatching delay have mainly been studied in salmonids. Low pH is known to delay hatching of these fish (Brown and Lynam, 1981; Swarts *et al.*, 1978; Peterson *et al.*, 1980). Chorionase is essential for hatching, because it digests the inner layer of the chorion. A pH of 5.2 reduced the activity of the hatching enzyme of rainbow trout embryos in vivo by 90 % (Hagenmayer, 1974). Peterson *et al.* (1980) found that the pH of the pvf more or less follows the pH of the ambient water. Thus, hatching delay could partly be attributed to a reduced break-down of the chorion. The final hatching action however is the mechanical rupture of the outer layer of the chorion by the moving embryo. This may be hampered by several factors. First, Haya and Waiwood (1981) observed that the outer mucopolysaccharide layer of the chorion became harder at low pH. Second, the frequency of the movements, necessary to rupture the chorion, was also lowered at low pH (Peterson and Martin-Robichaud, 1983). An additional effect of the reduced mobility could be a less



g. 7: Possible pathways leading to hatching time delay and deformation by low water pH with and without l. The results of studies on salmonids and non-salmonids (including our observations on carp) are combined.

efficient transport of the chorionase to the inner egg shell, where it exerts its action. Finally, delayed hatching could be partly attributed to a delay in development, as was noticed by Kwain and Rose (1985). We also found in carp, that the developmental rate is reduced at low pH (Chapter 2). In contrast, Runn *et al.* (1977) found neither delay in development nor a morphological difference in hatching glands when perch eggs were exposed to low pH, although hatching was delayed. In line with observations for salmonids however, they observed a reduced breakdown of the inner chorion at low pH.

In addition to delay of hatching, we found that exposure to Al resulted in a decreased trunk movement frequency. A correlation between hatching delay and reduced trunk movement was also found by Peterson and Martin-Robichaud (1983), who exposed eggs of Atlantic salmon to low water pH. Our observations suggest that coagulation of the pvf is an additional factor that might induce hatching delay. This could hamper trunk movement intensity and delay diffusion of the hatching enzyme towards the chorion.

The mechanisms by which the presence of either Al (at low pH) or H^+ can lead to hatching delay and deformation as observed by us and others, are summarized in Fig. 7. We conclude that increased chorionic strength, developmental delay and inhibition of chorionase activity directly lead to delay of hatching. Embryonic deformation, induced by Al or low water pH, or indirectly by reduction of the perivitelline space, will result in delay of hatching indirectly via reduced trunk movements and impaired distribution of chorionase. Via the same indirect mechanisms the coagulates in the perivitelline fluid may promote hatching delay. Finally, delay of hatching seems to increase the degree of deformation.

References

Bengtsson, B.E., 1979. Biological variables, especially skeletal deformations in fish, for monitoring marine pollution. Phil. Trans. R. Soc. London Ser. B 286: 457-464.

Brown, D.J.A. and S. Lynam, 1981. The effect of sodium and calcium concentrations on the hatching of eggs and the survival of the yolk sac fry of brown trout, *Salmo trutta* L. at low pH. J. Fish Biol. 19, 205-211.

Eddy, F.B., 1974. Osmotic properties of the perivitelline fluid and some properties of the chorion of Atlantic salmon eggs. J. Zool. Lond. 174: 237-243.

Eddy, F.B. and C. Talbot, 1983. Formation of the perivitelline fluid in Atlantic salmon eggs (*Salmo salar*) in freshwater and in solutions of metal ions. Comp. Biochem. Physiol. 75C: 1-4.

Eddy, F.B. and C. Talbot, 1985. Sodium balance in eggs and dechorionated embryos of the Atlantic salmon (*Salmo salar*) exposed to zinc, aluminium and acid waters. Comp. Biochem. Physiol. 81C: 259-266.

Hagenmaier, H.E., 1974. The hatching process of fish embryos-IV. The enzymological properties of a highly purified enzyme (chorionase) from the hatching fluid of the rainbow trout, *Salmo gairdneri* Rich.. Comp. Biochem. Physiol. 49B: 313-324.

Haya, K. and B.A. Waywood, 1981. Acid pH and chorionase activity of Atlantic salmon (*Salmo salar*) eggs. Bull. Environ. Contam. Toxicol. 27: 7-12.

Kudo, S., 1982. Ultrastructural modifications of fertilization envelope in the carp egg. Ann. Zool. Jap. 55: 224-235.

Kwain , W. and G.A. Rose, 1985. Growth of brook trout *Salvelinus fontinalis* subject to sudden reductions of pH during their early life history. Trans. Am. Fish. Soc.: 114-570.

Peterson, R.H., 1984. Influence of varying pH and some inorganic cations on the perivitelline potential of eggs of Atlantic salmon (*Salmo salar*). Can. J. Fish Aquat. Sci. 41: 1066-1069.

Peterson, R.H., Daye, P.G. and J.L. Metcalfe, 1980. Inhibition of Atlantic salmon hatching at low pH. Can. J. Fish. Aquat. Sci. 37: 770-774.

Peterson, R.H. and D.J. Martin-Robichaud, 1982. Water uptake by Atlantic salmon ova as affected by low pH. Trans. Am. Fish Soc. 111: 772-774.

Peterson, R.H. and D.J. Martin-Robichaud, 1988. Embryo movements of Atlantic salmon (*Salmo salar*) as influenced by pH, temperature, and state of development. Can. J. Fish. Aquat. Sci. 40: 777-782.

Peterson, R.H. and D.J. Martin-Robichaud, 1986. Growth and major inorganic cation budgets of Atlantic salmon alevins at three ambient acidities. Trans. Am. Fish. Soc. 115: 220-226.

Rask, M., 1983. The effect of low pH on perch, *Perca fluviatilis* L. I. Effects of low pH on the development of eggs of perch. Ann. Zool. Fennici 20: 73-76.

Rudy, P.P. and W.T.W. Potts, 1969. Sodium balance in the eggs of the Atlantic salmon, *Salmo salar*. J. Exp. Biol. 50: 239-246.

Runn, p., Johansson, N. and G. Milbrink, 1977. Some effects of low pH on the hatchability of eggs of perch, *Perca fluviatilis* L.. Zoon 5: 115-125.

Shephard, K.L., 1987. The influence of water pH on the perivitelline fluid of perch (*Perca fluviatilis*) eggs. Comp. Biochem. Physiol. 86C/2: 383-386.

Shephard, K.L., 1988. Aluminium and calcium affect the perivitelline fluid in fish eggs. Comp. Biochem. Physiol. 91C/2: 503-505.

Swarts, F.A., Dunson, W.A. and J.E. Wright, 1978. Genetic and environmental factors involved in increased resistance of brook trout to sulfuric acid solutions and mine acid polluted waters. Trans. Am. Fish. Soc. 107: 651-677.

Verbost, P.M., Lafeber, F.P.J.G., Spanings, F.A.T., Aarden, E.A. and S.E. Wendelaar Bonga, 1992. Inhibition of Ca^{2+} uptake in freshwater carp, *Cyprinus carpio*, during short-term exposure to aluminium. J. Exp. Zool. 262: 247-254.

CHAPTER 6

Low-pH mediated aluminium effects on body ion concentrations of early life stages of carp and their mitigation by humic acids.

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Low-pH mediated aluminium effects on body ion concentrations of early life stages of carp and their mitigation by humic acids.

Abstract

Early life stages from fertilization up to the end of yolk absorption (168 h) were studied in carp, *Cyprinus carpio*. The parameters determined were dry body weight, aluminium content of the chorion, and total body concentrations of Al, Ca, Na and Mg. In the pH range 7.5 - 4.8, the effects of 3.7 μmol aluminium (Al)/l and/or 33 mg humic acids/l were determined on eggs and larvae. Additionally, dose-response effects of Al (0, 1.85 and 3.7 μmol Al/l) as well as humic acids (11, 19 and 33 mg/l) were studied at pH 5.4. Samples were taken at 48 and 168 h after fertilization.

Already before hatching, the dry body weight was reduced in the presence of Al at pH 5.4 and lower. These effects were, although less pronounced, still present 168 h after fertilization. Addition of humic acids totally prevented these effects of Al.

Al accumulation in the egg shell was 25-100 times higher than in the encapsulated embryo. Accumulation of Al in both the egg shell and the embryo was related to the water Al concentration and was most pronounced between pH 6.0 and 5.0. At pH 4.8, Al accumulation was lower. The presence of humic acids reduced Al accumulation very effectively. At 168 h after fertilization, Al accumulation in hatched larvae showed a bimodal pattern with peaks at pH 6.0 and 5.0.

Total body concentrations of Ca, Na and Mg of larvae at 168 h were reduced when exposed to Al at water pH 5.4 and lower. At pH 4.8, this reduction was less severe than at pH 5.5-5.0. These Al effects were concentration-dependent. The presence of humic acids effectively prevented these effects of Al.

Introduction

During the early life stages of fish, a high accumulation rate of ions such as Ca^{2+}, Na^{+} from the ambient water is necessary for normal development (Zeitoun, *et al.*, 1976; Wood *et al.*, 1990; Chapter 3). This makes them extremely vulnerable to any factor disturbing ion regulatory processes. Peterson and Martin-Robichaud (1986) reported negative effects of low pH on the net uptake of Ca and Na from early life stages. Only few publications indicated that also the presence of Al disturbed this ion uptake (Gunn and Noakes, 1987; Wood *et al.*, 1990).

In this study we have investigated the effects of low water pH and of Al on the body weight and accumulation of Ca, Na and Mg during the early life stages of carp. Since Al toxicity is pH dependent, Al was tested in the pH range of 7.5-4.8. Part of this study was

focused on the question to what extent Al is accumulated by the chorion, and whether Al is able to penetrate and reach the developing embryo. We further examined if the effects of Al were concentration-dependent, and whether the presence of humic acids affected Al toxicity. Humic acids have a strong affinity for various metals, including Al (Schnitzer, 1969; Lind and Hem, 1975; Pott *et al.*, 1985), and are known to mitigate the impact of Al in acid water on adult fish (Witters *et al.*, 1990).

We have shown that low pH and Al increase egg mortality, delay hatching and increase deformation of freshly hatched larvae of carp, indicating that these factors influence early development even before hatching (Chapters 2 and 4). We therefore determined the above parameters at 48 h and 168 h after fertilization, i.e. a few hours before the normal hatching time, and around the time that yolk absorption is completed. This enabled us to study the effects of low pH and Al toxicity on early development both in the presence and absence of the protective egg membranes.

Materials and methods

Fertilization and incubation of eggs.

Techniques to obtain fertilized eggs and incubation methods were the same as those described in Chapter 2.

Exposure conditions

Eggs and larvae were exposed to the test conditions immediately after fertilization until 168 h afterwards. The pH levels studied were 7.5, 5.9, 5.4, 5.2, 5.0 and 4.8. In addition to every pH level, the following conditions were tested:

pH plus 33 mg/l humic acid
pH plus 3.7 μmol Al/l
pH plus 3.7 μmol Al/l plus 33 mg/l humic acids

The concentration-dependency of Al effects was studied at pH 5.4, when Al is expected to have negative effects without much interference of effects of pH itself. Eggs and larvae were exposed to Al levels of 0, 1.85 and 3.7 μmol/l.

At the same acidity level, different concentrations of humic acids were tested at concentrations of 0 mg/l, 11 mg/l, 19 mg/l and 33 mg/l, and in the presence of 3.7 μmol Al/l.

Freshwater was prepared by dissolving the following salts (in mmol/l) in demineralised water: NaCl, 3.8; KCl, 0.06; $CaCl_2$, 0.8; $MgSO_4$, 0.2 and $NaHCO_3$: 0.335. The protocols followed to maintain pH levels and humic acid and Al concentrations were described in Chapters 2, 3 and 4.

Parameters and analysis

The observed parameters were dry body weight, Al content of the egg membrane and total body concentrations of Al, Ca, Na and Mg (on dry weight basis).

To determine the dry weight at the different developing stages, four times 25 larvae were collected at 48 and 168 h after fertilization. They were dried at 70°C for 24 h. At 48 h after fertilization, and if necessary at 168, the larvae were removed from the chorion.

The Al concentration of the removed chorion was measured and expressed on dry weight basis, as were the total body concentrations of Al, Ca, Na and Mg. The same embryos and larvae sampled for dry weight measurements were used for this purpose. The samples were destructed during 24 h at 70°C with 65 % NHO_3 and after dilution analyzed with an Inductively Coupled Plasma Atomic Emission Spectrometer.

Statistics and mathematics

The effects of Al and humic acids were compared with the pH group and the differences tested for statistical significance by means of the Mann-Whitney-U test. Significance was accepted at 5 %. The same test was used for evaluating the concentration dependence of the effects of Al and humic acids.

Results

Effects on dry body weight.

Fig. 1 shows the effects of low pH, Al and humic acids on the dry weight of pre-hatching embryos (48 h). No effect of water acidity on dry weight was observed. At 3.7 μmol Al/l, no effects were visible at pH levels of 5.9 or higher. A small reduction was observed at pH 5.4, and a strong reduction (80 %) at pH 5.2. When Al was added together with humic acids, the body weights were no longer different from the controls. The presence of humic acids without Al had no effect on this parameter. During severe mortality, large cells of the vitelline membrane died during epiboly and were expelled into the perivitelline fluid. In Table 1, the concentration dependency of the Al effects at

pH 5.4 are shown. Reduction in embryonic body weight appeared related to water Al concentrations as well as to total body Al concentrations.

Fig. 2 shows the body weights of sac fry at the moment that yolk absorption was completed (168 h). The effects of acidity, Al and humic acids on dry body weight were similar to those found for the pre-hatching embryos. The Al-induced reduction in body weight at pH 5.2 was less severe (50 %).

Effects on the Al concentration of the chorion.

Absorption of Al by the chorion at different pH is shown in Fig. 3. Background levels of chorionic Al were measured in the entire pH range studied. When Al was added to the exposure media, it was bound by the chorion at all pH levels tested. Even in neutral water some Al binding occurred. At pH 5.9, where Al had no toxic effects on embryos and larvae, the amount of bound Al was considerable (78 % of that at pH 5.2). Maximum binding occurred at pH 5.2. At lower pH, Al binding by the chorion decreased sharply. The combined exposure of Al and humic acids resulted in a strong reduction of the chorionic Al absorption at pH levels lower than 7.5. A small absorption peak was seen at pH 5.2. When humic acids were present without Al, there was a slight binding of Al, which appeared to be pH independent. Since humic acids sticked to the chorion, this binding could be the result of some Al contamination of the humic acids. Fig. 3 (inset) shows a concentration related inhibition of Al binding to the egg shell by humic acids. The binding of Al to the chorion appeared related to the Al concentration of the water (Table 1).

Table 1: Effects of exposure to 0, 1.85 and 3.7 μmol Al/l at pH 5.4 on Al concentrations of the chorion, 48 h embryos and 168 h larvae, on dry body weights (Wb) of 48 h embryos and 168 h larvae (mg), and on the body concentrations of Ca, Na and Mg in 168 h larvae (μmol/g dry weight). Means and SD's are given; n = 4. * = $p<0.05$.

Al water	Al chorion 48 h	Al embryo 48 h	Al larvae 168 h	Wb embryo 48 h	Wb larvae 168 h	Ca larvae 168 h	Na larvae 168 h	Mg larvae 168 h
0	2.88 (1.0)	0.42 (0.16)	0.42 (0.11)	0.219 (0.03)	0.183 (0.01)	292 (13.1)	481 (15.3)	59.6 (2.7)
1.85	51.9* (4.4)	1.14* (0.17)	2.45* (0.32)	0.200 (0.02)	0.157 (0.03)	208* (8.2)	385* (16.9)	48.8* (1.5)
3.7	89.6* (6.6)	3.7* (0.79)	6.17* (0.56)	0.172* (0.02)	0.156* (0.02)	138* (1.4)	258* (18.2)	41.1* (2.3)

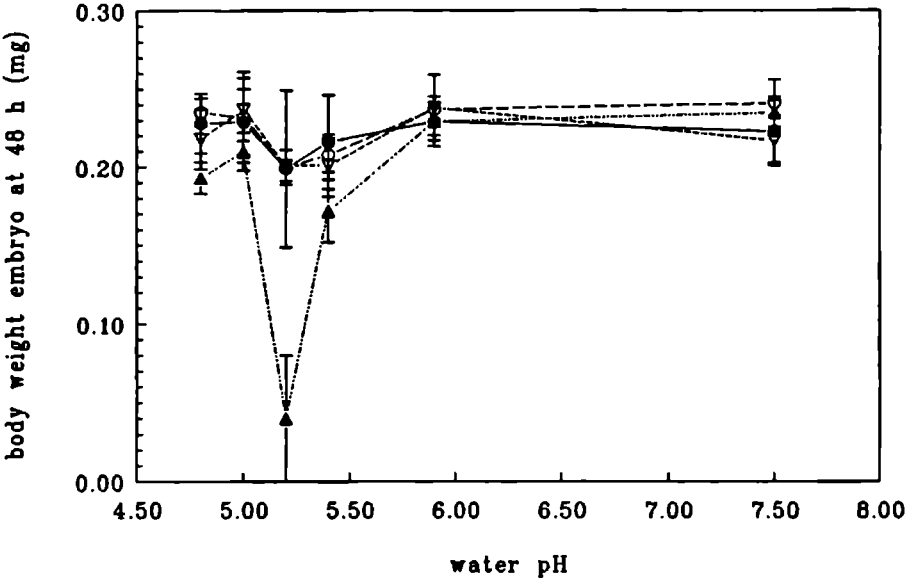

Fig. 1: Effects of exposure to different pH levels, in the presence or absence of 3.7 µmol Al/l, and of 33 mg humic acid/l, on the dry body weights of 48 h-embryos (mean values ± SD; n = 4-8; • = pH; o = pH + humic acid; ▲ = pH + Al; ▽ = pH + Al + humic acid).

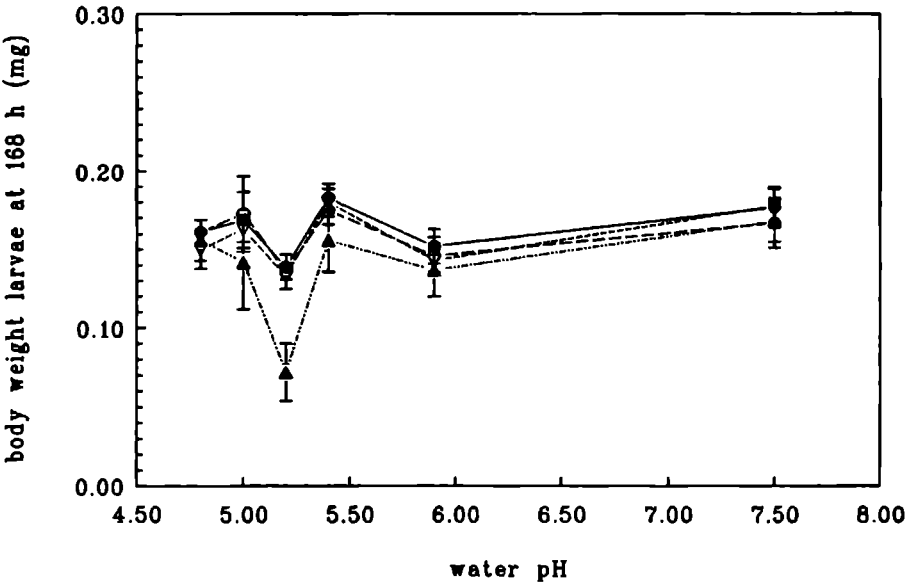

Fig. 2: Effects of exposure to different pH levels, in the presence or absence of 3.7 µmol Al/l, and of 33 mg humic acid/l, on the dry body weights of 168 h-embryos (mean values ± SD; n = 4-8; • = pH; o = pH + humic acid; ▲ = pH + Al; ▽ = pH + Al + humic acid).

Effects on the Al concentration of the 48 h-embryo.

When eggs were exposed to Al, it was partly accumulated into the developing embryo (Fig. 4). Accumulation was restricted to acidity levels below pH 5.9. Maximum accumulation occurred at pH 5.4 (3.7 μmol Al/l). At pH 5.0 and 4.8, accumulation of Al in the embryo was only slightly above control levels (1.1-1.2 μmol Al/l). Al concentrations in the larvae were 25-100 times lower than those in the egg shell (dry weight basis). When Al was added simultaneously with humic acids, no accumulation of Al took place: the Al contents did not differ from control levels. Complete inhibition of Al accumulation in the embryo by humic acids even took place at the lowest concentrations of humic acids (Fig. 4, inset). Embryonic accumulation of Al at pH 5.4 was directly related to the Al concentration of the water (Table 1).

Effects on the Al concentration of the sac fry (48 - 168 h).

In Fig. 5, the gain of the Al concentration between 48 h and 168 h after fertilization (the period that sac fry would normally be directly exposed to Al without the protection of the chorion) is presented for different pH levels. A bimodal pattern is shown with two peaks of maximum increase at pH 5.9 and 5.0. At pH 4.8, the Al accumulation was almost zero. The presence of 33 mg humic acids/l resulted into an almost complete inhibition of the Al accumulation by the sac fry. The inhibition of Al accumulation by low concentrations of humic acids was less effective in sac fry than in embryos (Fig. 5 inset). Like Al accumulation in embryos, Al accumulation in sac fry was directly related to the Al concentration of the ambient water (Table 1).

Effects on the Ca concentration of the 168 h-larvae.

The body Ca concentrations decreased at low pH levels (Fig. 6). When Al was added, no effects on the Ca levels were seen in the pH range 7.5-5.9. At lower pH levels, a reduction of the body Ca concentrations occurred when compared to effects of low pH alone. When humic acids were also added to the incubation media, the negative effects of Al on the Ca concentration disappeared, but the effects of water acidity remained. As shown in Fig. 6 (inset), relatively high concentrations of humic acids were necessary to prevent the negative effects of Al. At pH 5.4, the body Ca concentration was inversely related to the Al concentration of the incubation media and to the total body Al concentration of the 168 h larvae (Table 1).

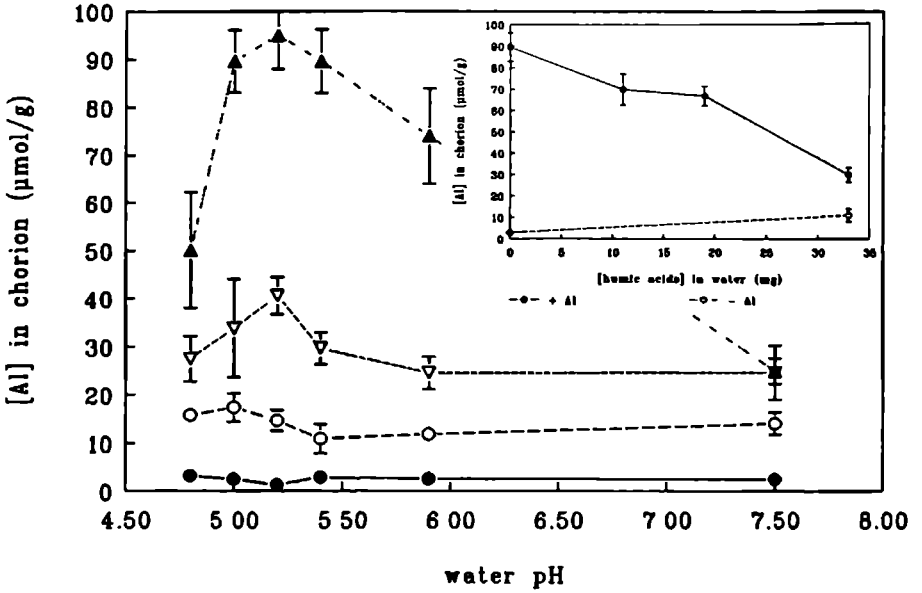

Fig. 3: Al concentration of the chorion of eggs exposed to different pH levels, in the presence or absence of 3.7 µmol Al/l and of 33 mg humic acid/l (mean values ± SD, n = 4-8, • = pH; ○ = pH + humic acid; ▲ = pH + Al; ▽ = pH + Al + humic acid) Inset: Effect of different humic acid concentrations on the Al content of the chorion in the presence of 3 7 µmol Al/l (mean values ± SD; n = 4).

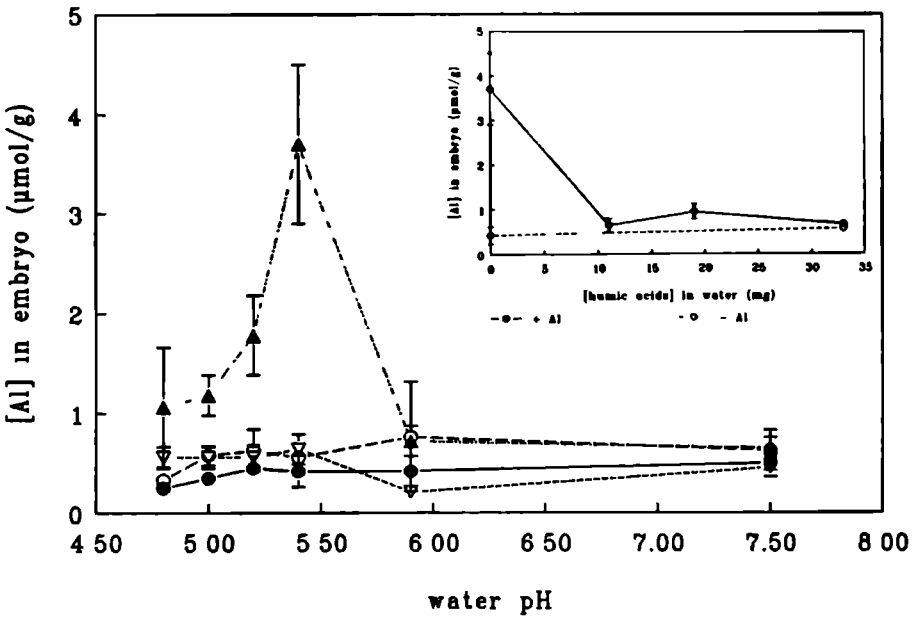

Fig. 4: Al concentration of the 48 h-embryo after exposure to different pH levels, in the presence or absence of 3.7 µmol Al/l and of 33 mg humic acid/l (mean values ± SD; n = 4-8, • = pH, ○ = pH + humic acid; ▲ = pH + Al, ▽ = pH + Al + humic acid). Inset: Effect of different humic acid concentrations on the Al content of the 48 h-embryo in the presence of 3.7 µmol Al/l (mean values ± SD, n = 4).

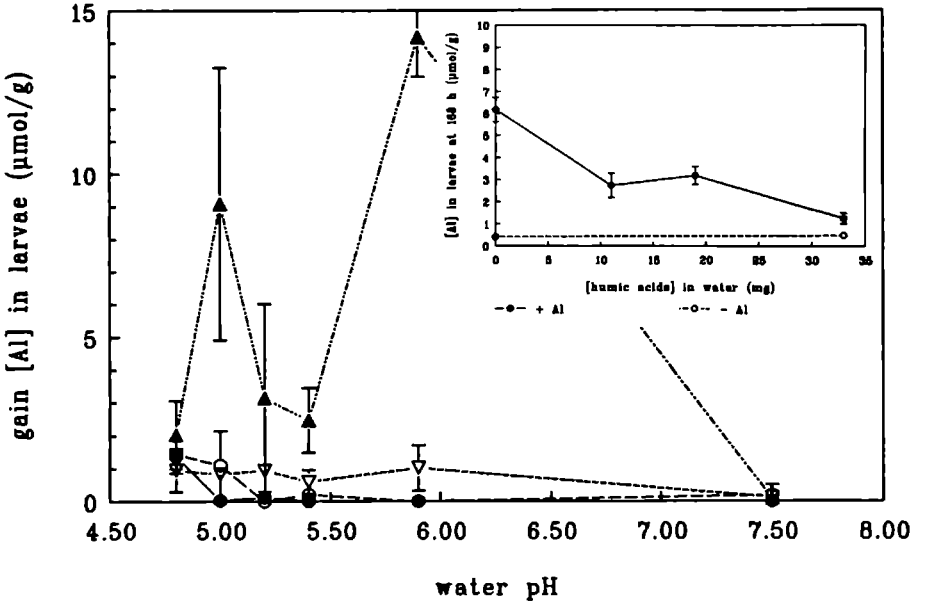

Fig. 5: Gain in Al concentration of the 168 h-larvae after exposure to different pH levels, in the presence or absence of 3.7 µmol Al/l and of 33 mg humic acid/l (mean values ± SD; n = 4-8; • = pH; o = pH + humic acid; ▲ = pH + Al; ▽ = pH + Al + humic acid). Inset: Effect of different humic acid concentrations on the Al content of the 168 h-larvae in the presence of 3.7 µmol Al/l (mean values ± SD; n = 4).

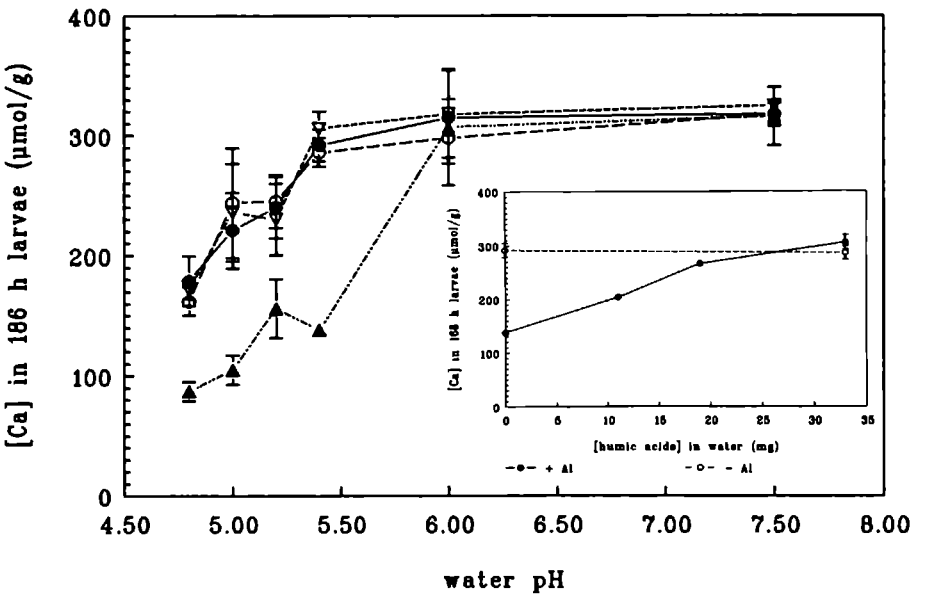

Fig. 6: Body Ca concentrations of 168 h-larvae after exposure to different pH levels, in the presence or absence of 3.7 µmol Al/l and of 33 mg humic acid/l (mean values ± SD; n = 4-8; • = pH; o = pH + humic acid; ▲ = pH + Al; ▽ = pH + Al + humic acid). Inset: Effect of different humic acid concentrations on the body Ca concentration of the 168 h-larvae in the presence of 3.7 µmol Al/l (mean values ± SD; n = 4). At 0, 0.11 and 0.19 mg humic acids with Al, SD's are hidden within the symbol.

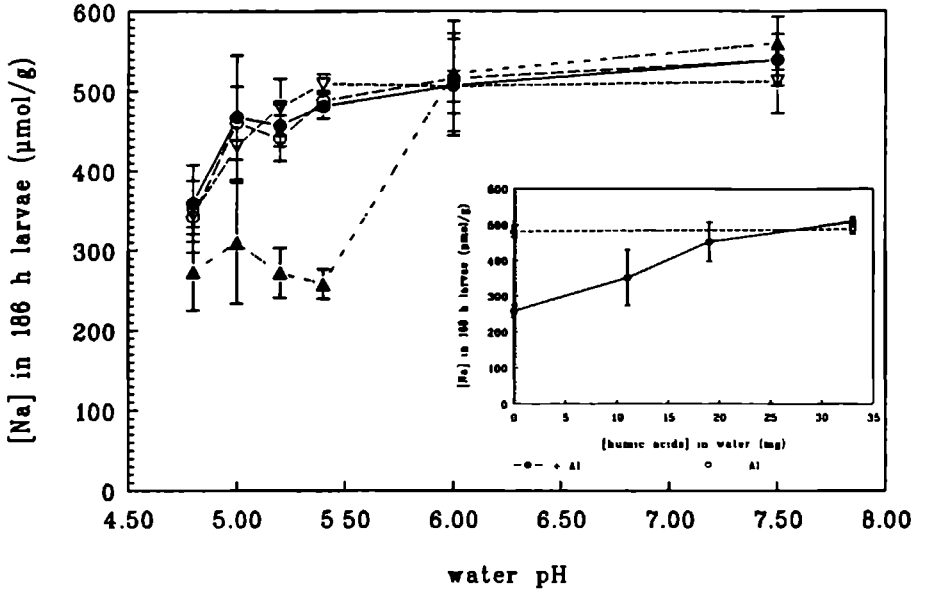

Fig. 7. Body Na concentrations of 168 h-larvae after exposure to different pH levels, in the presence or absence of 3.7 µmol Al/l and of 33 mg humic acid/l (mean values ± SD; n = 4-8; • = pH; o = pH + humic acid; ▲ = pH + Al; ▽ = pH + Al + humic acid). Inset: Effect of different humic acid concentrations on the body Na concentration of the 168 h-larvae in the presence of 3.7 µmol Al/l (mean values ± SD; n = 4; • = +Al; o = -Al).

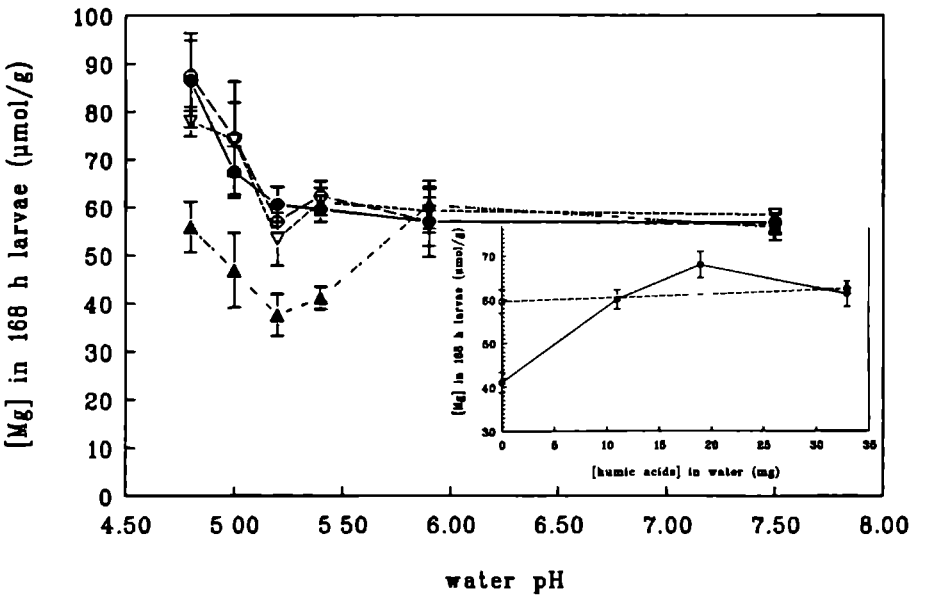

Fig. 8: Body Mg concentrations of 168 h larvae after exposure to different pH levels, in the presence or absence of 3.7 µmol Al/l and of 33 mg humic acid/l (mean values ± SD, n = 4-8; • = pH; o = pH + humic acid; ▲ = pH + Al; ▽ = pH + Al + humic acid). Inset: Effect of different humic acid concentrations on the body Mg concentration of the 168 h-larvae in the presence of 3.7 µmol Al/l (mean values ± SD; n = 4; • = +Al; o = -Al).

Effects on the Na concentration of the 168 h-larvae.

Exposure to low water pH resulted in a decrease of the body Na levels (Fig. 7). The presence of Al in the ambient water resulted in a further decrease of the body Na concentrations at pH of 5.4 and less, while at pH 7.5 - 5.9, no effects of Al were seen. Humic acids eliminated the negative Al effects completely. Fig. 7 (inset) shows that the negative effects of Al on the Na concentration decreased gradually with increasing humic acid concentrations. Exposure of the larvae to different Al levels at pH 5.4 revealed a concentration dependent decrease of the Na concentration. This decrease was related to the Al levels in the body (Table 1).

Effects on the Mg concentration of the 168 h-larvae.

In contrast to Ca and Na, the Mg concentrations of the body increased with lower pH values (Fig. 8). At water acidity levels of pH 7.5 - 5.9, no effects of Al on Mg regulation were apparent. In the range of pH 5.4 - 4.8, Mg concentrations were reduced when compared to the pH controls. The lowest level was found at pH 5.0; at pH 4.8 the level was not different from control values. In the presence of humic acids, all Al effects were absent. Fig. 8 (inset) shows that the effects of Al on the Mg concentration were absent even at lower humic acid concentrations. The total body concentrations of Mg were inversely related to the Al levels in the exposure media as well as to the larval Al concentrations (Table 1).

Discussion and conclusions

The effects of low pH will be discussed only briefly in this chapter. For a more detailed discussion of pH effects on body mineral concentrations of carp larvae, we refer to Chapter 3.

Effects on dry body weight.

Low water pH had no effect on dry body weight of embryos or larvae. The presence of Al reduced the dry body weights of the larvae between pH 5.9 and 4.8, the effect being most pronounced at pH 5.2. Forty-eight hours after fertilization, just before hatching, the reduction in body weight was more pronounced than after 168 h.

To our knowledge observations on Al-induced weight reductions before hatching have not been reported. We have no satisfactory explanation, but a contributing factor might be that the vitelline membrane did not encapsulate the total amount of yolk during epiboly.

During this stage, we observed that large cells of this membrane died and were expelled into the perivitelline fluid.

All measurements reported in the literature concern the dry weight at life stages after hatching. In neutral water, most authors observed no effects of Al on the dry body weight of non-fed fry of different salmonid species (Cleveland *et al.*, 1986; Hunn *et al.*, 1987; Ingersoll *et al.*, 1990; Wood *et al.*, 1990a,b). Reduction of body weight of non-fed larvae after Al exposure at pH 7 was only reported by Thomson *et al.* (1988). When exposure was continued and food was administered, the presence of Al reduced the increase in dry body weights (Cleveland *et al.*, 1986; Hunn *et al.*, 1987), indicating decreased appetite or a less efficient food conversion.

At water pH levels lower than 5.5, the available data on Al effects are less consistent or even contradictory. Hunn *et al.*, 1987), Ingersoll *et al.* (1990) and Wood *et al.* (1990a,b) reported no effects on body weight. Contrastingly, Gunn and Noakes (1987) and Thomsen *et al. (1988)* found reductions in length and dry body weight of fry. Reader *et al.* (1988) mentioned a delay in yolk sac absorption, whereas Cleveland *et al.* (1986) described a reduced growth of fed larvae. Thus, our observations on weight reduction of non-feeding carp larvae are in line with most of the observations on salmonids.

The rate of decrease in body weight during the yolk-absorbing larval stages of carp could be influenced by two opposing processes. Firstly, a delay of development might cause a delay of the weight loss associated with yolk absorption. This means a relative increase of dry weight of the exposed larvae when compared to the controls. Secondly, an increase of dry weight loss could result from an increase of metabolic activity. The result of both processes will determine the change in body weight. Since we observed dry weight reductions of larval carp in the presence of Al, the increase of metabolic activity is probably the dominating mechanism determining body weight under this condition.

Another factor that may contribute to the difference in body weight is that after 48 h of Al exposure, when the embryos are already smaller than normal, a selective mortality occurs in the smaller larvae. This could explain the less pronounced Al effect on the mean body weight at the end of the experiment.

Effects of pH on the Al-concentration of the chorion, the 48 h-embryo and the 168 h-larvae.

Al binds to the chorion at all pH levels tested. Binding was most pronounced in the pH range 5.9-5.0 and lower at pH 7.5. No literature on this subject was found. Ohzu and Kusa (1981) mentioned that the chorion is rich in negatively charged glutamic acids to which Al can bind. Zotin (1958) reported that the chorion binds Ca^{2+}. This Ca could be forced from its binding sites by Al. According to Rombough (1984), metals with a highly

positive electrode potential bind to these negatively charges and hence, the chorion might protect to toxic metals. Peterson (1984) suggested that Al absorption by the chorion diminishes the chorionic permeability to this metal. Contrastingly, Rombough (1984) suggested that metals with a strong negative electrode potential (like Al^{3+}) will be less strongly bound to the chorion and thus easier accessable to the embryo.

Our observations show that Al is indeed able to reach the developing embryo through the chorion. Al binding by the embryo appeared to be pH dependent. No Al accumulated into the embryo at pH values of 5.9 and higher, while maximum uptake occurred at pH 5.4. The Al concentration was much lower in the embryo than in the chorion, which suggests some protection of the embryo by the chorion. Peterson and Martin-Robichaud (1986) reported that the cadmium concentration in the embryo was also lower than that of the chorion. Al may pass the chorion via channels, which are connecting the ambient water with the perivitelline fluid during the hydration phase just after fertilization and during the time just before hatching. It is assumed that Al^{3+} can pass the chorion on the basis of the electrode potential of the ion (Rombough, 1984).

The accumulation of Al by the post-hatch larvae showed a minimum at a pH level where Al accumulation by the embryo was at its maximum. Accumulation of Al by post-hatch larvae showed two maxima: one at pH 5.9 and one at pH 5.0. These peaks, which were observed in the time-period between 48 and 168 h after fertilization, could be explained by Al-induced hatching delay (Chapter 4) at pH levels (5.4-5.2) where Al absorption is low. Since the chorion partly prevents Al accumulation in the embryo, a shorter exposure period (the post-hatch period) will result in lower Al uptake. Accumulation of Al by larvae was also reported by Wood *et al.* (1990), who found that Al absorption at pH 5.2 was higher than at pH 4.8 and 4.4. They observed no bimodal response and suggested that Al adhered mainly to the surface, since it quickly disappeared at neutral pH.

In the chorion Al is accumulated over a broader pH range than in the embryo, implying that the protective properties of the chorion vary with pH. The speciation of $Al(OH)_x$ is pH dependent and the relative occurrence of Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^+$ and $Al(OH)_4^-$ varies accordingly (Smith and Hem, 1972). As the potential of a metal to pass the chorion is determined by its electrochemical properties (Rombough, 1984), one might expect that the various Al species differ in their potential to pass the chorion. Assuming that one species passes the chorion selectively, speciation equilibrium is still expected to be in accordance with the pH of the perivitelline fluid. When the pH dependent embryonic Al accumulation curve was compared with the relative occurrence of the different $Al(OH)_x$ species at various pH levels in our experimental conditions, a striking similarity becomes apparent with the concentration of $Al(OH)_2^+$. Thus, starting from the Al-speciation approach, we suggest that $Al(OH)_2^+$ is the toxic agent that can explain the pH-dependency

during the pre-hatching period of Al-effects such as mortality, deformation rate, and hatching time delay (Chapter 4), but also Al accumulation in the embryo.

Another approach to explain the accumulation of Al in salmonid gills was formulated by Neville and Campbell (1988). They assumed that the Al toxicity is mediated by Al^{3+} binding to the surface of the exposed tissue. They included competition between Al^{3+} and H^+ for binding sites (L) in their model, using the following equations:

$$Al^{3+} + \text{L-gill} \rightleftarrows \text{Al-L-gill}$$

$$H^+ + \text{L-gill} \rightleftarrows \text{H-L-gill}$$

$$2H^+ + \text{L-gill} \rightleftarrows H_2\text{-L-gill}$$

On the basis of pK_a values for gills, they calculated the [Al-L-gill] at different pH levels. No interaction between Al^{3+} and gills at $pH > 6.5$ will occur. Maximum Al-L-gill binding is expected at pH 5.0, while the Al-L-gill complexation decreases at lower pH due to competition with H^+. The pattern we found for the Al-chorion binding at the different pH levels is consistent with these calculations. The pH dependent Al accumulation in the chorion therefore can aslo be explained by this model.

After hatching, metabolic processes intensify, and this could lead to an elevated pH of the body boundary layer, resulting in Al precipitation (Exley *et al.*, 1991). The free Al^{3+} ion model with H^+ competition also predicts a bimodal Al absorption pattern if this phenomenon is taken into account, and gives therefore an other explanation for the bimodal larval Al absorption pattern than the hatching time delay mentioned before.

Effects on the Ca concentration of the 168 h larvae.

Water acidity lowered the Ca concentration of the larvae. Negative effects of low water pH on Ca concentrations were also observed in salmonid larvae by Peterson *et al.* (1982), Peterson and Martin-Robichaud (1986), Reader *et al.* (1988) and Wood *et al.* (1990a).

Al reduced total body Ca concentrations at pH 5.4 and lower. In accordance with our results, Gunn and Noakes (1987) and Thomsen *et al.* (1988) reported reductions in whole body Ca levels of embryos and larvae of *Salvelinus namaycush* and *Salmo gairdneri* reared at pH 5.0 with 3.7-18.5 μmol Al/l. A decreased skeletal deposition of Ca and a decreased total body Ca concentration resulted from exposure of *Salmo trutta* sac fry to 8 μmol Al/l at pH 5.4 and 4.5 (Reader *et al.*, 1988). These Al effects were less pronounced at pH 4.5. Wood *et al.* (1990) reported a negative effect of Al on the Ca level only when sac fry of *Salvelinus fontinalis* were exposed to Al concentrations of 12.3 and 37 μmol/l

up from hatching. At lower concentrations (1.4-3 μmol/l), Al increased body Ca levels. They found this positive effect at all Al concentrations when exposure started at the time of fertilization, and it appeared to be independent of pH. Although we observed no effect of Al at neutral pH, a small negative effect of Al was noticed at pH 7 by Thomsen *et al.*(1988). Thus, most of the available data support our observations of reduced total body Ca concentrations in the presence of Al at low pH.

Effects on the Na concentration of 168 h-larvae.

Body Na concentrations of 168 h-larvae decreased when water acidity levels were lower than pH 5.4. Similar effects of pH on body Na concentrations were noticed by Wood *et al.* (1990), Reader *et al.* (1988), Peterson *et al.* (1982) and Peterson and Martin-Robichaud (1986).

At pH 5.4 and lower, Al induced a severe additional decrease in body Na concentrations. Similarly, Reader *et al.* (1988) found a reduction in Na concentrations of sac fry of *Salmo trutta* exposed to 8 μmol Al/l at pH 5.4. At pH 4.5, this effect was less severe than at pH 5.4. Embryos of *Salvelinus namaycush* showed a decreased Na concentration when reared at pH 5.0 in the presence of 3.7 and 7.4 μmol Al/l, while no effect of Al was found during the yolk stage after hatching (Gunn and Noakes, 1987). In sac fry of *Salvelinus fontinalis* exposed to Al up from fertilization, Na levels were lower at high Al concentrations (12.3-7 μmol/l), while at low Al levels (1.4-4.1 μmol/l), a stimulating effect of Al on the Na concentration was recorded (Wood *et al.*, 1990a,b). When the eggs were exposed up from fertilization, Al had positive effects at all concentration levels.

Effects on the Mg concentration of 168 h-larvae.

While the Ca and Na concentrations decreased, Mg levels increased at low pH. This observation confirms the results of Wood *et al.* (1990a), who found a small increase of body Mg concentrations on wet weight basis when early life stages of brook trout were exposed to low pH after hatching. In contrast, neither Gunn and Noakes (1985) nor Reader *et al.* (1988) found an effect of acid exposure on Mg concentrations of larval lake trout and brown trout.

The body Mg concentration was not influenced by Al at pH 5.9 and higher. When compared to low water pH without Al, the presence of Al at pH levels below 5.9 resulted in a more or less pH-related reduction of the Mg concentration. Compared to pH 7.5 without Al however, the Al exposed larvae had a significantly lower Mg concentration at pH 5.4 - 5.0. Reader *et al.* (1988) and Gunn and Noakes (1987) also found reductions in

the Mg concentration when yolk fry of *Salmo trutta* and alevins of *Salvelinus namaycush* were reared in the pH range of 5.4 - 4.5 with 3.7-8 μmol Al/l. Slight negative effects of Al on the Mg body concentration were observed by Wood *et al.* (1990b) for *Salvelinus fontinalis* sac fry when exposed after hatching. This effect was not noticeable when exposure started immediately after fertilization.

Reduction of Ca and Na concentrations in larvae as induced by Al at low pH levels may be caused by impaired ion regulation or by developmental effects. Since Al is able to damage the integument (Tandjung, 1982; Segener *et al.*, 1988), the major site of ion exchange in fish larvae, disturbance of ion regulation is likely. Wood *et al.* (1990b) also concluded that ion regulation of brook trout larvae was negatively influenced by Al. We showed earlier that the net uptake of Ca^{2+} and Na^{+} from the ambient water occurred mainly after hatching (Chapter 3). The pH-dependent Al effects on this process however, parallels more the pH-dependency of Al accumulation during the pre-hatching period than during the post-hatching life stages. The perivitelline fluid surrounding the embryo has an ability to accumulate cations. This property is lost in the presence of H^{+} and Al^{3+}. The resulting reduced cation levels in the perivitelline could be the primary cause of developmental disturbances i.e. delay in development and deformation. At the same pH levels where Al affects Ca and Na balances, Al also induces high spinal cord deformation rates and delayed hatching (Chapter 4). Reduced mobility, due to body deformation, might impair the replacement of the body surface diffusion layer, leading to lowered ion concentrations at the sites of ion uptake. Delay in hatching may also affect the uptake of Ca^{2+} and Na^{+} since uptake of these ions occurs mainly after hatching.

During larval stages Mg is suggested to be stored in excess in the yolk and Mg uptake occurs only via the food (Reader *et al.*, 1988; Gunn and Noakes, 1987). Wood *et al.* (1990a) mentioned that Mg levels on wet weight basis in the yolk are much higher than those in the embryo. This results in a decrease of the Mg concentration during development because larval tissue contains more water than yolk material. Therefore, the body Mg concentration on a wet weight basis is a reliable indicator of larval development: higher Mg levels indicate delayed development. Contrastingly, on dry weight basis, the total body Mg concentrations increase with the developmental stage due to loss of body weight during yolk conversion (Chapter 3). However, the increase of the Mg concentration on dry weight basis as observed at low pH, can not be explained by a less efficient utilization of the yolk organic matter, because no weight reductions were found. An alternative explanation is indicated by the results of Wood *et al.* (1990) and Van der Velden *et al.* (1991), who found evidence that Mg^{2+} and Ca^{2+} compete for uptake. Thus, a reduction of the Ca^{2+} uptake could result in an increased Mg^{2+} uptake and this is in line with our observations. This interpretation conflicts with the assumption that sac fry do not

accumulate Mg^{2+} out of the water.

The effects of Al on the Mg concentration in the larvae are opposite to those of water pH. A decreased Mg concentration on dry weight basis is predicted when development is delayed, and as will be shown in Chapter 7, Al induces such delays. Another explanation might be that the integument is damaged to such an extent, that ions, including Mg^{2+} are leaking out of the embryo. Indeed, skin damage due to acid water and Al have been reported by Daye and Garside (1976) and Segener *et al.* (1988).

Effects of humic acids.

Humic acids effectively prevented the negative effects of Al on dry body weight and on the concentrations of Ca, Na and Mg. Accumulation of Al in the (pre-hatch) embryo and the (post-hatch) larvae was inhibited by humic acids, while Al accumulation in the chorion was greatly reduced. The little Al found on the chorion in the presence of humic acids is likely caused by the adherence of Al-humic acid complexes to the egg membranes. Other studies at our laboratory showed that Al effects on mortality, deformation rate and hatching delay were also reduced by humic acids (Chapter 4). These observations are supported by literature. According to Snitzer (1969) and Lind & Hem (1975), humic acids bind Al very strongly, thus reducing its bioavailability. Several authors (Baker and Schofield, 1980; Driscoll *et al.*, 1980; Hutchinson and Sprague, 1987; Witters *et al.*, 1990) reported that complexation of Al with humic acids or citrate reduces the toxicity of Al for fish.

References

Baker, J.P. and C.L. Schofield, 1980. Aluminium toxicity to fish as related to acid precipitation and Adirondack surface water quality. Ecol. Imp. Acid Precip. Proc. Int. Conf., Sandefjord, Norway, 1980, 292-293.

Cleveland, L., Little, E.E., Hamilton, S.J., Buckler, D.R. and J.B. Hunn. 1986. Interactive toxicity of aluminium and acidity to early life stages of brook trout. Trans. Am. Fish. Soc. 115, 610-620.

Daye, P.G. and E.T. Garside, 1976. Histopathologic changes in surficial tissues of brook trout, *Salvelinus fontinalis* (Mitchill), exposed to acute and chronic levels of pH. Can. J. Zool. 54: 2140-2155.

Driscoll, C.T., J.P. Baker, J.J. Bigsoni, Jr. and C.L. Schofield, 1980. Effect of aluminium speciation on fish in dilute acidic waters. Nature 284: 161-164.

Driscoll, C.T., Baker, J.P., Bisogni, J.J., and C.L. Schofield, 1980. Effect of aluminium speciation on fish in acidified waters. Nature 284: 161-164.

Exley, C., J. S. Chappell and J.D. Birchall, 1991. A mechanism for acute aluminium toxicity in fish. J. Theor. Biol. 151: 417-428.

Gunn, J.M. and D.L.G. Noakes, 1987. Latent effects of pulse exposure to aluminium and low pH on size, ionic composition, and feeding efficiency of lake trout (*Salvelinus namaycush*) alevins. Can. J. Fish. Aquat. Sci. 44: 1418-1424.

Hunn, J.B., Cleveland, L. and E.E. Litle, 1987. Influence of pH and aluminium on developing brook trout in a low calcium water. Environ. Pollut. 43: 63-73.

Hutchinson, N.J., and J.B. Sprague, 1987. Reduced lethality of Al, Zn and Cu mixtures to American flagfish by complexation with humic substances in acidified soft waters. Environ. Tox. Chem. 6: 755-765.

Ingersoll, C.G., Sanchez, D.A., Meyer, J.S., Gully, D.D. and J.E. Tietge, 1990. Epidermal response to pH, aluminum, and calcium exposure in brook trout (*Salvelinus fontinalis*) fry. Can. J. Fish. Aquat. Sci. 47: 1616-1622.

Lind, L.J. and J.D. Hem, 1975. Chemistry of aluminium in natural water. Effects of organic solutes on chemical reactions of aluminium. U.S. Geol. Surv. Wat. Suppl. Pap. 1827 G: G1-G83.

McDonald, D.G., Wood, C.M., Rhem, R.G., Mueller, M.E., Mount, D.R. and H.L. Bergman, 1991. Nature and time course of acclimation to aluminium in juvenile brook trout (*Salvelinus fontinalis*). I. Physiology. Can. J. Fish. Aquat. Sci. 48: 2006-2015.

Neville, C.M. and P.G.C. Campbell, 1988. Possible mechanisms of aluminium toxicity in a dilute, acidic environment to fingerlings and older life stages of salmonids. Water Air Soil Pollut. 42 (3/4): 311-321.

Ohzu, E. and Kusa, M. Amino acid composition of the egg chorion of rainbow trout. Annot. Zool. Jap. 54: 241-244.

Peterson, R.H., 1984. Influence of varying pH and some inorganic cations on the perivitelline potential of eggs of Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 41: 1066-1069.

Peterson, R.H., Daye, P.G., Lacroix, G.L. and E.T Garside, 1982. Reproduction in fish experiencing acid and metal stress, p. 177-196. in R.E. Johnson [ed.] Acid Rain/Fisheries, American Fisheries Society. Bethesda, M.D.

Peterson, R.H., and D.J. Martin-Robichaud. 1986. Growth and major inorganic cation budgets of Atlantic salmon alevins at three ambient acidities. Trans. Am. Fish. Soc. 115: 220-226.

Playle, R.C., Goss, G.G. and C.M. Wood, 1989. Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminium exposures in soft water of two calcium concentrations. Can. J. Zool. 67: 314-324.

Pott, D.B., Alberts, J.J. and A.W. Elzerman, 1985. The influence of pH on the binding capacity of the conditional stability constants of aluminium and naturally occurring organoc matter. Chem. Geol. 48: 293-304.

Reader, J.P., Dalziel, T.R.K. and R. Morris, 1988. Growth, mineral uptake and skeletal calcium deposition in brown trout, *Salmo trutta* L., yolksac fry exposed to aluminium and manganese in soft acidic water. J. Fish Biol. 32: 607-624.

Smith , R.W. and J.D. Hem, 1972. Effect of aging on aluminium hydroxide complexes in dilute aqueous solutions. Geological Survey Water-supply Paper 1827-D.

Schnitzer, M., 1969. Reactions between fulvic acid, a soil humic component and inorganic constituents. Soil Sci. Soc. Amer. Proc. 33, 75-81.

Segner, H., Marthaler, R. and M. Linnenbach, 1988. Growth, aluminium uptake and mucus cell morphometrics of early life stages of brown trout, *Salmo trutta*, in low pH water. Envir. Biol. Fish. 21: 153-159.

Tandjung, S.D., 1982. The acute toxicity and histopathology of brook trout (*Salvelinus fontinalis*, Mitchill) exposed to aluminium in acid water. PhD dissertation. Fordham University, New York, NY. (213 pp.)

Thomsen, A., Korsgaard, B. and J. Joensen, 1988. Effect of aluminium and calcium ions on survival and physiology of rainbow trout *Salmo gairdneri* (Richardson) eggs and larvae exposed to acid stress. Aquat. Toxicol.: 291-300.

Velden vd, J.A., Spanings, F.A.T., Flik, G. and S.E. Wendelaar Bonga, 1991. Early stages of carp (*Cyprinus carpio* L.) depend on ambient magnesium for their development. J. Exp. Biol. 158: 431-438.

Witters, H.E., Van Puymbroeck, S., Vangenechten, J.H.D. and O.L.J. Vanderborgt, 1990. The effect of humic substances on the toxicity of aluminium to adult rainbow trout, *Oncorhynchus mykiss* (Walboum). J. Fish Biol. 37: 43-53.

Wood, C.M., McDonald, D.G., Ingersoll, C.G., Mount, D.R., Johansson, O.E., Landsberger, S. and H.L. Bergman, 1990a. Effects of water acidity, calcium, and aluminium on whole body ions of brook trout (*Salvelinus fontinalis*) continuously exposed from fertilization to swim-up: A study by instrumental neutron activation analysis. Can. J. Fish. Aquat. Sci. 47: 1593-1603.

Wood, C.M., and McDonald, D.G., Ingersoll, C.G., Mount, D.R., Johansson, O.E., Landsberger, S. and H.L. Bergman, 1990b. Whole body ions of brook trout (*Salvelinus fontinalis*) alevins: responses of yolk-sac and swim-up stages to water acidity, calcium, and aluminium, and recovery effects. Can. J. Fish. Aquat. Sci. 47: 1604-1615.

Zeitoun, I.H., D.E. Ullrey, W.G. Bergen, and W.T. Magee, 1976. Mineral metabolism during the ontogenesis of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Bd Can. 33: 2587-2591.

Zotin, A.I., 1958. The mechanism of hardening of the salmonid egg membrane after fertilization or spontanuous activation. J. Exp. Morph. 6: 546-568.

CHAPTER 7

Aluminium in acid water: effects on chloride cell numbers and body ion concentrations of sac fry of carp.

F.G.F. Oyen. E.H. v Ham and S.E. Wendelaar Bonga

Aluminium at acid conditions: Effects on chloride cell numbers and body ion concentrations of sac fry of carp.

Abstract

Effects of 2 μmol/l aluminium (Al) on extrabranchial chloride cell proliferation were studied in carp larvae at pH 5.4. This was done during the mineralisation phase, between hatching (72 h) and the end of yolk absorption (168 h). In this period, body length and gain of Ca, Na and Mg on dry weight basis was measured and related to chloride cell densities.

At pH 5.4, the proliferation of the chloride cells in the skin (the cells involved in Ca^{2+} and Na^{+} uptake) was reduced in larvae exposed to Al as compared to controls. The development of the pronephros and the lateral line system was impaired and the increase in body length was reduced. The chloride cell density was related to body length under all conditions. Al reduced the body concentrations of Ca and Na. The ratio of the net gain of Na and Ca and the chloride cell density was higher in the Al-exposed larvae than in the controls. For Mg the ratios were similar in both groups. The most important effect of Al determined in this study was the delay in body growth and development. No notable effects of Al on ion regulation and mineral balance could be demonstrated.

Introduction

Elevated Al concentrations as commonly found in acid water disturb ion regulation in juvenile and adult fish (Chapter 1). Uptake of Ca^{2+} and Na^{+} from the water is reduced in the presence of Al (Peterson and Martin-Robichaud, 1986, Verbost *et al.*, 1992). Part of the Ca^{2+} and Na^{+} uptake can be attributed to the branchial chloride cells. Low water pH as well as Al have been reported to induce hyperplasia and hypertrophy of these cells (Wendelaar Bonga *et al.*, 1987; Youson and Neville, 1987). A reduction of Ca^{2+} and Na^{+} uptake by Al has also been found in sac fry of salmonids (Wood *et al.*, 1990; Peterson *et al.*, 1982; Reader *et al.*, 1988).

In fish embryos, the development of chloride cells in the integument appears to signal the beginning of active ion regulation (Alderdice, 1987). The first chloride cells appear outside the branchial area. Later on, they become restricted to the gills. From hatching to the end of yolk absorption, carp larvae show an intensive mineralisation (Chapter 3). During this time, branchial chloride cells are still not present, which leaves the extra-branchial skin as the major site of ion uptake from the ambient water. According to Daye

and Garside (1976), damage to this tissue may be one of the causes of death during early life stages of fish when exposed to toxic pollutants. We investigated whether or not the reduced ion uptake from the water induced by the presence of Al was reflected by a change in the extrabranchial chloride cell density of post-hatching carp larvae.

The effects of Al were studied at pH 5.4. In particular at this pH, Al is known to have negative effects on body ion concentrations of carp larvae (Chapter 6). The effects of 2 μmol Al/l on chloride cell proliferation were studied at this pH. Body length was measured to see whether differences in chloride cell density between the experimental groups could be attributed to a specific effect of Al on these cells, or to effects of Al on development in general. To find out whether differences in chloride cell density could be correlated with disturbances in net ion uptake in the period after hatching, body Ca, Na and Mg concentrations were measured.

Materials and methods

Fertilization and incubation of eggs.

For a description of the techniques used to obtain fertilized eggs, and for the incubation protocols, we refer to Chapters 2 and 3. Methods for controlling water pH and Al concentrations are described in Chapters 2 and 4.

Parameters.

Chloride cell density.

Chloride cells of 5 larvae either reared at pH 5.4 or pH 5.4 with 2 μmol Al/l were examined at 72 h (around hatching), 96 h, 120 h, 144 h and 168 h (end of yolk absorption) after fertilization. Larvae were stained for 1.5 h with Rhodamine 123, a fluorescent vital dye which accumulates in mitochondria-rich cells. Stained larvae were examined under a confocal laserscanning fluorescence microscope and photographed. The distribution of chloride cells over the skin varied strongly, making standardisation of the sampling areas necessary. Per larva, 5 areas of 0.14 mm^2 were counted. These areas were situated at regular intervals along the body axis between the eye and above the anal opening.

Body length.

The total body length of the larvae was measured by means of the photographs made under the confocal laserscanning fluorescence microscope.

Body mineral concentrations.

At 72 h, 116 h and 168 h after fertilization, four times 25 larvae were collected and dried at 70°C for 24 h. After destruction during 24 h in 65 % HNO_3 and appropriate dilution with distilled water, they were analyzed with an Inductively Coupled Plasma Atomic Emission Spectrometer. The gain of the body Ca, Na and Mg concentrations in the period after hatching (72 h after fertilization) was calculated.

Statistical analysis.

The statistical significance of Al effects on chloride cell density and larval body length were tested by means of the Student's t-test. Significance was accepted at the 5 % level.

Results

Developmental features of control animals at pH 5.4

Organogenesis.

In addition to chloride cells, other mitochondria-rich cells were stained, mostly sensory cells of the lateral line system, the nose epithelium and the taste buds in the mouth region (Fig. 1). The first two sensory systems were already visible at 72 h, while the taste buds only became apparent at 120 h. They could be easily distinguished from the chloride cells because of size difference. Up from 144 h, control animals showed stained mitochondria-rich cells of the pronephros and the ductus deferens.

Body length.

After hatching (72 h), mean total body length was 5.7 mm. During yolk sac absorption length increased in time until 7.2 mm was reached at 168 h after fertilization (Fig. 2).

Chloride cells

Immediately after hatching, chloride cells in the skin epithelium were already present. The chloride cell density was highest on the head and the anterior part of the trunk (Fig. 1). At the surface of the yolk sac, the density was much lower, while in the tail region behind the anal opening almost no chloride cells were present. With time, chloride cell densities increased (Fig. 3). Chloride cells were not observed in the branchial area.

Body mineral concentrations.

Total body concentrations of Ca and Na increased strongly between 72 and 168 h after fertilization. Mg concentrations increased only slightly during this period (Table 1).

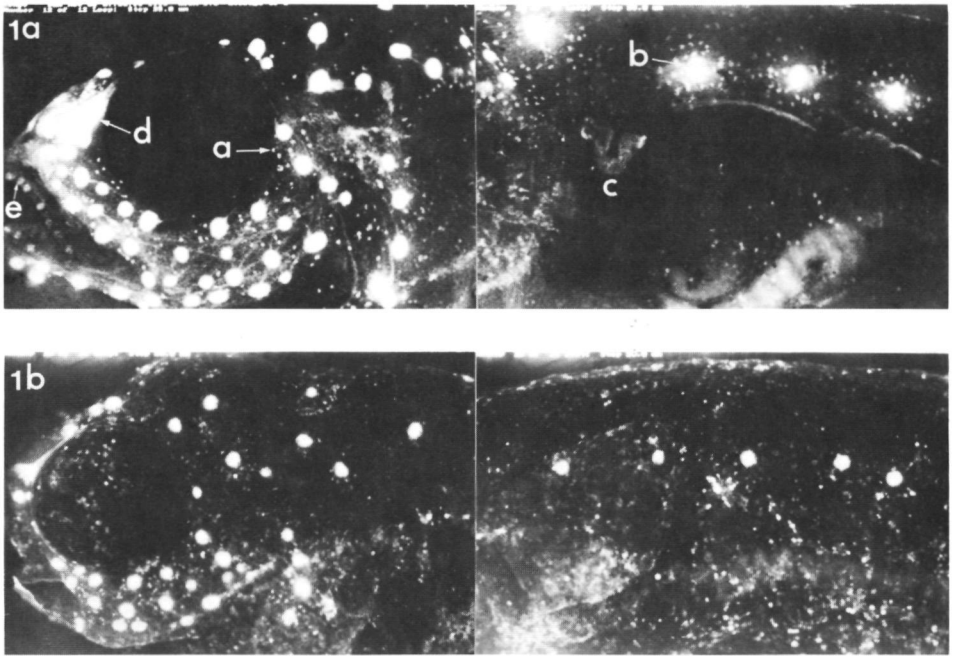

Fig. 1: Photographs of rhodamine-stained anterior part of a 168 h larva reared at pH 5.4 (Fig. 1a) and a 168 h larva exposed to pH 5.4 + 2 μmol Al/l (Fig. 1b) using a confocal laser scanning fluorescence microscope (40 x). a = chloride cell, b = lateral line system, c = pronephros, d = nose epithelium and e = taste buds.

Effects of Al at pH 5.4

Organogenesis

In larvae exposed to Al at pH 5.4, the pronephros was not visible during the entire experiment. The lateral line system and nose epithelium were far less developed than those of control larvae. Another notable feature of larvae exposed to Al was the staining of their skin epithelium with the dye, probably because of its affinity for dead or damaged cells. Nevertheless, the chloride cells could still be distinguished on the basis of their typical size and morphology.

Body length

Body lengths of Al exposed larvae were significantly ($P < 0.05$) lower at all sampling-times (Fig. 2). The net difference in length between the larvae of the experimental groups and those of controls (pH 5.4) was already established at 72 h; the net gain in length between 72 and 168 h was not influenced by Al.

Table 1: Effects of exposure to 0 and 2 μmol Al/l at pH 5.4 on body concentrations of Ca, Na and Mg in 168 h larvae (μmol/g dry weight). Means and SD's (numbers between brackets) are given; * = significant at $p < 0.05$; n = 4 groups of 25.

sample	Ca		Na		Mg	
time	-Al	+Al	-Al	+Al	-Al	+Al
72 h	32	24 *	210	171 *	52	42 *
	(5.2)	(1.6)	(13)	(22)	(0.9)	(4.2)
116 h	118	68 *	330	223 *	68	44 *
	(2.0)	(3.4)	(9.2)	(13)	(0.3)	(1.1)
168 h	236	144 *	353	395 *	74	53 *
	(11)	(9.8)	(26)	(10)	(3.1)	(2.7)

Chloride cells

The differences in chloride cell density of the skin between the experimental groups are presented in Fig. 3. The density was significantly ($P < 0.05$) lower in larvae reared in the presence of Al. This was already found at 72 h, but the difference increased with time.

Chloride cell density and body length were positively correlated (Fig. 4). Chloride cell densities of larvae with the same body length of both groups were similar.

Body mineral concentrations.

Ca: The body Ca concentration was lower in the presence of Al (Table 1). When the gain in dry body Ca concentration was plotted against the chloride cell density it appeared that the ratio of the gain in the Ca concentrations and chloride cell density was higher in Al-exposed larvae (Fig. 5).

Na: Exposure to Al also led to a lower Na concentration (Table 1), and an increase in the ratio of the gain in Na and the chloride cell density (Fig. 6).

Mg: The presence of Al resulted into a lower Mg concentration on a dry weight basis (Table 1). However, when the ratio of the gain in the Mg concentration and the chloride cell density was not changed (Fig. 7).

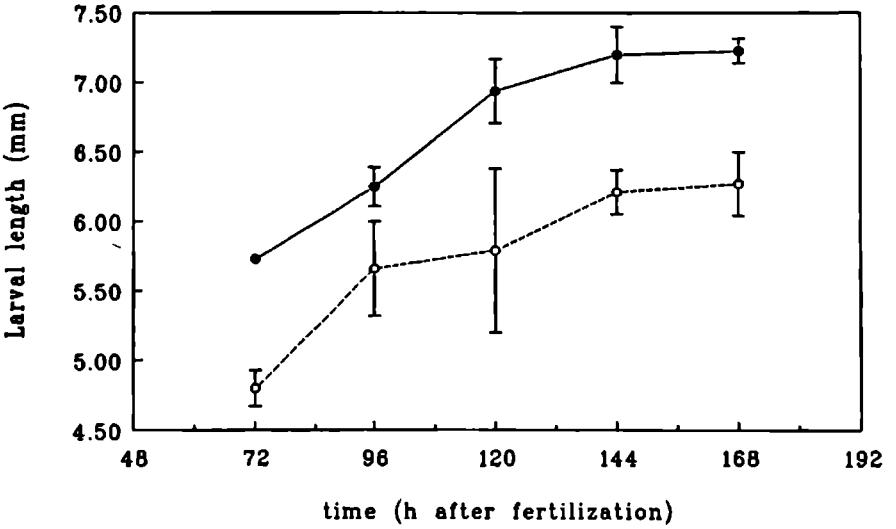

Fig. 2: Effects of 2 µmol Al/l at pH 5.4 on body length (mean values ± SD) during the mineralization phase (• = pH 5.4; o = pH 5.4 + 2 µmol Al/l).

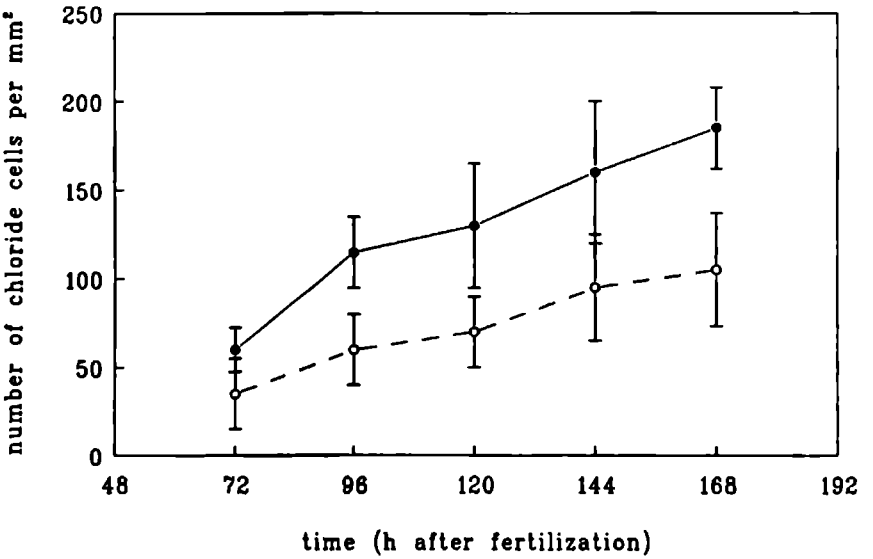

Fig. 3: Influence of 2 µmol Al/l at pH 5.4 on extrabranchial larval chloride cell density during the mineralization phase (mean values ± SD; • = pH 5.4; o = pH 5.4 + 2 µmol Al/l).

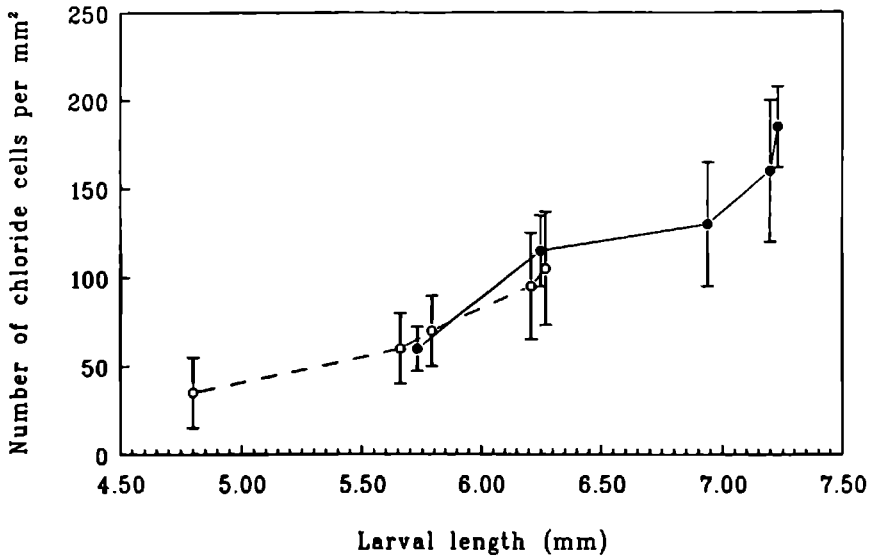

Fig. 4: Relationship between larval body length (mean values) and chloride cell density (mean values ± SD) of larvae reared at pH 5.4 (•) or at pH 5.4 + 2 µmol Al/l (○).

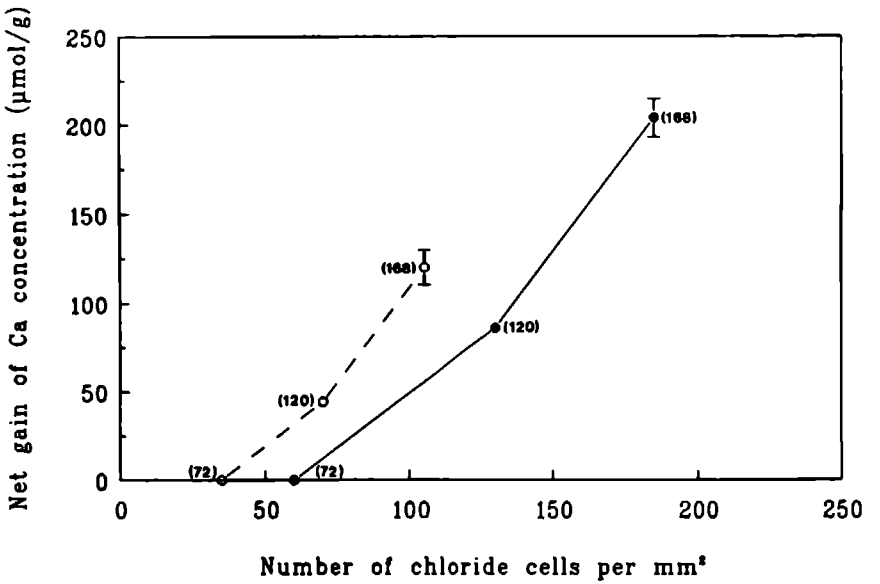

Fig. 5: Gain in dry body Ca concentration after hatching (mean values ± SD) in relation to chloride cell densities (mean values) of larvae reared at pH 5.4 (•) or pH 5.4 + 2 µmol Al/l (○). Numbers between brackets indicate h after fertilization.

Discussion

Ion regulation by larvae at pH 5.4

We found that the chloride cells were located primarily on the head and anterior part of the trunk, already prior to hatching. Similar observations were reported for embryos and larvae of seawater and freshwater teleost species (Alderdice, 1987). Cells are generally concentrated in the skin covering the epicardial region, on the yolk sac and in the tail region of the trunk (Alderdice, 1987). In embryos of sea water species, these extrabranchial chloride cells are assumed to maintain primarily the ionic balance by Na^+ and Cl^- excretion (Guggino, 1980). In freshwater species, chloride cells of adult fish contribute to the active uptake of Na^+ and Ca^{2+} from the environment (Flik *et al.*, 1985), and one may expect a similar function of these cells in larval carp. Our observations indicate that the capacity of selective ion uptake from the water in carp larvae develops around hatching. The renal ion-regulation apparently develops around 144 h after fertilization.

Effects of Al at pH 5.4

Body length and organogenesis

Exposure to Al resulted in a decrease in body length. Early development was also delayed by Al. Indications for this were observed on the lateral line system, nose epithelium and other mitochondria-rich sensory cells, which were far less developed when compared to larvae reared at pH 5.4 without Al. This Al induced developmental delay was also reflected in the formation of the pronephros and ductus deferens. We found no literature reports about effects of Al on larval growth and rate of development.

Chloride cell density

In contrast to most observations on juvenile and adult fish (Neville and Campbell, 1988; Youson and Neville, 1987; Wendelaar Bonga *et al.*, 1987), we observed a decline in chloride cell numbers as a result of Al exposure. This reducing effect of Al on chloride cell numbers has also been described for adult fish (Karlsson-Norrgren *et al.*, 1986). Large amounts of Al accumulate in chloride cells and this may lead to cellular necrosis (Karlsson-Norrgren *et al.*, 1986). Furthermore, active ion influx through the chloride cells is inhibited by Al while passive losses through paracellular routes are stimulated. Probably as a response to this ionic disturbance by low Al concentrations, a compensatory mechanism is activated leading to chloride cell hyperplasia and hypertrophy (Evans *et al.*,

1988; Karlsson-Norrgren *et al.*, 1986; Wendelaar Bonga *et al.*, 1987). Another explanation of the chloride cell reductions observed in our study in the Al-exposed larvae is the reduction of the larval development rate. We found that the positive relation between chloride cell density and body length was similar in the presence or the absence of Al (Fig. 4). Thus, just as body length, the chloride cell density is directly related to larval development.

Body mineral concentration

Our earlier observations on carp larvae have demonstrated that body concentrations of Ca, Na and Mg were lower in the presence of Al (Chapter 6), and this is confirmed in the present study. Similar observations for Ca and Na were done on other fish species, while no effects of Al on Mg were measured (Wood *et al.*, 1990; Peterson *et al.*, 1982; Peterson and Martin-Robichaud, 1986; Reader *et al.*, 1988).

One possible mechanism leading to the lower levels of Ca and Na by Al might be Al-induced developmental delay and hence interference of Al with the intensive mineralisation associated with normal development. Since uptake of Ca and Na occurs mainly after hatching (Chapter 3), a delay in hatching time reduces the time-span during which this accumulation can occur.

Another possible mechanism is direct interference of Al with the transport capacity of the chloride cells. Ca^{2+} and Na^{+} are both transported via the chloride cells from the surrounding water into the body, and the presence of more functional chloride cells could lead to an increased uptake of Ca and Na. Since Na^{+} K^{+}-ATPase activity and Ca^{2+} transport in adult fish are negatively affected by Al (Staurnes *et al.*, 1984; Verbost *et al.*, 1992), a reduction of the transport capacity of the chloride cells may be expected. In addition to relating the body ion concentrations to time at fixed time-intervals, we also related them also to chloride cell density (e.g. developmental stage). Then, it appeared that the ratios of Ca/Na gain and chloride cell density are higher in the presence of Al. From this we can deduce that the gain in Ca and Na was higher in larvae exposed to Al than in those exposed to pH 5.4 only. This suggests that Al improves the transport capacity of the chloride cells despite the fact that Al is known to reduce the activity of Ca and Na transporting enzymes. However, when larvae of the same chloride cell density are compared, those of the Al-exposed group, although being in the same developmental stage, are much older than the control larvae. This explains that although the higher observed Ca and Na concentrations because these larvae had more time for mineral accumulation.

The present observation that the Mg concentration is lower when larvae are exposed to Al supports our earlier observations (Chapter 6). In Chapter 3 we demonstrated that Mg

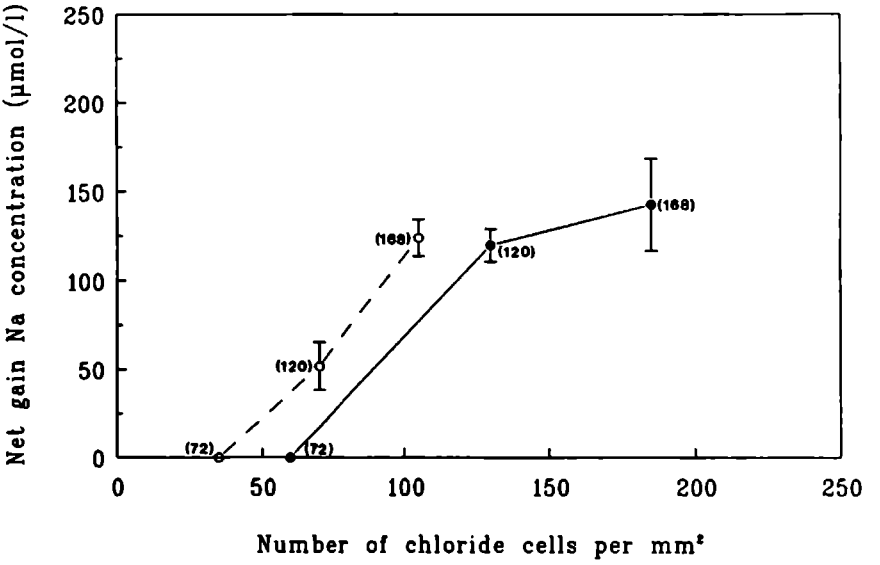

Fig. 6: Gain in dry body Na concentration after hatching (mean values ± SD) in relation to chloride cell densities (mean values) of larvae reared at pH 5.4 (•) or pH 5.4 + 2 µmol Al/l (○). Numbers between brackets indicate h after fertilization.

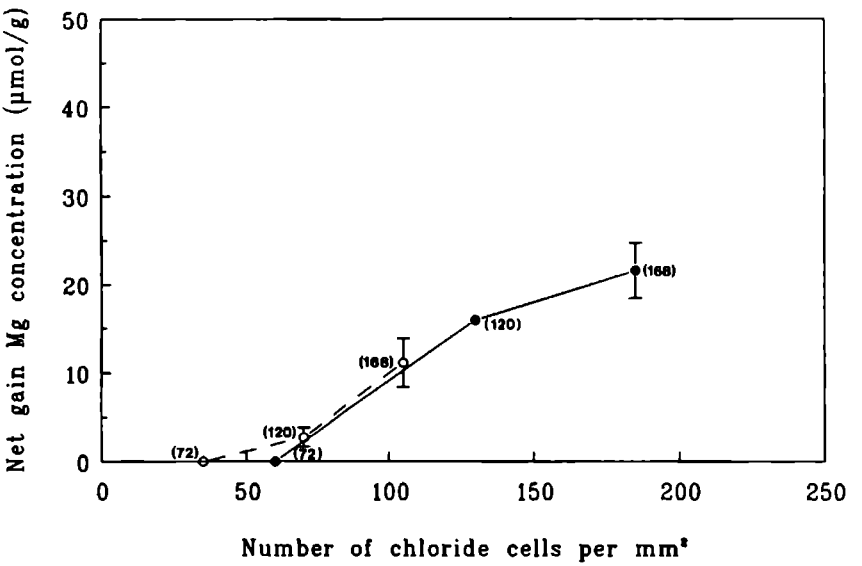

Fig. 7: Gain in dry body Mg concentration after hatching (mean values ± SD) in relation to chloride cell densities (mean values) of larvae reared at pH 5.4 (•) or pH 5.4 + 2 µmol Al/l (○). Numbers between brackets indicate h after fertilization.

concentrations increased with larval development, due to loss of body weight and not because of uptake of Mg from the environment. Therefore the positive correlation between the chloride cell density and the gain in Mg concentration does not imply a functional relationship (Wood *et al.*, 1990). The positive correlation between the chloride cell density and the gain in Mg concentrations can be considered as indirect since, like the chloride cell density, the Mg concentration is assumed to be a marker of development (Chapter 3).

Epithelial skin damage

Disturbance of ion balance can not only be induced by chloride cell dysfunction, but also by leakage of ions through the integument (McDonald, 1983). Our observations on carp larvae showed a remarkable integumental damage, that could easily lead to ion losses. In adult *Oreochromis mossambicus*, water pH 4.0 induced necrosis of superficially located cells of the skin (Wendelaar Bonga *et al.*, 1987). On the other hand, Segner *et al.* (1988) observed no histopathological changes of the epidermis of larval *Salmo trutta* after exposure to low water pH and Al.

In summary we conclude that the appearance of chloride cells is delayed by the presence of Al. This is likely the result of a general delay in larval development, which is reflected in other parameters such as body length and development of the kidney and the lateral line system. The observed reductions in the total body Ca and Na concentrations in the presence of Al may also be the result of delayed larval development.

References

Alderdice, D.F., 1988. Osmotic and ionic regulation in teleost eggs and larvae: pp 163-251. In: Fish Physiology Vol. XI, W.S. Hoar and D.J. Randall (eds), Academic Press Inc. (London).

Daye, P.G. and E.T. Garside, 1976. Histopathologic changes in surficial tissues of brook trout, *Salvelinus fontinalis* (Mitchill), exposed to acute and chronic levels of pH. Can. J. Zool. 54: 2140-2155.

Evans, R.E., Brown, S.B. and T.J. Hara, 1988. The effects of aluminium and acid on the gill morphology in rainbow trout, *Salmo gairdneri*. Env. Biol. Fishes 22: 299-311.

Flik, G., Van Rijs, J.H. and S.E. Wendelaar Bonga, 1985. Evidence for high affinity Ca^{2+} ATPase activity and ATP driven Ca^{2+} transport in membrane preparation of the gill epithelium of the ciclid fish *Oreochromis mossambicus*. J. Exp. Biol. 119: 335-347.

Guggino, W.B., 1980. Water balance in embryos of *Fundulus heteroclitus* and *Fundulus bermudae* adapted to sea water. Am. J. Physiol. 238: R42-R49.

Gunn, J.M. and D.L.G. Noakes, 1987. Latent effects of pulse exposure to aluminium and low pH on size, ionic composition, and feeding efficiency of lake trout (*Salvelinus namaycush*) alevins. Can. J. Fish. Aquat. Sci. 44: 1418-1424.

Karlsson-Norrgren, L., Dickson, W., Ljungberg, O. and P. Runn, 1986. Acid water and aluminium exposure: gill lesions and aluminium accumulation in farmed brown trout, *Salmo trutta* L. J. Fish Diseases 9: 1-9.

Leuven, R.S.E.W., 1988. Impact of acidification on aquatic ecosystems in the Netherlands. Ph. D. thesis, Catholic University Nijmegen, The Netherlands.

Nelson, J.A., 1982. Physiological observations on developing rainbow trout, *Salmo gairdneri* (Richardson), exposed to low pH and varied calcium ion concentrations. J. Fish Biol. 20: 359-372.

Neville, C.M. and P.G.C. Campbell, 1988. Possible mechanisms of aluminium toxicity in a dilute, acidic environment to fingerlings and older life stages of salmonids. Water Air Soil Pollut. 42: 311-321.

Peterson, R.H., Daye, P.G., Lacroix, G.L. and E.T. Garside, 1982. Reproduction in fish experiencing acid and metal stress: pp 177-196 In: R.E. Johnson (ed.) Acid Rain/Fisheries American Fisheries Society Bethesda, M.D.

Peterson, R.H., and D.J. Martin-Robichaud, 1986. Growth and major inorganic cation budgets of Atlantic salmon alevins at three ambient acidities. Trans. Am. Fish. Soc. 115: 220-226.

Playle, R.C., Goss, G.G. and C.M. Wood, 1989. Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminium exposures in soft water of two calcium concentrations. Can. J. Zool. 67: 314-324.

Reader, J.P., Dalziel, T.R.K. and R. Morris, 1988. Growth, mineral uptake and skeletal calcium deposition in brown trout, *Salmo trutta* L., yolk-sac fry exposed to aluminium and manganese in soft acidic water. J. Fish Biol. 32: 607-624.

Reid, S.D., McDonald, D.G. and R.R. Hem, 1991. Acclimation to sublethal aluminium: Modifications of metal-gill surface interactions of juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 48: 1996-2005.

Segner, H., Marthaler, R. and M. Linnenbach, 1988 Growth, aluminium uptake and mucus cell morphometries of early life stages of brown trout, *Salmo trutta*, in low pH water. Envir. Biol. Fish. 21: 153-159.

Staurnes, M., Sigholt, T. and O.B. Reite, 1984. Reduced carbonic anhydrase and Na-K-ATPase activity in gills of salmonids exposed to aluminium-containing acid water. Experientia 40: 226-227

Verbost, P.M., Lafeber, F.P.J.G., Spanings, T.A.T., Aarden, E.A. and S.E. Wendelaar Bonga, 1992. Inhibition of Ca^{2+} uptake in freshwater carp, *Cyprinus carpio*, during short-term exposure to aluminium. J. Exp. Zool. 262: 247-254.

Wendelaar Bonga, S.E., Flik, G. and P.H.M. Balm, 1987. Physiological adaptation to acid stress in fish. Ann. Soc. Roy. Zool. Belg. 117: 243-254.

Wood, C.M., McDonald, D.G., Ingersoll, C.G., Mount, D.R., Johansson, O.E., Landsberger, S. and H.L. Bergman, 1990. Effects of water acidity, calcium, and aluminium on whole body ions of brook trout (*Salvelinus fontinalis*) continuously exposed from fertilization to swim-up: a study by instrumental neutron analysis. Can. J. Fish. Aquat. Sci. 47: 1593-1603.

Youson, J.H. and C.M. Neville, 1987. Deposition of aluminium in the gill epithelium of rainbow trout (*Salmo gairdneri* Richardson) subjected to sublethal concentrations of the metal. Can. J. Zool. 65: 647-656.

CHAPTER 8

Effects of acid water on growth and energy metabolism of juvenile carp.

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submitted to Aquatic Toxicology

Effects of acid water on growth and energy metabolism of juvenile carp.

Abstract

Juvenile carp were reared in a flow-through system for 8 weeks at pH values of 8.0; 5.7 and 5.3. Growth, relative growth rate, food conversion and the energy required for basal metabolism (= sustaining energy) were determined. The relative growth rate was affected by pH and decreased from 1.42 % body weight/day at pH 8.0 to only 0.46 % at pH 5.3, while the food conversion was elevated from 1.9 to 9.3. The sustaining energy at pH 5.3 increased with 29 % relative to control levels. Similar effects, although less pronounced, occurred at pH 5.7.

Introduction

Field observations on the effect of acid water on growth of fish, are not consistent. Decreased as well as increased growth rates have been reported (Fromm, 1980; Harvey, 1982). Interference with factors such as inter- and intraspecies competition, food availability and the presence of additional stressors such as aluminium, are likely responsible for this apparent discrepancy. The aim of this study is to determine the effect of low water pH on growth and energy metabolism of juvenile carp under controlled conditions. Growth is only possible when the available energy in the food exceeds that of the sustaining energy. Sustaining energy is defined as the energy need of fish to maintain a constant body weight. Growth is the net gain of weight as a result of food uptake and conversion, minus the loss of weight due to metabolic activity. At low pH osmoregulatory processes demand more energy, leaving less energy for growth. We calculated the increase in sustaining energy in acid water on the basis of the observed growth reductions in this condition.

Materials and methods

Juvenile carp of about 10 gram were exposed in flow-through systems at pH 8.0 (control), 5.7 and 5.3 after acclimation for 2 weeks under control conditions. Every experimental group consisted of 120 fish, distributed over 6 aquaria (N = 6) of 200 l each. Water inflow was 30 l/h for each aquarium and the temperature was regulated at 23 $\pm$ 1°C. The water in the aquaria was aerated and the pH controlled by a pH stat system. It could be maintained within 0.1 pH unit. CO_2 formed during the acidification process was allowed to escape before the fish were exposed to the water. Nijmegen tap water was

used. Fish were fed once a day with commercial carp pellets (Trouvit K30, Trouw Nederland BV). The feeding level was kept at 2 % of the metabolic weight (kg food = $0.02*(W_b \text{ in kg})^{0.8}$). At this feeding level, all the food was consumed. The body weight (W_b) was measured every two weeks and the amount of food was adjusted accordingly.

The parameters determined were W_b, mean relative growth rate, food conversion (=1/growth efficiency or $1/K_1$) and percentage increase in sustaining energy level. Growth was defined as gain in biomass, including the gonads. The relative growth rate was calculated as the mean of (kg gain body weight/day)/(kg initial body weight) every two-week period. Food conversion was defined as (kg food ingested)/(kg gain body weight).

In juvenile animals, the energy left for growth processes is equivalent to the available food energy minus sustaining energy. Winberg (1956) formulated the following "balanced energy equation":

$\Delta W = pR - T$

ΔW = energy of gain body weight/unit of time
p = % available energy in food
R = energy in food/unit of time
T = heat production/unit of time

Approaching our observations with this formula requires a few assumptions:
1) The digestion and uptake efficiency of the food is not influenced by pH exposure.
2) The ratio of the synthesis of protein and fat remains constant under the different experimental conditions, so gain in body weight can be used as an indication for energy gain.

The sustaining food level under control conditions was calculated as:

$\Delta W = 0$, which means

$0 = pR_{\text{sust. pH 7.5}} - T_{\text{pH 7.5}}$ or $pR_{\text{sust. pH 7.5}} = T_{\text{pH 7.5}}$

Because the sustaining food level is dependent on size and other factors such as crowding, this food level was determined for our experimental conditions. This value was used for energy calculations. Groups of 20 carp, weighing 10 gram each, were fed at 0.75, 1.0, 1.5 and 2% of the metabolic weight. Extrapolation of the specific growth rate curve to the point where no growth occurred indicated the sustaining food level.

For the different pH groups, a 2 % feeding level was used, so the available food energy (pR) was the same for these groups. This implied that changed growth rates (change in ΔW) as a result of acid stress will indicate a changed level of sustaining energy or:

if $\Delta W_{pH\,7.5} \neq \Delta W_{pH\,5.3}$, then $T_{pH\,7.5} \neq T_{pH\,5.3}$.

$\Delta W_{pH\,7.5} = pR_{2\%} - T_{pH\,7.5} = pR_{2\%} - pR_{mnt.\,pH\,7.5}$

$\Delta W_{pH\,5.3} = pR_{2\%} - T_{pH\,5.3}$

$\Delta W_{pH\,7.5} - \Delta W_{pH\,5.3} = -T_{pH\,7.5} + T_{pH\,5.3} = -pR_{mnt.\,pH\,7.5} + T_{pH\,5.3}$ thus

$T_{pH\,5.3} = pR_{mnt.\,pH\,7.5} + (\Delta W_{pH\,7.5} - \Delta W_{pH\,5.3})$.

For calculating the percentage change of the sustaining energy during acid stress relative to the sustaining energy under normal conditions, it is sufficient to approach growth rates as a result of energy addition in terms of percentual feeding levels (it is not necessary to know the absolute energy values).

For statistical analysis, the Mann-Whitney-U-Test was applied.

Results

The growth of young carp exposed to pH 8.0, 5.7 and 5.3 is shown in Fig. 1. Growth was strongly affected at pH 5.3, and to a slightly lesser extent at pH 5.7. The mean relative growth rate at the different pH levels is presented in Fig. 2. At pH 5.3, the relative growth rate was only 34 % of that at pH 8.0. The influence of water pH on food conversion is illustrated in Fig. 3. It shows that for producing one kg of biomass, the required amount of food at pH 5.3 is 9.3 kg, compared to 1.9 kg at pH 8.0. Fig. 4 gives the growth rate at pH 8.0 in relation to the food level. The intercept of the curve with the horizontal axis indicates that the sustaining food level at this pH is 1.24 % of the metabolic body weight. Since the standard deviations of the growth rates are relatively high compared to the angle of the curve at the intercept, also the value of the sustaining food level varies considerable. This value was used to calculate the percentage gain in sustaining energy at low water pH. Energy demands for sustaining processes increased with 24 % at pH 5.7 and with 29 % at pH 5.3.

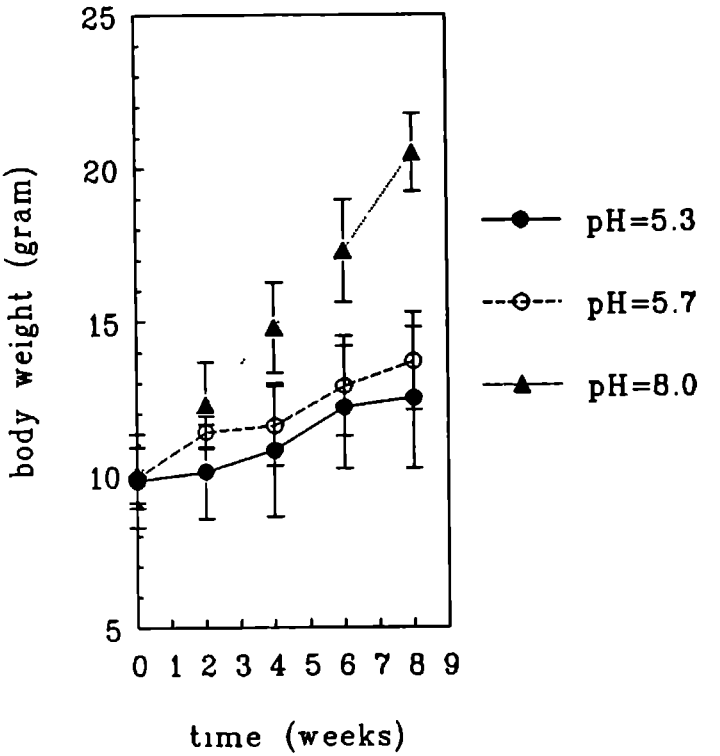

Fig. 1. Increase in body weight of juvenile carp subjected for 8 weeks to different water pH levels during 8 weeks (means ± SD; n = 6).

Discussion

Our results clearly showed a decrease in growth rate when young carp were exposed to acid stress. Indications for similar effects of low pH on growth rates were found by others. For example, Menendez (1976) reared alevins of brook trout (*Salvelinus fontinalis*) at pH values in the range of pH 7.1 to pH 5.0, and observed at pH 6.5 and lower decreasing growth rates. At pH 5.5, growth was about 50 % of controls. Extrapolating our observations on growth reductions of carp indicates a pH value of 5.65 for 50 % growth reduction. In contrast to our study, Menendez administered food ad libitum. Reduced food uptake in acid water could therefore be a factor contributing to the growth rate differences. The growth rate of rainbow trout and arctic char was not affected at pH 6.2 and 5.5, whereas a 8 % decrease was found at pH 4.8. The growth of brown trout was not affected at any of these pH levels (Gjedrem, 1980). These fish were also

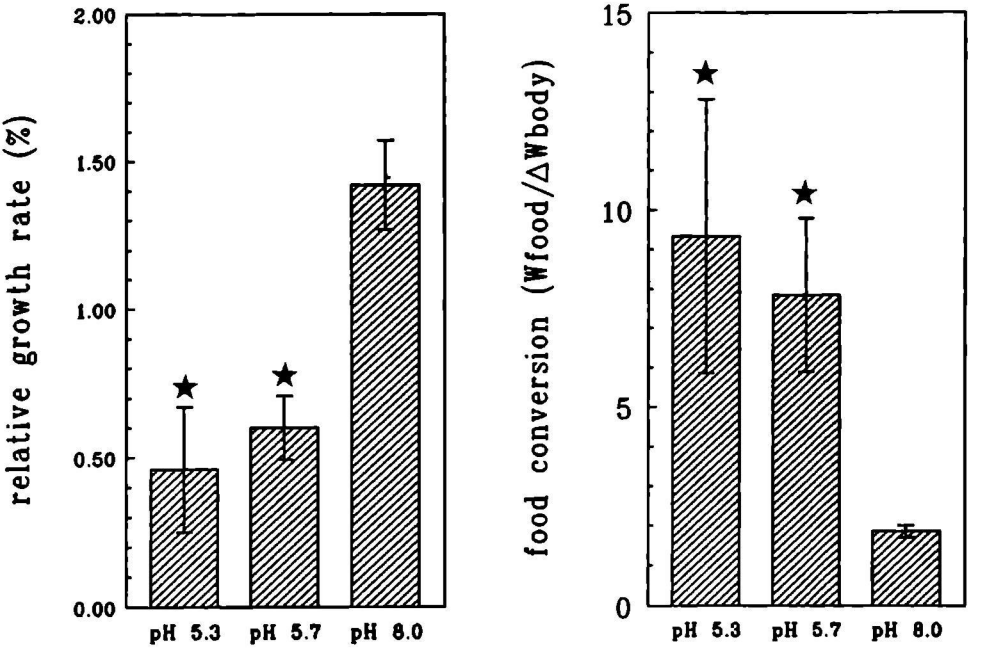

Fig. 2. Relative growth rate of juvenile carp (10 g body weight) kept for 8 weeks at different pH levels (means ± SD; n = 6; * = $p<0.001$).

Fig. 3. Effect of water pH on food conversion of juvenile carp (10 g body weight; means ± SD; n = 6; * = $p<0.001$).

fed ad libitum

During the following growth experiments fish were fed fixed diets. Rodgers (1984) exposed brook trout to pH 6.5 and 5.3 at a feeding level of 1.1 % of their body weight. Reduction of growth at the lower pH was about 40 % of that at pH 6.5. Tam and Payson (1986) found for the same species that at pH 4.5 and pH 5.15 growth was retarded with about 30 %, while exposure at pH 5.5 had no influence when compared to the controls at pH 7.4. Exposure of brown trout (*Salmo trutta*) to a pH range of 6.3-4.7 did not affect growth rates, but at pH 4.3 growth was only 43 % of that at pH 6.3 (Sadler and Lynam, 1987). Jacobson (1977) observed no differences in growth rates after exposing brown trout to pH 5.0, 5.44 and 6.25, but Sadler and Lynam (1987) found that decreased growth for this species occurred only at pH levels lower than 4.7. Finally, growth of Atlantic salmon (*Salmo salar*) reared at pH 5.2 was 22 % of that of fish kept at pH 7.0 (Perry, 1990). We conclude that growth of carp is affected at pH levels similar to those which affect growth of salmonid species.

An increased value for food conversion at low water pH, as observed in our

experiment, was also noted by Svenson et al (1989). They lowered the pH of a small lake from pH 6.1 to pH 5.5, and found that the food conversion of largemouth bass (*Micropterus dolomieu*) increased from 4.1 to 7.0. We found values for carp of 1.9 at pH 8 and 7.8 at pH 5.7. Svenson *et al.* further mentioned a growth reduction of about 35 % in bass at pH 5.5 when compared to pH 6.1.

Reports on changes in sustaining energy as a result of acid stress are not known to us. To calculate the increase of this energy, we determined the sustaining food level of 10 g carp, which appeared to be 1.24 % of the metabolic weight. Huisman (1977) found a similar sustaining food level for carp of 50 g (1.2 %).

Since the variance of the estimated sustaining food level was substantial, and a small difference in this level leads to marked differences in the calculated sustaining food levels at low pH, the latter values must be considered as a rough estimate. At relatively mild acidity (pH 5.7), the extra energy demand already amounted 29 % of the sustaining energy at neutral pH. Our unpublished observations on carp showed that it could survive pH 4.0. This most likely means a large increase of energy need for sustaining activity under these conditions. At relatively low concentrations, Ca^{2+} are protective against the effects of low water pH, by reducing the disturbance of water and ion balance (Wood and Mc Donald, 1982). According to Rodgers (1984), elevation of Ca^{2+} concentrations of the surrounding water reduced the growth reduction experienced by brown trout in acid

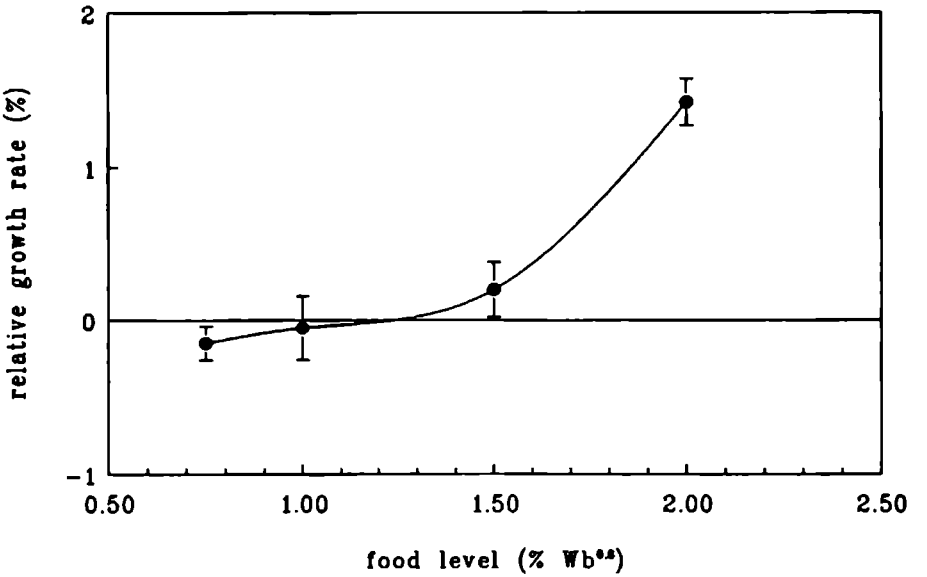

Fig. 4. Relative growth rate (%) of carp (10 g body weight) as function of the administered food level (% $W_b^{0.8}$), mean values ± SD (n = 6).

water. This is an indication that energy used for growth in neutral water is, at least partially, used for compensation of the osmoregulatory imbalance in acid water.

Reduced growth of carp as a result of low water pH will have severe consequences for the population. In Chapter 2 we showed that successful hatching of carp eggs is limited at pH levels lower than pH 5.2-5.0. Our present results demonstrate that at these pH levels growth of juveniles is strongly affected. A minimum body size is required to survive the winter season. If this minimum size has not been reached as result of reduced growth through acid stress, or if the increased sustaining energy need consumes too much stored energy, the population becomes endangered. In addition, fish egg production may become affected, because egg production is related to body weight.

References

Fromm, P.O., 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Environm. Biol. Fishes 5, 79-93.

Gjedrem, T., 1980. Growth and survival of fingerlings in acidic water. Proc. Int. Conf. Ecol. Impact Acid Precip., Norway 1980, SNSF Project: P 30.

Harvey, H.H., 1982. Population responses of fishes in acidified waters. Pages 227-242 in Johnson (1982).

Huisman, E.A., 1976. Sauerstoff als Umweltfactor fur die Fischproduktion. H.H. Reichenbach-Klinke (Ed.), Fisch und Umwelt 2, 135-144.

Jacobsen, O.J., 1977. Brown trout (*Salmo trutta* L.) growth at reduced pH. Aquaculture 11, 81-84.

Menendez, R., 1976. Chronic effects of reduced pH on brook trout (*Salvelinus fontinalis*). L. Fish. Res. Bd Can. 33, 118-123.

Perry. M.C., 1990. Chronic effects of low pH on length and weight of Atlantic salmon, *Salmo salar*. Environm. Biol. Fishes 27, 153-155.

Rodgers, D.W., 1984. Ambient pH and calcium concentration as modifiers of growth and calcium dynamics of brook trout, *Salvelinus fontinalis*. Can J. Fish. Aquat. Sci. 41, 1774-1780.

Sadler, K. and S. Lynam, 1987. Some effects on the growth of brown trout from exposure to aluminium at different pH levels. J. Fish Biol. 31, 209-219.

Svenson, A.W., J.H. McCormick, T.D. Simonson, K.M. Jensen and J.G. Keaton, 1989. Experimental acidification of Little Rock Lake (Wisconsin): fish research approach and early responses. Arch. Environ. Contam. Toxicol. 18, 167-174.

Tam, W.H. and P.D. Payson, 1986. Effects of chronic exposure to sublethal pH on growth, egg production, and ovulation in brook trout, *Salvelinus fontinalis*. Can. J. Fish. Aquat. Sci. 43, 275-280.

Wood, C.M. and D.G. McDonald, 1982. Physiological mechanisms of acid toxicity to fish. Acid Rain/Fisheries. Proc. Int. Symp. Acidic Prec. Fish. Imp. Northern North America., New York, 1981; pp. 197-225.

CHAPTER 9

Responses of juvenile carp to acid water and aluminium: gill histology and blood parameters.

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Responses of juvenile carp to acid water and aluminium: gill histology and blood parameters.

Abstract

Juvenile carp were exposed to pH 7.5, pH 5.4 and pH 5.4 + 7.5 μmol/l aluminium (Al) in a flow-through system. They were sampled after 0, 1, 3, 7, 15, 30 and 60 days. The parameters studied were haematocrit, blood plasma osmolarity, chloride cell density in the opercular epithelium, and number and size of mucous cells in the branchial epithelium. Exposure to low pH did not affect the hematocrit. The osmolarity of the blood plasma was lower, chloride cells increased in numbers, and mucous cells increased in numbers and size during the first 4 days of exposure. Afterwards restoration occurred, and after 60 days most effects had disappeared. Only blood osmolarity remained below control levels. The presence of Al in acid water resulted in increased osmolarity, elevated hematocrit levels, increased numbers of chloride cells, and increased numbers and size of mucous cells when compared to control fish exposed to acid water. All these changes were transient. The drop of blood plasma osmolarity was less pronounced than in the Al-free controls.

Introduction

Effects of sublethal low pH levels are mainly restricted to iono-regulatory disturbances, while exposure of fish to low pH and Al may lead to disturbance of ion regulation and respiration (Ultsch *et al.*, 1980; Muniz and Leivestad, 1980; Howells *et al.*, 1990). These effects point to the gills as the main target organ, and this has been supported by morphological studies (Mueller *et al.*, 1991). The maintenance of ion regulation in fish is to a high extent dependent on the chloride cells in the gills. Low water pH and Al are both known to affect these cells, and this is reflected in the ion composition of the blood (Staurnes *et al.*, 1984; Chevalier & Gauthier, 1985; Playle *et al.*, 1989, Wendelaar Bonga *et al.*, 1990). Respiration is affected by Al-induced gill damage and mucus accumulation on the gills, leading to impaired gas diffusion (Malte, 1986).

In this study we exposed juvenile carp for different periods of time (up to 60 days) to sublethal low pH levels and Al concentrations, and studied the effects on gill morphology and on some blood parameters.

Materials and methods

Experimental animals and exposure protocols.

Juvenile carp of ± 75 g body weight were used. Every experimental group consisted of 35 fish. Water temperature was kept at 23°C. Fish were fed pellets in a ration of about 2 % of the metabolic body weight, $(Wb \text{ in } kg)^{0.8}$, per day. This ration was just above the maintenance food level, to restrict complexation of Al with food residues or excretion products.

Groups of fishes were exposed to pH 7.5, pH 5.4 or pH 5.4 with 7.5 μmol Al/l, in three separate flow-through systems. At pH 5.4, Al is known to be toxic (Chapters 4 and 6). About 80 % of the total Al concentration appeared to be in the inorganic form, as measured by the method of Driscoll (1984). Prior to exposure, fish were acclimated to the holding conditions (200 l tanks) for two weeks. Water flow was 3 l/min. Nijmegen tap water was used (ion concentration of the water in mmol/l: Ca, 0.8; Na, 1.9; K, 0.05; Cl, 3.1; Mg, 0.2).

Before the water entered the exposure tanks, it was mixed in a separate tank with the required amounts of sulfuric acid and a stock solution of $Al_2(SO_4)_3$ added by means of tubular pumps (Masterflex). Monomeric Al concentrations were determined colorimetrically on pyrocatechol violet (Wilson and Sergeant, 1963). Water pH was maintained with a pH-stat system. Fish were gradually exposed to low pH and Al: final pH and Al concentrations were reached 6 h after the start of the experiment.

Tissue preparation and examination.

Samples of 5 fish each were taken after 0. 1, 3, 7, 15, 30 and 60 days of exposure. Fish were anaesthetized with a buffered solution of 1 g MS 222/l. Parameters studied were chloride cell and mucous cell densities, mucous cell diameter, blood hematocrit (volume percent of erythrocytes) and osmotic value of the blood plasma.

Blood from the cut-off tail end was collected in heparinized microcapillaries. Haematocrit values were measured after centrifugation for 3 min. Blood plasma was collected and its osmotic value established with a micro-osmometer.

Chloride cell densities were determined in the epithelium covering the inner side of the right operculum. The opercula were stained for 2 h in DASPEI, a fluorescent vital dye concentrating in mitochondria (Wendelaar Bonga *et al.*, 1990). Opercula were examined for chloride cells under a fluorescence microscope. Cells were counted randomly in 25 areas (0.06 mm^2 each) per fish; epithelial areas around large blood vessels were excluded.

The middle part of the first right gill arch was taken for histological examination of the

mucous cells. They were fixed in Bouin's solution and embedded in paraplast after dehydration in a graded series of ethanol and xylene. Cross sections of 10 μm thickness were cut, mounted on glass slides, and rehydrated. Staining followed for 30 min. with Alcian Blue and for 1 min with eosin, respectively. Of every fish, 30 fields (0.34 mm^2 each) in the gill sections were examined to determine the mucous cell density. Diameters were measured for those mucous cells which were in contact with the outer epithelial surface. The longest cell axis was measured of 30 cells per fish.

Statistical analysis.

Data (n=5) are reported as mean values $\pm$ SD. The low pH group and the low pH with Al group were compared with the control (pH 7.5), and with each other, with the Mann-Whitney-U-test. A 5 % significance level was accepted for significance.

Results

No mortality was observed during the experiment. Exposure to Al, and to a lesser degree to low pH, induced behavioural changes. Fish remained passively just above the bottom of the exposure tank, and their fins were contracted against the body. Ventilatory rates increased and "coughing" occurred at irregular intervals.

Haematocrit.

Water acidification to pH 5.4 had no significant effect on the hematocrit (Fig. 1). In the presence of 7.5 μmol Al/l, the hematocrit was elevated significantly ($p<0.05$) when compared to control values (pH 7.5) and the values measured at low pH without Al. Highest levels were observed at day 1 and 3. After 5 days, the elevation was less pronounced (although still significant), and eventually the hematocrit returned back to control levels.

Plasma osmolarity.

Exposure to pH 5.4 only and to pH 5.4 with Al resulted in a significant ($p<0.05$) reduction of plasma osmolarity (Fig. 2). This effect was less pronounced in the presence of Al. Lowest values were observed at day 3. In carp exposed to low pH only, the values increased at day 7 although complete recovery did not occur. Plasma osmolarity of animals exposed to pH 5.4 with Al gradually returned to control values at day 60.

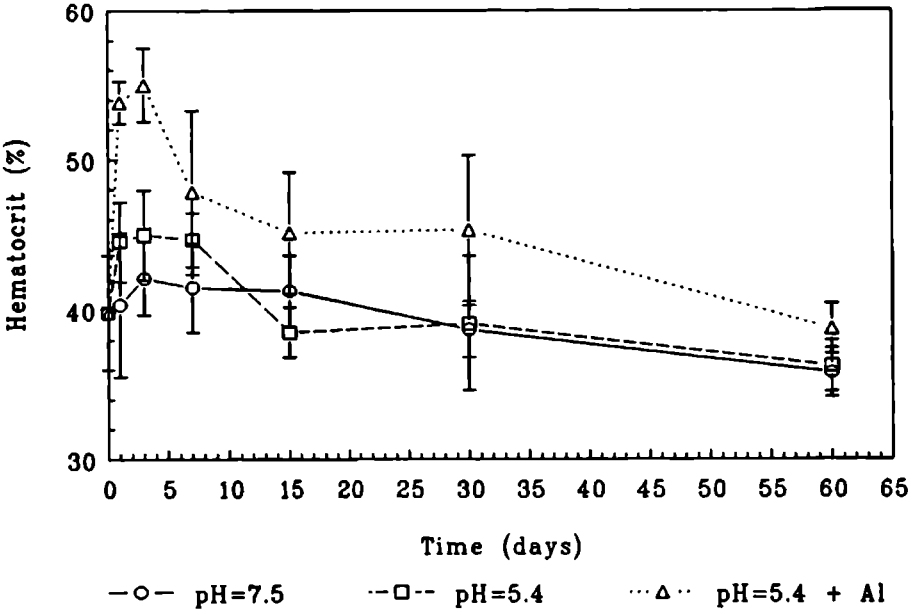

Fig. 1: Haematocrit of fish exposed to water of pH 7.5, pH 5.4 and pH 5.4 + 7.5 µmol Al/l (mean values ± SD).

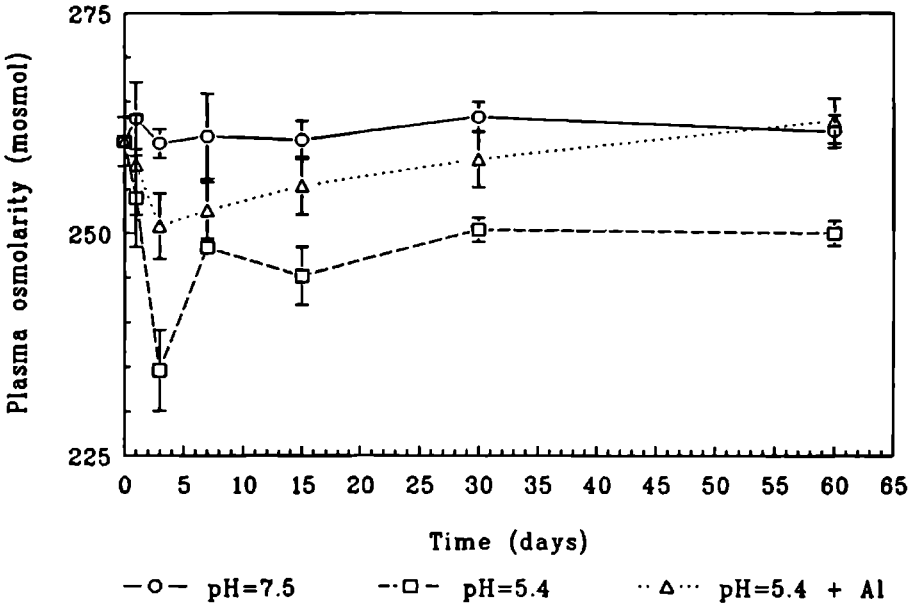

Fig. 2: Blood plasma osmolarity of carp kept at pH 7.5, pH 5.4 and pH 5.4 + 7.5 µmol Al/l (mean values ± SD).

Chloride cell densities

Chloride cell densities increased in fish kept at pH 5.4 with or without Al (Fig. 3). The response was already apparent at day 1 and was strongest in the presence of Al. Maximum values for both exposure conditions were found between 15 to 30 days of exposure, when a 4-fold increase was found in the pH 5.4 group, and a 6-fold increase in the pH 5.4 with Al group. At day 60, chloride cell densities of both groups had declined, although they were still above control levels.

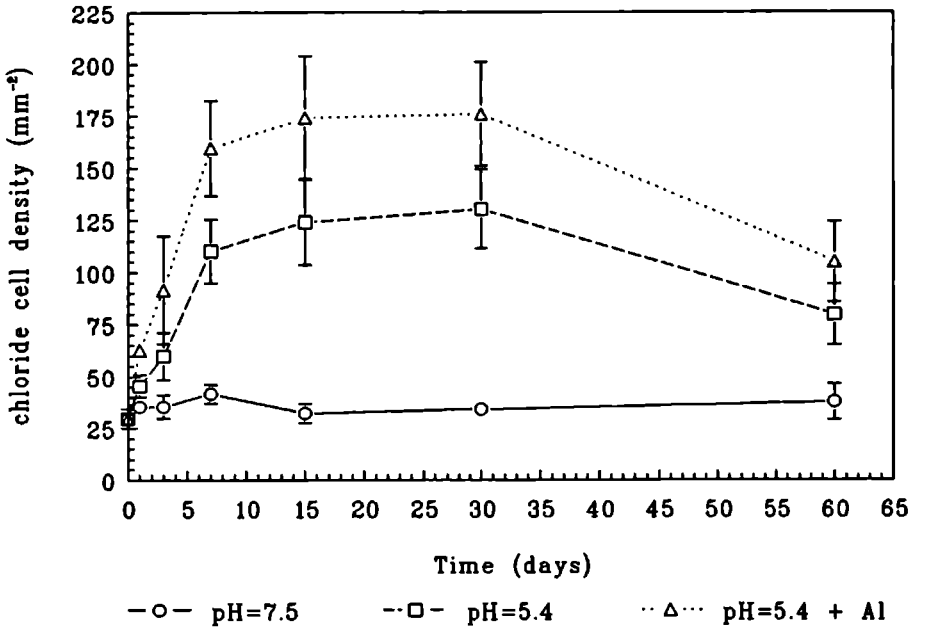

Fig. 3: Chloride cell density in the opercular epithelia of carp kept at pH 7.5, pH 5.4 and pH 5.4 + 7.5 μmol Al/l (mean values ± SD).

Mucous cell diameters.

Exposure to pH 5.4 and to pH 5.4 + Al increased mucous cell densities in the gills for a period of 30 days (Fig. 4). These elevations were most pronounced at days 3 and 7. The densities in fish exposed to low pH only were 80 % higher than at pH 7.5, while exposure at pH 5.4 with Al resulted into an increase of 110 %. The differences between the pH 5.4 group and the pH 5.4 with Al group were statistically significant ($P < 0.05$) during this period. At day 30 and later differences between the experimental groups were no longer apparent.

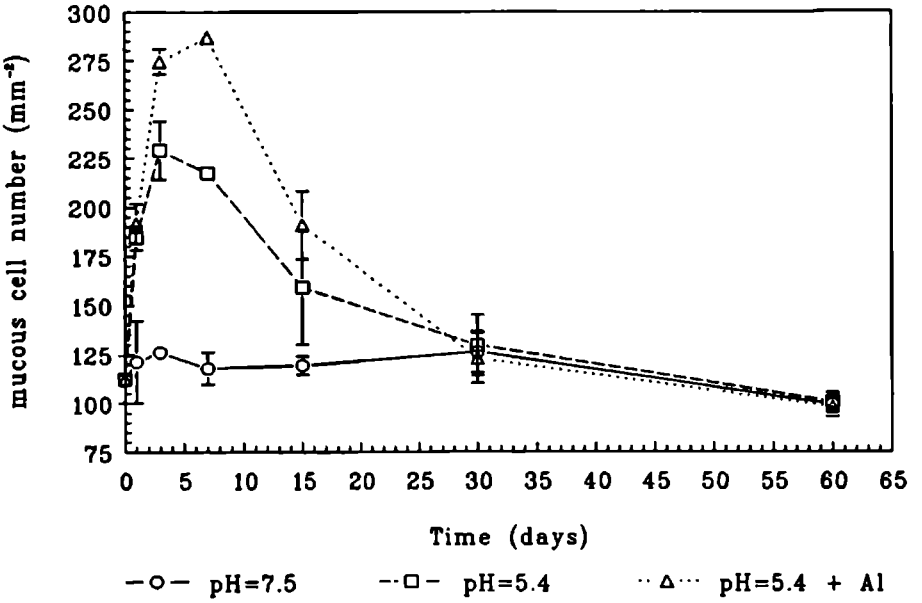

Fig. 4: The effect of exposure to pH 5.4 or to pH 5.4 + 7.5 µmol Al/l on branchial gill mucous cell density (mean values ± SD).

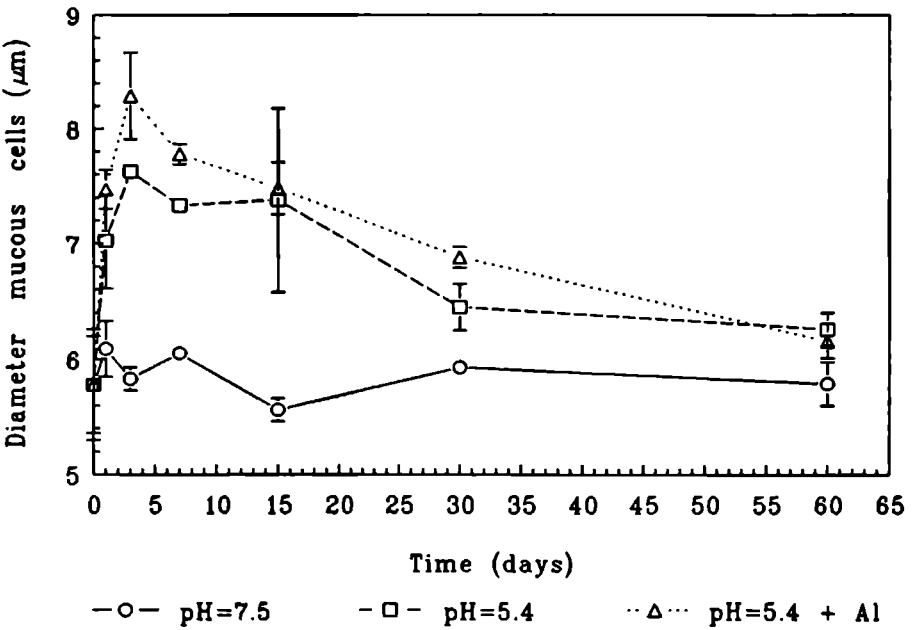

Fig. 5: Branchial mucous cell size of fish exposed to pH 7.5, pH 5.4 and pH 5.4 + 7.5 µmol Al/l (mean values ± SD).

Mucous cell densities.

Already at day 1 hypertrophy became apparent in the pH 5.4 group (Fig. 5). Peak values during the first week were followed by a gradual decline to normal values at day 60. In the pH 5.4 with Al group the cell diameters were slightly, but significantly ($p<0.05$) higher than in the pH 5.4 group; this difference disappeared at day 60.

Discussion

Hematocrit.

We observed a transient increase in the hematocrit values of carp exposed to pH 5.4 as well as pH 5.4 with Al. This is in accordance with Witters *et al.* (1987), who observed an increase in hematocrit in *Oncorhynchus mykiss* (rainbow trout) after 3 days at pH 5.0. No effects on hematocrit were reported by Reid *et al.* (1991), who exposed *Oncorhynchus mykiss* to pH 5.2 (8 days, 21 days), or by Wood *et al.* (1988) and Mount *et al.* (1988), who exposed *Salvelinus fontinalis* (brook trout) for 70 days at pH 5.2 and for 41 and 193 days at pH 5.9 respectively. The absence of a response as reported by the latter authors may be attributed to the fact that most measurements were done after a relatively long exposure time, when recovery might have occurred. These data indicate that the rise in hematocrit of *Oncorhynchus mykiss* is transient at moderate, sublethal pH levels. We observed recovery already after 7 days.

Playle *et al.* (1989) observed a rise in hematocrit value of *Oncorhynchus mykiss* after 66 h of exposure to 3.9 μmol Al/l at pH 4.4, 4.8 and 5.2 when compared to the low pH controls. Witters *et al.* (1990) studied the same species and observed that the presence of 6.7 μmol Al/l at pH 4.7 doubled the hematocrit even after 10 days of exposure. At pH 5.2, *Oncorhynchus mykiss* exposed to only 1 μmol Al/l showed elevated hematocrit values of 12.5 % after 8 days, but recovery was observed after 21 days (Reid *et al.*, 1991). Long-term exposure experiments to Al were also done by Wood *et al.* (1988) and Mount *et al.* (1988) with *Salvelinus fontinalis*. At pH 5.2, addition of 3.7 μmol Al/l resulted in an increase of hematocrit by 12 % after 4 exposure days; afterwards it stabilized at $\pm$ 6-7 % above the control value. No effects of 1.3 μmol Al/l at pH 5.0 were observed in the period of 41-193 days after the experiment started.

The rise of the hematocrit at low pH conditions with or without Al, has been attributed to red cell swelling and loss of plasma volume, as a result of a drop in blood osmolarity, and a fluid shift out of the plasma into the tissues and the red blood cells (Milligan and Wood, 1982; Witters *et al.*, 1987). However, this explanation can only be supported when an inverse relation between hematocrit and plasma osmolarity is found. In our

experiments houwever, such a relation was not observed. The highest hematocrit was observed in the pH 5.4 with Al-group, whereas the reduction in osmolarity was maximal in fish from pH 5.4 without Al. An explanation may be that not the drop in osmolarity but the drop in Na^+ and Cl^-, which under normal conditions makes up 90 % of plasma osmolarity, is the cause of the increase in haematocrit. Wood *et al.* (1988b) found that in *Salvelinus fontinalis* plasma Na^+ and Cl^- concentrations decreased while plasma osmolarity was unchanged. This was explained by the mobilisation of non-ionic osmolytes such as glucose and taurine (Giles *et al.*, 1984; Scherer *et al.*, 1986; Witters *et. al,* 1987) in the blood in the presence of Al. Another explanation of the increased hematocrit in our carp could be the release of erythrocytes by the spleen. This might have been induced by hypoxia resulting from gill damage and mucification of the gills. Indeed, this may account for the higher hematocrit in the pH with Al group, despite of the fact that these fish show higher plasma osmolarity than fish exposed to low pH alone.

Plasma osmolarity

Plasma osmolarity dropped during the first 4 days when carp were exposed to pH 5.4 and pH 5.4 with Al. After 4 days, the fish showed total (pH 5.4 with Al) or partial (pH 5.4 without Al) recovery. Witters *et al.* (1987) demonstrated that plasma osmolarity of *Oncorhynchus mykiss* decreased after 3 days of exposure to pH 5.0, an effect that disappeared after 11 days. According to Mount *et al.* (1988), plasma osmolarity was lower after 4 days of exposure to pH 5.0, but restoration was observed after 79 days. Keeping *Salmo trutta* for 7 days at pH 4.2 induced a 15 % drop of plasma osmolarity (Edwards *et al.*, 1987). A study on non-salmonid species was done by McCormick *et al.* (1987), who exposed *Perca flavescens*, *Ambloplites rupestris*, *Pomoxis nigromaculatus* and *Micropterus salmoides* at pH 4.5 and 4.0 for 30 days. All species could maintain blood osmolarity at pH 4.5, but at the lower pH level it dropped and all fish except *Perca flavescens* died within 30 days. The extremely acid-resistant *Umbra pygmaea* suffered osmoregulatory stress at a water acidity as low as pH 3.2 (Wendelaar Bonga *et al.*, 1990). Thus, our observation of a drop in plasma osmolarity in acid water is supported by the literature. Indications that the drop becomes less severe in time at relatively mild acidity levels, as in our study, are also found in literature.

We found that the drop in osmolarity in water of pH 5.4 with Al was less pronounced than in water of pH 5.4 without Al. This contrasts with literature, which is mainly restricted to salmonids. For example for *Oncorhynchus mykiss*, Witters *et al.*, 1987 reported that plasma osmolarity was lower in fish exposed to water of pH 5.0 with 7.4 μmol Al/l for 3 days than in fish from pH 5.0 without Al. This is in line with observations of Playle *et al.* (1989) on the same species. A reduction of plasma Na^+ at

low pH with Al below the already reduced level of low pH alone was further reported by Wood *et al.* (1988a,b). The finding that osmolarity returned to normal after 60 days at pH 5.4 with Al contrasts with the low osmolarity observed at pH 5.4 without Al. An explanation might be provided by observations of McDonald and Milligan (1988). These authors studied effects of Al and pH on Na^+ influx of *Salvelinus fontinalis*. After 70 days of exposure to pH 5.2, influx was decreased, but this could be reversed by addition of Al. They suggested that Al evokes a compensatory response. The previously mentioned release of glucose into the blood in the presence of Al might also be a factor explaining the observed mitigating effect of Al on plasma osmolarity. Wendelaar Bonga *et al.* (1987) suggested an adaptive hormonal regulation mechanism to low pH for *Oreochromis mossambicus*, with cortisol promoting Na^+-uptake and prolactin restoring branchial osmotic permeability to water and ions. Such a hormonal response can explain the observed partial and total recovery of blood osmolarity in carp.

Chloride cells

Chloride cell proliferation in opercula of carp was induced by low pH as well as by Al. Data on effects of low pH on chloride cells in literature are very consistent and in line with our results. Mueller *et al.* (1991) observed proliferation of chloride cells after exposing *Salvelinus fontinalis* for 4 days to pH 5.2. A stimulating effect of low pH on chloride cell numbers in gills of *Oncorhynchus mykiss* (10 days) was also reported by Youson and Neville (1987). Numbers were still increased at pH 4.8 after 91 days for *Salmo salar* (Jagoe & Haines, 1990). Even after 147 days at pH 4.9, chloride cell numbers remained elevated in *Salvelinus fontinalis* (Tietge *et al.*, 1988). Non-salmonid fish species showing chloride cell hyperplasia at pH 4.5 and 4.0 are *Perca flavescens*, *Ambloplites rupestris*, *Pomoxis nigromaculatus*, *Oreochromis mossambicus* and *Micropterus salmoides* (McCormick *et al.*, 1987; Wendelaar Bonga *et al.*, 1990). Proliferation of chloride cells was also observed after exposure for as long as 129 days of *Pimephalus promelas* to pH 5.5 and lower (Leino & McCormick, 1984).

Literature data on the effects of Al on chloride cell proliferation are less consistent. Chevalier *et al.* (1985) found in the gills of *Salvelinus fontinalis* from lakes with water of pH 5.2-5.5 and Al levels of 7.4-11.1 μmol/l, 80 % more chloride cells than in trout from non-acidified lakes. In the same species, exposure for 24 days to 2 μmol Al/l at pH 5.2 induced proliferation of chloride cells. However, Al levels higher than 10 μmol/l induced strong necrotic effects and a decrease in cell numbers in *Oncorhynchus mykiss* (Evans *et al.*, 1988). An increased proliferation of chloride cells can reflect a compensatory response to a disturbance of ion regulation. Skin wounding, fungus infection, deionized water and low water pH have all been reported to increase chloride cell numbers in the

gill epithelium (De Renzis & Maetz, 1973; Laurent & Dunel, 1980; Leino & McCormick, 1984). Chloride cell proliferation and degeneration often occur when fish are exposed to toxicants, where necrosis is the direct result of the toxicant and increased proliferation the response of the animal (Wendelaar Bonga and Lock, 1992). Wendelaar Bonga *et al.* (1990) exposed *Oreochromis mossambicus* to low pH and found that the increase in chloride cell numbers could mainly be ascribed to immature and apoptotic cells. The number of mature cells remained constant and the ion transport capacity of the gills increased only slightly. This means that an increase in chloride cell numbers as we observed at low pH and Al is not necessarily parallelled by a proportional increase in ion transport capacity.

The return of chloride cell numbers to control values, as we found in the present study, has not been published earlier for low pH and for Al. For Cd however, recovery of chloride cell numbers in *Oreochromis mossambicus* was observed after 14 and 35 days of exposure (Fu *et al.*, 1990; Pratap and Wendelaar Bonga, in press).

Mucous cells.

In our experiments the mucous cells increased transiently in number and in size as a result of exposure to both low pH and Al. Stimulation of mucus secretion in acid water has frequently been reported, in particular for salmonids (see review by Fromm, 1984). More recently, stimulation of mucus secretion by low pH has been reported for *Salmo trutta* (Linnenbach *et al.* (1987). An increase in mucus production and in mucous cell numbers was noted by Jagoe and Haines (1990) in *Salmo salar* kept for 91 days at pH 4.8; our carp already showed complete recovery after 30 days. The size of the mucous cells of salmon however, was reduced after these 91 days of exposure. No effect on mucus secretion or mucous cell numbers was found in *Salvelinus fontinalis* exposed to pH 5.2 (McDonald *et al.*, 1991; Mueller *et al.*, 1991), a species that is considered rather resistant to acid water (Howells *et al.*, 1990).

Induction of hypertrophy and hyperplasia of mucous cells by Al is also well known (see Mueller *et al.*, 1991). Mucification of external gill surfaces often occurs in the presence of toxic agents (Mallat, 1985). Irritation of the gill epithelium can result in a release of catecholamines, which stimulate mucus secretion, into the blood (Wood & McDonald, 1982). A thick mucous layer covering the gills might interfere with oxygen transfer (McDonald, 1983; Ultsch *et al.*, 1980). This phenomenon is associated more directly with Al than with low pH. As mucus has a high binding capacity for Al (Booth *et al.*, 1988), increased production and release of mucus could be a primary defence mechanism to remove this metal from the gills.

Of all parameters determined we observed an initial disturbance phase, followed by a phase of gradual recovery. This was observed for low water pH as well as for Al. As an exception, blood osmolarity of acid exposed fish remained at reduced levels. The survival of carp in moderately acid water confirms the results of earlier studies in our laboratory. In these experiments we showed an increase of the energy required to maintain homeostasis at low pH (Chapter 8).

References

Booth. C.E., McDonald, D.G., Simons, B.P. and C.M. Wood, 1988. Effects of aluminium and low pH on net ion fluxes and ion balance in the brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1562-1574.

Chevalier, G., Gauthier, L. and G Moreau, 1985. Histopathological and electron microscopic studies of gills of brook trout, *Salvelinus fontinalis*, from acidified lakes. Can. J. Zool. 63: 2062-2070.

Daye, P.G. and E.T. Garside, 1976. Histopathologic changes in surficial tissues of brook trout, *Salvelinus fontinalis* (Mitchill), exposed to acute and chronic levels of pH. Can. J. Zool. 54: 2140-2155.

Driscoll, C.T., 1984. A procedure for the fractionation of aqueous aluminium in dilute acidic waters. Int. J. Environ. Anal. Chem. 16: 267-283.

de Renzis, G., and J. Maetz. 1973. Studies on the mechanism of chloride absorption by the goldfish gill. Relation with acid-base regulation. J. Exp. Biol. 59: 339-358.

Edwards, D., Brown, J.A. and C. Whitehead, 1987. Endocrine and other physiological indicators of acid stress in the brown trout. Annls. Soc. Roy. Zool. Belg. 117: 331-342.

Evans, R.E., Brown, S.B. and T.J. Hara, 1988. The effects of aluminium and acid on the gill morphology in rainbow trout, *Salmo gairdneri*. Env. Biol. Fishes 22/4: 299-311.

Fromm, P.O., 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Environ. Biol. Fish. 5: 79-93.

Fu, H., Steinebach, O.M., Hamer, C.J.A. van den, Balm, P.H.M. and R.A.C. Lock, 1990. Involvement of cortisol and metallothionein-like proteins in the physiological responses of tilapia (*Oreochromis mossambicus*) to sublethal cadmium stress. Aquat. Toxicol. 16: 257-270.

Giles, M.A., Majewsku, H.S. and B. Hobden, 1984. Osmoregulatory and haematological responses of rainbow trout (*Salmo gairdneri*) to extended environmental acidification. Can. J. Fish. Aquat. Sci. 41: 1686-1694.

Howells, G., Dalziel, T.R.K., Reader, J.P. and J.F. Solbe, 1990. EIFAC water quality criteria for european freshwater fish: report on aluminium. Chem. and Ecol. 4: 117-173.

Jagoe, C.H. and T.A. Haines, 1990. Morphometric effects of low pH and limed water on the gills of Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 47· 2451-2460.

Jensen, K.W. and E. Snekvik, 1972. Low pH levels wipe out salmon and trout populations in southern Norway. Ambio 1, 223-225.

Karlsson-Norrgren, L., Dickson, W., Ljungberg, O. and P. Runn, 1986. Acid water and aluminium exposure: gill lesions and aluminium accumulation in farmed brown trout, *Salmo trutta* L. J. Fish Diseas. 9: 1-9.

Kirschner, L.B., 1978. External charged layer and Na^+ regulation. In Alfred Benzon Symposium XI. Osmotic and volume regulation. Academic Press, New York. pp. 310-321.

Laurent, P. and S. Dunel, 1980. Morphology of gill epithelia in fish. Am. J. Physiol. 238: R147-R159.

Leivestad, H. and I P Muniz, 1976. Fish kill at low pH in a Norwegian river Nature (London) 259: 391-392.

Leino, R.L. and J.H. McCormick, 1984. Morphological and morphometrical changes in chloride cells of the gills of *Pimephales promelas* after chronic exposure to acid water. Cell Tiss. Res. 236: 121-128.

Linnenbach, M., Marthaler, R. and H. Gebhardt, 1987. Effects of acid water on gills and epidermis in brown trout (*Salmo trutta* L.) and in tadpoles of the common frog (*Rana temporaria* L.). Annls. Soc. Roy. Zool. Belg. 117: 365-374.

Mallat, J., 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can J. Fish. Aquat. Sci. 42. 630-648.

Malte, H., 1986. Effects of aluminium in hard, acid water on metabolic rate, blood gas tensions and ionic status in the rainbow trout. J. Fish Biol. 29: 187-198.

McCahon, C.P., Pascoe, D. and C. McKavanagh, 1987. Histochemical observations on the salmonids *Salmo salar* L. and *Salmo trutta* L and the ephemeropterans *Baetis rhodani* (pict) and *Ecdyonurus venosus* (Fabr.) following a simulated episode of acidity in an upland stream. Hydrobiologia 153: 3-12.

McCormick, J H., Jensen, K.M., Leino, R.L. and G.N. Stokes, 1987. Fish blood osmolarity, gill histology and oocyte atresia as early warning acid stress indicators Annls Soc. Roy Zool. Belg. 117: 309-391.

McDonald, D.G., and C.L. Milligan, 1988 Sodium transport in the brook trout, *Salvelinus fontinalis*: effects of prolonged low pH exposure in the presence and absence of aluminium. Can. J. Fish. Aquat. Sci. 45: 1606-1613.

Milligan, C.L., and C.M. Wood, 1982. Disturbances in hematology, fluid volume distribution and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. J. Exp. Biol. 99: 397-415.

Mount. D.R., Hockett, J.R. and W.A. Gern., 1988. Effect of long-term exposure to acid, aluminium, and low calcium on adult brook trout *Salvelinus fontinalis*. 2 Vitellogenesis and osmoregulation. Can. J. Fish. Aquat. Sci 45: 1633-1642

Mueller, M.E., Sanchez, D.A., Bergman, H L , McDonald, D.G., Rhem, R.G and C M. Wood, 1991. Nature and time course of acclimation to aluminium in juvenile brook trout (*Salvelinus fontinalis*). II Gill histology. Can. J. Fish. Aquat. Sci. 48: 2016-2027.

Muniz, I.P., and H. Leivestad, 1980. Acidification effects on freshwater fish. Proc. Int. Conf. Ecol. Impact Acid Precip. SNSF project 84-92, Norway.

Playle, R.C., Goss, G.G. and C.M. Wood, 1989. Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminium exposures in soft water of two calcium concentrations. Can. J. Zool. 67: 314-324.

Pratap, H.B. and S.E. Wendelaar Bonga, 1993. Effect of ambient and dietary cadmium on pavement cells, chloride cells, and ($Na^+ + K^+$) ATPase activity in the gills of the freshwater teleost (*Oreochromis mossambicus* at normal and high calcium levels in the ambient water. in press.

Reid, S.D., McDonald, D.G. and R.R. Hem, 1991. Acclimation to sublethal aluminium: Modifications of metal-gill surface interactions of juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 48: 1996-2005.

Scherer, E., Harrison, S.E. and S.B. Brown, 1986. Locomotor activity and blood plasma parameters in acid-exposed lake whitefish, *Coregonus clupeaformis*. Can. J. Fish. Aquat. Sci. 43: 1556-1561.

Skogheim, O.K., Rosseland, B.O. and I. Sevaldrud, 1984. Deaths of spawners of Atlantic salmon in river Ogna, S. W. Norway caused by acidified aluminium rich water. Inst. Freshw. Res. Drottningholm 61: 194-202.

Staurnes, M., Sigholt, T. and O.B. Reite, 1984. Reduced carbonic anhydrase and Na-K-ATPase activity in gills of salmonids exposed to aluminium-containing acid water. Experienta 40: 226-227

Tietge, J.E., Johnson, R.D. and H.L. Bergman, 1988. Morphometric changes in gill secondary lamellae of brook trout (*Salvelinus fontinalis*) after long-term exposure to acid and aluminium. Can. J. Fish. Aquat. Sci. 45: 1643-1648.

Ultsch, G.R., Ott, M.E. and N. Heisler, 1981. Acid-base and electrolyte status in carp (*Cyprinus carpio*) exposed to low environmental pH. J. Exp. Biol. 93: 65-80.

Ultsch, G.R., Ott, M.E. and N. Heisler, 1980. Standard metabolic rate, critical oxygen tension and aerobic scope for spontaneous activity of trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) in acidified water. Comp. Biochem. Physiol. A 67: 329-335.

Walker, R.L., Wood, C.M. and H.L Bergman, 1991. Effects of long-term preexposure to sublethal concentrations of acid and aluminium on the ventilatory response to aluminium challenge in brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 48: 1989-1995.

Wendelaar Bonga, S.E., 1978. The effects of changes in external sodium, calcium, and magnesium concentrations on prolactin cells, skin and plasma electrolytes of *Gasterosteus aculeatus*. Gen. Comp. Endocrinol. 34: 265-275.

Wendelaar Bonga, S.E., Flik, G. and P.H.M. Balm, 1987. Physiological adaptation to acid stress in fish. Ann. Soc. Roy. Zool. Belg. 117: 243-254.

Wendelaar Bonga, S.E., Flik, G., van der Velde, J.A. and Z. Kolar, 1990. Prolactin cell activity and sodium balance in the acid-tolerant mudminnow *Umbra pygmaea* in acid and neutral water. Gen. Comp. Endocrin. 78: 421-432.

Wendelaar Bonga, S.E., Flik, G., Balm, P.H.M. and J.C.A. van der Meij, 1990. The ultrastructure of chloride cells in the gills of the teleost *Oreochromus mossambicus* during exposure to acidified water. Cell Tissue Res. 259: 575-585.

Wendelaar Bonga, S.E. and R.A.C. Lock, 1992. Toxicants and osmoregulation in fish. Neth. J. Zool. 42: 478-493.

Witters, H.E., Vangenegten, J.H.D., Van Puymbroeck, S. and O.L.J. Vanderborght, 1987. Ionoregulatory and haematological responses of rainbow trout, *Salmo gairdneri*, to chronic acid and aluminium stress. Ann. Soc. Roy. Zool. Belg. 117/1: 411-420.

Witters, H.E., Van Puymbroeck, S., Vangenechten, J.H.D. and O.L.J. Vanderborght, 1990. The effect of humic substances on the toxicity of aluminium to adult rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Biol. 37: 43-53.

Wollast, R.; Chou, L.; Deddaut, V., Wollast, A. and J. Zobrist, 1984. The influence of acid rains on the geochemistry of aluminium. Proc. "Acid deposition and sulphur cycle" Brussels, June 1984, 277-286.

Wood, C.M. and D.G. McDonald, 1982. Physiologigal mechanisms of acid toxicity to fish. in: R.E. Johnson (ed) Proc. Int. Symp. Acidic Precip. Fish., Amer. Fish. Soc. Northeastern Division, Batesda, Maryland: 197-225.

Wood, C.M., McDonald, D.G., Booth, C.E., Simons, B.P., Ingersoll, C.G. and H.L. Bergman, 1988a. Physiological evidence of acclimation to acid-aluminium stress in adult brook trout (*Salvelinus fontinalis*). 1. Blood composition and net sodium fluxes. Can. J. Fish. Aquat. Sci. 45: 1587-1596.

Wood, C.M., Simons, B.P., Mount, D.R. and H.L. Bergman, 1988b. Physiological evidence of acclimation to acid-aluminium stress in adult brook trout (*Salvelinus fontinalis*). 2. Blood parameters by cannulation. Can. J. Fish. Aquat. Sci. 45: 1597-1605.

Youson, J.H. and C.M. Neville, 1987. Deposition of aluminium in the gill epithelium of rainbow trout (*Salmo gairdneri* Richardson) subjected to sublethal concentrations of the metal. Can. J. Zool. 65: 647-656.

CHAPTER 10

Summary and general discussion

Summary and general discussion

Summary

Al and water acidity affect mortality, rate of embryonic development, hatching time and deformation rate of carp eggs. In summary, our observations lead to the following conclusions:

- In the absence of Al, reproduction is impaired at a water acidity of pH 5.2 and less (Chapter 2).

- Between pH 7.5 and pH 5.9, inorganic Al levels up to 7.4 μmol/l have no negative effects on early life stages (Chapter 4).

- In the range of pH 5.9 - pH 5.1, concentrations of only 1.3 μmol Al/l increase larval deformation and delay hatching. At higher concentrations, Al increases mortality (Chapter 4).

- In the range of pH 5.1 to pH 4.8, Al protects the eggs against the effects of low pH at concentrations up to 13.5 μmol/l. These Al levels become toxic after hatching. However, higher Al concentrations are toxic to eggs as well as larvae (Chapter 4).

- The pH dependent pattern of Al toxicity (Chapter 4) coincides with the pH dependent pattern of Al accumulation by the embryo (Chapter 6).

- Embryonic deformation, caused by low water pH, Al, and reduction of the perivitelline space, leads to delayed hatching via reduced trunk movements and, possibly impaired distribution of chorionase. Coagulation of proteins in the perivitelline fluid by low water pH and by Al results in delayed hatching via the same mechanism. Developmental delay, inhibition of chorionase activity and increased chorionic strength may further contribute to hatching delay, leading to an increased degree of deformation.

- Humic acids do not off-set the impact of low water pH on eggs and larvae of carp (Chapter 2), but reduce Al toxicity very effectively (Chapter 4).

Between hatching and yolk sac absorption, larval carp accumulate Ca^{2+} and Na^{+} from the ambient water at high rates (Chapter 3). They probably do so via chloride cells in the skin. This accumulation of ions can rapidly be disturbed by low water pH and by Al:

- At low water pH without Al, net uptake of Ca^{2+} and Na^{+} by the larvae is impaired at pH values lower than 5.2 and 5.0 respectively, while the Mg concentration increases at pH lower than 5.0 (Chapter 3).

- In the presence of Al, reductions of the net uptake of Ca^{2+} and Na^{+} by larval carp are severe at pH 5.4 and lower, while at pH 5.9 and higher, Al has no effect. The changes of the total body Mg concentration shows a similar pattern (Chapter 6).

- The reducing effect of Al on the body concentrations of Ca, Na and Mg is positively correlated with the Al concentration in the water and in the embryo (Chapter 6).

- The chorion accumulates Al to a much higher extent than the embryo, and plays a protective role (Chapter 6).

- The presence of humic acids does not influence the effects of low water pH on ion uptake, but prevents effects of Al on these processes by interfering with Al accumulation by the embryo and the chorion (Chapter 6).

- The negative effects of low water pH and of Al on body ion concentrations can mainly be attributed to a delay in development (Chapters 2 and 7).

Juvenile and adult carp are more resistent to low pH and to Al than early life stages. Where eggs already are affected at pH 4.8-5.2 and at 1.3 μmol Al/l, older life stages tolerate pH 4.0 and 15 μmol Al/l (Chapters 2, 4, 8 and 9). Responses of juvenile carp to low pH and Al are generally the same as those of other fish species.

- Growth rates of carp are reduced at low water pH. For growth to be unaffected, water pH should be above pH 5.7. At lower water pH, the conversion of food is less efficiently because energy, normally utilised for growth, is necessary for homeostatic control (Chapter 8).

- Within certain limits, juvenile carp are able to adapt to low water pH and to elevated Al levels, provided they survive an initial disturbance phase. This disturbance is reflected by an increased number of opercular chloride cells and number and size of mucous cells in the gills, as well as by an increase in hematocrit value. Low water pH induces a drop in blood osmolality, an effect that is mitigated by Al. Most effects are however transient (Chapter 9).

Comparing responses of carp with those of salmonids to low pH and Al.

Larval development is delayed by low pH and Al in carp as well as in salmonids (Chapters 2 and 7). The pH dependent Al-toxicity as reflected by mortality is also similar for both types of fish (Chapter 4), although a pH shift might occur as result of temperature differences. Exposure to low pH induces hatching delay in both, but the presence of Al induces a much longer hatching time delay in carp than has been reported for salmonids. Similarly, the very high Al-induced deformation rates as encountered in our studies has never been reported for salmonid species (Chapter 4). Another difference between carp and salmonids is the occurrence of coagulations in the perivitelline fluid of carp eggs after exposure to low pH or Al, which has never been reported before (Chapter 5). Reductions in total body concentrations of Ca and Na in larvae in the presence of low pH or Al as found for carp have also been reported for salmonids, as have increases in Mg concentrations by low pH and reductions in Mg concentrations by Al (Chapters 3 and 6).

Older life stages of carp and salmonids are able to compensate the effects of moderate levels of water acidity or Al. In carp as well as in salmonids, chloride cells and mucous cells proliferate, and hematocrit increases after exposure to sublethal combinations of pH and Al. These changes are transient (Chapter 9). In addition, both types of fish show a reduced growth rate and an increase in food conversion when exposed to low pH (Chapter 8). A difference between carp and salmonids manifested itself in blood osmolarity values. Low pH suppresses osmolarity in both, but although in salmonid fishes values return to normal after prolonged exposure, osmolarity in carp remains low. Also in contrast to salmonids, exposure to Al mitigates the negative effect of low pH on blood osmolarity (Chapter 9).

In general, responses of carp to both environmental stressors show a similar pattern as in salmonids. Although juvenile and adult salmonids are more vulnerable to low pH and elevated environmental Al concentrations than carp, the opposite is the case with the early life stages of these fish.

Ecological relevance of the effects of low pH and Al, and recommendations for water quality criteria.

Extrapolation of our results to field situations should be done with caution. In Belgium and The Netherlands, total Al concentrations of 30 respectively 27 μmol/l have been reported (Howells *et al.*, 1990). The average Al concentrations tested in this study (3.7-7.4 μmol/l) are well within ecologically relevant limits. Our experiments were performed in water containing 0.8 mmol Ca^{2+}/l. Because Ca^{2+} ions ameliorate the effects of low pH and Al, our data can only be extrapolated to waters with similar Ca^{2+} levels. When extrapolating our findings to field conditions it should be kept in mind that in natural waters Al may not only form inorganic complexes with hydroxides, but also with fluoride, sulphate and silicate. We have tested the Al hydroxides, but the toxicity of the other complexes has not been established. Moreover, the toxicity of Al will depend on the pH and the temperature of the water, because the speciation of Al hydroxides is pH- and temperature dependent (Howells *et al.*, 1990). This implies that seasonal temperature changes effect Al toxicity in natural waters. Our experiments were done at 23°C, when $Al(OH)_2^+$, considered to be the most toxic Al species according to the Al-speciation theory (Chapter 4), prevails in the pH range 5.0-6.0 (Smith and Hem, 1972). At lower temperatures the formation of this toxic complex shifts to higher pH levels.

We observed that humic acids at a concentration of 10 mg/l decreased Al toxicity very effectively. When humic acid levels exceed this concentration, most Al will be in a non-toxic complexed form (Chapter 6). Although above average, this humic acid level is still within the range occurring in natural waters. Not only humic acids, but also other organic substances such as fulvic acids bind Al (Schnitzer, 1969) and thus will have a neutralizing effect on Al toxicity. Therefore, the toxicity of Al in natural waters is dependent on the concentration of organic matter. Thus, fish in water containing high levels of organic materials are less affected by a certain total Al concentration than those in water with low levels of organic matter.

Delay of hatching prolongs the period that eggs are vulnerable to predation or infections. Spinal cord deformation undoubtly affects survival. Deformed larvae are unlikely to escape predators, and will have difficulties in catching their food. Deformation occurs at much lower Al concentrations than mortality (Chapter 4) and thus, since deformation also leads to death in field situations, deformation rather than mortality should be taken as parameter for determining the critical Al levels in field situations. Early life stages of carp are more sensitive to low pH and Al than adults. Eggs die at pH 5.2-4.8, while juveniles can cope with pH 4.0 (Chapter 2). Similarly, 1.3 μmol Al/l at pH 5.4 increases the rate of larval deformation, while 7.4 μmol Al/l lies within the tolerance limits of juvenile carp (Chapters 7 and 9). Thus the egg stage of carp

determines the tolerance limits for water pH and Al for the population. On the other hand, carp populations can survive episodes of pH levels and Al concentrations lethal to eggs and larvae, provided they do occur outside the reproductive period.

Table 1: Effects of Al concentrations (μmol/l) on early life stages of different fish species in the pH range 5.0-6.0.

fish species	[Al]	effect	reference
salmonid species			
eggs of			
Oncorhynchus clarki stomias	3.7	none	Woodward *et al.*, 1991
Salmo salar	4.8	none	Skogheim and Rosseland, 1986
Salmo trutta fario	4.8	none	Skogheim and Rosseland, 1984
Salmo trutta	11.1	none	Hunn *et al.*, 1987
	7.4	negative	Baker and Schofield, 1982
Salvelinus fontinalis	12.9	protective	Ingersoll, 1989
	11.1	none	Hunn *et al.*, 1987
	18.5	negative	Baker and Schofield, 1982
larvae of			
Oncorhynchus clarki stomias	3.7	negative	Woodward *et al.*, 1991
Salmo salar	4.1	negative	Dalziel and Brown, 1984
Salmo trutta	6.0	negative	Reader *et al.*, 1988
Salvelinus fontinalis	7.4	negative	Baker and Schofield, 1982
non-salmonid species			
eggs of			
Cyprinus carpio	1.3	negative	this thesis
Catostomus commersoni	7.4	negative	Baker and Schofield, 1982
larvae of			
Catostomus commersoni	3.7	negative	Baker and Schofield, 1982
Micropterus dolomieu	7.4	negative	Kane and Rabeni, 1987
Alosa aestivalis	15.4	negative	Klauda, 1987
elvers of			
Anguilla anguilla	6.3	none	Fjellheim *et al.*, 1985
	8.5	negative	Fjellheim *et al.*, 1985

Considering low pH alone, it appears that the eggs and larvae of carp are more sensitive (pH 4.8-5.2) than those of salmonids and most of the non-salmonid species studied (Chapter 2). Adult carp could survive till pH 4.0 although at pH 5.7, growth is already affected (Chapter 8).

During early life stages, carp is more sensitive to Al than salmonid species and other non-salmonid species (Table 1). Howells *et al.* (1990) rank different salmonid species according to the increasing resistance of juveniles and adults against Al as follows: *Oncorhynchus mykiss* < *Salmo salar* < *Salmo trutta* < *Salmo trutta fario* < *Salvelinus fontinalis*. Reid *et al.* (1991) reported 4.4 μmol Al/l at pH 5.2 to be acutely lethal for adult *Oncorhynchus mykiss*, while adult *Salvelinus fontinalis* acclimates to 5.6 μmol Al/l and dies at 12.3 μmol Al/l (Wood *et al.*, 1988). In Chapter 9 we demonstrated that juvenile carp can tolerate 7.4 μmol Al/l. Field observations showed survival of adult carp at 15 μmol Al/l at pH 5.2, while species like *Abramis brama, Rutilus rutilus* and *Scardinius erythropthalmus* died. Thus, older life stages of carp seem to be more resistant to Al than the other fish species studied, including salmonids.

Since the Al concentration and the Al toxicity are pH dependent, it is obvious that this environmental problem will occur in aquatic ecosystems sensitive to acidification, or with a natural low water pH. The sensitivity of aquatic ecosystems to acidification depends on the buffering capacity of their soils, and on their dependency on rainfall. When acidification actually occurs, the Al content of the soil is an important factor determining Al water concentrations. Most of these sensitive ecosystems have poor nutrient levels leading to low concentrations of organic matter. Thus, low-alkaline waters, being vulnerable to acidification, often lack high concentrations of organic matter which protect against Al toxicity. Sometimes, naturally acid bogs which are often dominated by *Sphagnum* species become disturbed by an input of alkaline substances. As a result, large amounts of humic acids are released in the water. Although the thick organic soil, often occurring in this type of ecosystem, is unlikely to release large amounts of Al, any dissolved Al will be completely complexed by the humic acids. In parts of The Netherlands and of Belgium, this type of ecosystems are inhabited by the very acid-tolerant fish *Umbra pygmaea*, which is thus protected against Al. On the basis of our observations for carp, and provided that humic acids are absent, the following recommendations for water quality with respect to pH and Al can be given for carp:

- In the absence of toxic metals such as Al, the critical water pH is pH 5.2. Below this pH, reproduction is impaired and thus, carp populations are unlikely to survive. Unaffected growth takes place at water pH above pH 5.7.

- Between pH 7.5 and 5.9, natural Al concentrations are not expected to have negative effects.

- Between pH 5.9 and 5.1, concentrations as low as 1.3 μmol Al/l already have severe negative effects. Safe limits will be below this concentration.

- Between pH 5.1 and 4.8, Al protects against water acidity at concentrations lower than 13.5 μmol/l; higher concentrations are toxic. In this pH range protecting is transient, and has therefore only ecological significance when short acid-pulses occur. The effects of water acidity alone on these early life stages are already severe to an extent that the presence of Al makes no difference for the survival of carp populations.

REFERENCES

Baker, J.P. and C.L. Schofield, 1982. Aluminium toxicity to fish in acidic waters. Water Air Soil Pollut. 18: 289-309.

Fjellheim, A., Raddum, G.G. and T. Sagen, 1985. Effect of aluminium at low pH on the mortality of elvers (*Anguilla anguilla*), a laboratory experiment. Verh. Internat. Verein. Limnol. 22: 2544-2547.

Howells, G., Dalziel, T.R.K., Reader, J.P. and J.F. Solbe, 1990. EIFAC water quality criteria for European freshwater fish: report on aluminium. Chem. Ecol. 4: 117-173.

Hunn, J.B., Cleveland, L. and E.E. Litle, 1987. Influence of pH and aluminium on developing brook trout in a low calcium water. Environ. Pollut. 43:63-73.

Kane, D.A. and C.F. Rabeni, 1987. Effects of aluminium and pH on the early life stages of smallmouth bass (*Micropterus dolomieu*). Water Res. 21, 633-640.

Klauda, R.J., Palmer, R.E. and M.J. Lenkevich, 1987. Sensitivity of early life stages of blueback herring to moderate acidity and aluminium in soft freshwater. Estuaries 10, 44-53.

Reid, S.D., McDonald, D.G. and R.R. Rhem, 1991. Acclimation to sublethal aluminium· Modifications of metal-gill surface interactions of juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 48: 1996-2005.

Reader, J.P., J.P., Dalziel, T.R.K. and R. Morris, 1988. Growth, mineral uptake and skeletal calcium deposition in brown trout, *Salmo trutta* L., yolk-sac fry exposed to aluminium and manganese in soft acidic water. J. Fish Biol. 32: 607-624.

Smith, R.W. and J.D. Hem, 1972. Effect of aging on aluminium hydroxide complexes in dilute aqueous solutions. Geological Survey Water-supply Paper 1827-D.

Schnitzer, M., 1969. Reactions between fulvic acid, a soil humic component and inorganic constituents. Soil Sci. Soc. Amer. Proc. 33: 75-81.

Skogheim, O.K. and B.O. Rosseland, 1984. A comparative study on salmonid fish species in acid aluminium-rich water I. Mortality of eggs and alevins. Rep. Inst. Freshwat. Res. 61: 177-185.

Wendelaar Bonga, S.E., Flik, G., van der Velde, J.A. and Z. Kolar, 1990. Prolactin cell activity and sodium balance in the acid-tolerant mudminnow *Umbra pygmaea* in acid and neutral water. Gen. Comp. Endocrinol 78: 421-432.

Wood, C.M., McDonald, D.G., Ingersoll, C.G., Mount, D.R., Johansson, O.E., Landsberger, S. and H.L. Bergman, 1990. Effects of water acivity, calcium, and aluminium on whole body ions of brook trout (*Salvelinus fontinalis*) continuously exposed from fertilization to swim-up: A study by instrumental neutron activity analysis. Can. J. Fish. Aquat. Sci. 47: 1593-1603.

Woodward, D.F., Farag, A.M., Little, E.E., Steadman, B. and R. Yancik, 1991. Sensitivity of Greenback Cutthroat Trout to acidic and elevated aluminium. Trans. Am. Fish. Soc. 120: 34-42.

SAMENVATTING

Summary in Dutch

Samenvatting

De groeiende menselijke activiteit en het consumptieve levenspatroon heeft geleid tot een enorme energiebehoefte waarin voor het merendeel voorzien wordt door het berbruik van fossiele brandstoffen. De hierbij gevormde verzurende stoffen, in combinatie met stoffen die worden geproduceerd door de intensieve veehouderij, leiden onder meer tot verzuring van aquatische ecosystemen.

Waterverzuring wordt vaak vergezeld door een verhoogde aluminium concentratie. Aluminium is het meest voorkomende metaal in de aardkorst, en wordt in gebonden vorm in bijna alle mineralen aangetroffen. Mobilisatie, en dus biologische beschikbaarheid, van dit gebonden aluminium is het gevolg van uitwisseling met H^+ ionen afkomstig van zure depositie. De oplosbaarheid van aluminium neemt toe naarmate de water-pH daalt. Eenmaal in oplossing vormt aluminium verbindingen met OH^-, F^-, SO_4^- en organische liganden zoals humuszuren. Deze verbindingen verschillen onderling in toxiciteit. Tussen pH 5 en 6 domineren de aluminium hydroxides. Hiervan zijn drie chemische vormen bekend, namelijk, $Al(OH)^{2+}$, $Al(OH)_2^+$ en $Al(OH)_4^-$, waarvan de relatieve dominantie pH afhankelijk is.

Zowel een lage water pH als een verhoogde aluminium concentratie worden gezien als oorzaak van het verdwijnen van vispopulaties. Dit kan plotseling gebeuren als gevolg van massale vissterfte, maar ook geleidelijk doordat succesvolle voortplanting wordt verhinderd. Als directe oorzaak van de vissterfte wordt vaak verstoring van de osmoregulatie en, in ernstige gevallen, van de zuurstof opname genoemd.

Bodems in Noord-Amerika en Scandinavië worden gekenmerkt door moeilijk verwerende gesteenten met weinig buffercapaciteit. De verzuringsproblematiek manifesteerde zich in deze regio's dan ook het eerste. Als gevolg hiervan is het meeste onderzoek met betrekking tot dit onderwerp gedaan aan het in deze gebieden voorkomende type oecosysteem, namelijk nutrient-arme wateren welke gedomineerd worden door zalmachtigen.

Nu waterverzuring in toenemende mate in regio's met een meer gebufferde bodem zoals Midden- en West-Europa ook gesignaleerd wordt, is de noodzaak ontstaan om meer inzicht te krijgen in de manier waarop de vissoorten die hier voorkomen op deze verstoring van het milieu reageren. De cypriniden zijn de dominerende visfamilie van de meer gebufferde wateren in Midden en West Europa. Van deze cypriniden is de gewone karper, *Cyprinus carpio*, uitgekozen om dit onderzoek aan te verrichten. Hierbij is de aandacht vooral uitgegaan naar de vroege ontwikkelingsstadia, omdat deze relatief het meest gevoelig zijn voor toxische stoffen en zo de bestaansvoorwaarden van de populatie in hoge mate bepalen. Tevens werd de verandering van de aluminium-toxiciteit onder invloed van de pH van het water en van humus zuren die in het water kunnen voorkomen

in het onderzoek betrokken.

Allereerst werden alleen de effecten van lage pH op de eieren en larvale stadia bestudeerd (hoofdstuk 2 en 3). Blootstelling aan een pH < 5,35 resulteerde in een verhoogd sterfte- en misvormingspercentage, een vertraagde ontwikkelingssnelheid en een vertraging van het uitkomen van de eieren. Tussen pH 4,8 en 5,2 werd de intensiteit van deze effecten zodanig dat succesvolle voortplanting vrijwel uitgesloten was. In de periode na het uitkomen maken de larven normaal een sterke mineralisatie fase door die gekenmerkt wordt door ondermeer een hoge opname van calcium en natrium uit het water. De inwendige magnesium concentratie veranderde echter in veel mindere mate gedurende deze periode. Blootstelling van larven aan een lage pH resulteerde in een sterke reductie van de opname van calcium en natrium, terwijl de inwendige magnesium concentratie toenam. Het is bekend dat calcium de negatieve effecten van water verzuring vermindert. Dit metaal wordt tevens in zekere mate door humuszuren gebonden. Het bleek echter dat de aanwezigheid van humuszuren de gevoeligheid van eieren en larven voor verzuring niet verhoogde.

Vervolgens werden de effecten van aluminiumhydroxyde op deze vroege levensstadia nagegaan. Daarbij werden tevens de invloed van de pH en de aanwezigheid van humuszuren op de aluminiumtoxiciteit onderzocht. Net als bij verzuring leidde de aanwezigheid van aluminium tot een verhoogd voorkomen van sterfte en misvorming, een vertraagde ontwikkelingssnelheid en een vertraagd uitkomen van de eieren (hoofdstuk 4 en 7). De aanwezigheid van humuszuren, welke aluminium binden, voorkwam de toxiciteit van aluminium volledig (hoofdstuk 4 en 6). De bij pH waarden > 5,9 gevormde aluminiumhydroxiden bleken niet toxisch bij de geteste concentraties. Tussen pH 5,1 en 5,9 was aluminium wel toxisch, terwijl, afhankelijk van de concentratie, bij lagere pH waarden de toxiciteit kon variëren. Hoge aluminium concentraties bleken giftig, terwijl lage aluminium concentraties een beschermende werking hadden tegen een lage pH. Deze beschermende werking viel echter weg na het uitkomen van de eieren. Dit patroon van pH-afhankelijke aluminiumtoxiciteit weerspiegelde zich ook in de ionconcentraties van calcium, natrium en magnesium in de larve. Boven pH 5,9 had de aanwezigheid van aluminium geen effect, maar tussen pH 5,0 en 5,9 leidde deze tot een verlaging van de concentraties van deze ionen. Chloridecellen zijn betrokken bij de opname van calcium en natrium ionen, een functie die wordt aangetast door lage pH en aluminium. Verwacht werd dat een reductie van de opname van deze ionen in verband te brengen zou zijn met veranderingen in de chlorideceldichtheid in de huid van de larven. De dichtheid van deze chloridecellen nam toe met de ontwikkeling van de larve, en kon gerelateerd worden aan de lengte van de dieren. De aanwezigheid van aluminium resulteerde in een lagere chloridecel dichtheid (hoofdstuk 7). Naar alle waarschijnlijkheid was dit echter geen direct gevolg van aluminium, maar van een door aluminium geïnduceerde vertraging van

de ontwikkeling. Deze vertraging kwam ook tot uiting in de ontwikkeling van de pronephros en het zijlijnsysteem. Het is aannemelijk dat de verlaging van de calcium- en natriumconcentraties in de larve voornamelijk samenhangen met deze vertraagde ontwikkeling.

Er zijn twee benaderingen in omloop om de pH-afhankelijke aluminiumtoxiciteit te verklaren. Ten eerste is er het aluminium-speciatiemodel dat ervan uitgaat dat de diverse aluminiumhydroxide-vormen elk hun eigen toxiciteit hebben. Deze toxiciteit zal zich, overeenkomstig de relatieve aanwezigheid van deze aluminiumvormen zoals die door de pH gedicteerd wordt, manifesteren. De andere benadering is die van de vrije-ion theorie, welke stelt dat de mate waarin het vrije aluminiumion (Al^{3+}) zich aan het weefsel bindt evenredig is met de toxiciteit. Deze Al^{3+} binding kan gemedieerd worden door competitie met H^+. De aluminium-toxiciteit die in onze experimenten tot uiting kwam via de sterfte kon gecorreleerd worden met de aanwezigheid van de $Al(OH)_2^+$ vorm. Dit pleit voor de eerste theorie, maar zoals blijkt uit hoofdstuk 4, kan ook de vrije-ion-theorie deze waarnemingen verklaren. De aluminiumabsorptie door het embryo binnen de eischaal bleek maximaal te zijn bij die pH's waar aluminium het meest toxisch bleek. Bij pH > 5,9 waar aluminium niet toxisch was, was ook geen aluminium aan het embryo gebonden. De binding van aluminium aan de eischaal gaf eenzelfde pH-afhankelijkheid te zien. De hoeveelheid geabsorbeerd aluminium aan de eischaal was 25-100 maal zo hoog als die van het embryo, wat op een beschermende rol van de eischaal duidt (hoofdstuk 6). Ook deze aluminiumbindingsstudies konden niet doorslaggevend één van de beide toxiciteitstheorieën ondersteunen. Wel werd duidelijk dat de beschermende werking van humuszuren is gelegen in het wegvangen van aluminium, omdat in de aanwezigheid van deze organische stoffen de aluminium-binding aan het embryo en de eischaal sterk tot geheel gereduceerd werd.

De manier waarop een lage pH en aluminium tot misvorming en vertraging in het uitkomen van de eieren kunnen leiden werd bestudeerd in hoofdstuk 5. Het bleek dat de larven al misvormd waren voordat van een vertraging van het uitkomen van de eieren sprake kon zijn. In de perivitelline ruimte van de eieren werden eiwit-coagulaten aangetroffen waarvan verwacht kan worden dat ze door middel van mechanische compressie tot misvorming van het embryo kunnen leiden. Deze misvorming bemoeilijkt het mechanisch openscheuren van de eischaal door het embryo. Dit laatste wordt nog eens versterkt door het feit dat aluminium een remmende invloed bleek te hebben op de frequenties van de staartbewegingen van het embryo in het ei.

Naast vroege stadia werden ook juveniele dieren aan de invloeden van lage pH en aluminium onderworpen. Blootstelling aan een pH van 5,7 en lager leiden tot vertraagde groei en een minder efficiënte omzetting van voedsel in lichaamseigen stoffen. Ook nam de energiebehoefte voor onderhoudsactiviteiten toe (hoofdstuk 8). Deze extra energie is

waarschijnlijk nodig voor processen die de effecten van een lage pH compenseren. Zowel pH 5,4 als pH 5,4 + 7,4 μAl/l stimuleerden de aanmaak van chloridecellen, hetgeen wijst op osmoregulatorische stress. Andere indicaties hiervoor die werden waargenomen waren een verhoogde haematocriet en een verlaagde osmotische waarde van het bloedplasma. Daarnaast nam het aantal en de grootte van de slijmcellen toe (hoofdstuk 9). In het algemeen waren de waarden na 60 dagen blootstelling weer terug op hun normale niveau, hetgeen wijst op tolerantie van en adaptatie aan de geteste pH- en aluminium-niveaus.

Samengevat leidde het onderzoek tot de volgende conclusies:

- In vergelijking met zalmachtigen zijn juveniele en volwassen karpers toleranter, maar hun eieren en larven daarentegen gevoeliger voor waterverzuring en aluminium.

- Bij een water pH lager dan 5,2 en in de afwezigheid van toxische metalen zoals aluminium wordt het voortplantingssucces dermate verminderd door sterfte, misvormingen en een vertraagde ontwikkeling en een vertraagd uitkomen van de eieren, dat karperpopulaties in hun bestaan bedreigd worden. Wil vertraging van de groei geheel vermeden worden dan dient de water pH zelfs hoger dan 5,7 te zijn.

- Tussen pH 7,5 en 5,9 heeft een aluminiumconcentratie tot 7,4 μmol/l geen negatieve invloed op de vroege ontwikkelingsstadia en kan daarom als veilig beschouwd worden. Aluminium wordt in dit pH traject niet of nauwelijks gebonden aan het embryo.

- Tussen pH 5,9 en 5,1 is aluminium erg toxisch voor eieren en larven; concentraties van slechts 1,3 μmol Al/l hebben al een negatief effect. Deze toxiciteit uit zich onder meer in verhoogde mortaliteits- en misvormingspercentages en een vertraging van de ontwikkeling en het uitkomen van de eieren. Dit kan gerelateerd worden aan de binding van aluminium aan het embryo. De eischaal heeft een beschermende werking.

- Tussen pH 5,1 en 4,8 beschermen lage aluminium concentraties de eieren tegen de toxische invloed van de lage water-pH, terwijl hogere concentraties giftig zijn. Deze beschermende invloed verandert in een toxisch effect na het uitkomen. De effecten van de lage pH alleen zijn reeds zo groot dat de aanwezigheid van aluminium geen extra invloed meer heeft op de overleving van karperpopulaties.

- De verlagende werking die zowel H^+ als aluminium (in het pH traject waar het toxisch is) hebben op de concentraties van calcium en natrium in de larve zijn naar alle waarschijnlijkheid het gevolg van de vertraagde ontwikkeling die door een lage pH en aluminium geïnduceerd wordt.

- Het vertraagde uitkomen van de eieren zoals geïnduceerd door lage pH en de aanwezigheid van aluminium wordt voor een deel veroorzaakt door de misvorming en de gereduceerde bewegingsfrequentie van het embryo. Ook de coagulaten zoals die onder deze condities in de perivitelline vloeistof ontstaan zijn hier mede debet aan.

- De aanwezigheid van humuszuren heeft geen effect op de gevoeligheid van eieren en larven voor lage pH, maar doet de aluminiumtoxiciteit geheel teniet bij een concentratie van 10 mg humuszuur/l. Humuszuren verhinderen de binding van aluminium aan het embryo en de larve.

- Zowel pH 5,4, als 7,4 μmol Al/l bij pH 5,4, brengen bij juveniele karpers een fysiologische verstoring teweeg. Deze kan echter door de dieren gecompenseerd worden. Deze compensatie gaat ten koste van de energie die anders voor groei gebruikt zou worden.

- Aangezien de toxiciteit van aluminium vooral wordt veroorzaakt door verzuring van het milieu, moeten de oorzaken van deze verzuring worden aangepakt.

DANKWOORD

Degenen die voor het proefschrift het grootste offer hebben gebracht zijn de vele naamloze eieren, larven en vissen, die hun leven gegeven hebben zonder zich ook maar bewust te zijn geweest waarvoor dit diende. Toch was dit proefschrift niet tot stand gekomen als minder naamloze, en gelukkig ook wat minder vergaande hulp van vele waardevolle collega's, vrienden en kenissen er niet geweest was. Een aantal van hen verdienen het speciaal om hier naar voren geschoven te worden.

Allereerst Sjoerd Wendelaar Bonga: "Sjoerd; heb dank voor de heerlijke discussie's tijdens onze gemeenschappelijke autoreizen, waardoor als gevolg van vele gemiste afslagen mijn kijk op het Nederlandse natuurgebeuren aanzienlijk verruimd werd". Contacten van eenzelfde filosofisch gehalte, maar met minder drastische geografische gevolgen, zoals ik die met Coen v d Mey deelde heb ik als erg verrijkend ervaren. Eigenschappen als eindeloos geduld mocht ik waar nemen bij Jelle Eygenstein en Theo Schoenmakers, die mijn hardleersheid als een muur moeten hebben ervaren als het ging om het onder de knie krijgen van de ICP en de PC. Als het gaat om het uitdrukken van de katholieke identiteit van de KUN mag naar Rob Lock verwezen worden, wiens onbaatzuchtige helpende hand zonder meer sierend genoemd mag worden, en waaraan slechts in geringe mate afbreuk gedaan wordt door zijn ondeugendheid. Ferry Derksen van de technische dienst heeft mij op zodanige wijze ingewijd in de geheimen van de zelf-bouw dat de stelling "De beste stellingen zijn van Ferd" een begrip op de afdeling geworden is. Tommie Palings, beste vrind, veel vreugde heb ik beleefd aan jouw zieke geest en jouw serieus-zijn, ons swingend stappen en ons doorbijten in de Canadese natuur. Dat je daarnaast ook nog mijn vissen verzorgd hebt valt hierbij eigenlijk in het niet.

Studenten behoren bij elk promotie onderzoek, en die welke ik heb mogen vergezellen in en fase van hun studie waren een gouden greep uit de schatkist, ofschoon ik soms twijfelde of het niet de doos van Pandora geweest kon zijn. Peter, Marga, Erich, Xander en Karen, ieder van jullie bedankt voor de bijdragen en jullie eigenheid. Ik wens jullie in de verdere carriere evenveel plezier toe als in onze tijd samen, waarin is gelachen, liefdes verdriet is gedeeld en duurexperimenten gedaan zijn totdat we er letterlijk bij neervielen.

Een goede sfeer op de werkvloer is vaak productiebepalend. Daarom mijn waardering voor alle mensen die ieder op hun eigen wijze een steen hebben bij gedragen aan de gemeenschappelijke activiteiten zoals labuitstapjes en andere activiteiten.

Energie vrij maken om een promotie succesvol te volbrengen wordt pas mogelijk met een goede levenskwaliteit en onmisbaar hiervoor zijn mijn vrienden, waarvan in het bijzonder Lo Camps, die voor mij het levende bewijs is dat de meest gepolariseerde eigenschappen tot een integer geheel zijn te combineren, en mijn familie en diegenen die ik als zodanig beschouw. Ik spreek bij deze de wens uit dat we elkaar met zoveel liefde

en inzichten mogen blijven inspireren als tot nog toe gebeurd is.

Ferdinand

CURRICULUM VITAE

erd Oyen zag het levenslicht op 19 mei 1959 te Roermond. Omdat de aandacht meer uitgin aar plantjes en beestjes kostte het doorworstelen van het VWO aan het Bisschoppelij 'ollege Broekhin te Roermond hem enige tijd, maar behaalde hij toch in 1978 zijn einddipl(ia Atheneum B. Tegen de uitdrukkelijke wil van vaders in werd direct hieropvolgend aan d iologiestudie aan de Katholieke Universiteit te Nijmegen begonnen, alwaar hij in 1983 h(andidaatsexamen behaalde. Het doctoraalexamen "oude stijl" werd in januari 1987 afgeleg iet als hoofdvakken Dierfysiologie (Prof. dr S.E. Wendelaar Bonga) en Aquatisch)ecologie (Prof. dr C. Den Hartog) en als negenmaands vak Visteelt aan de Landbou' Universiteit te Wageningen (Dr. C.J.J. Richter). Vervolgens was hij gedurende 5 maanden i ienst van Natuur, Milieu en Fauna beheer te Utrecht, alwaar hij na inventariseren nderzoek een visstandbeheersplan formuleerde. Van begin 1988 tot begin 1992 werd d :hrijver dezes te werk gesteld aan de Katholieke Universiteit van Nijmegen als Assistent i)pleiding bij de Vakgroep Experimentele Dierkunde. Gedurende deze periode verrichtte h nder toeziend oog van Prof. Dr S.E. Wendelaar Bonga het in dit proefschrift beschreve nderzoek. Naast het verrichten van onderzoek is tevens een bijdrage geleverd aan h(octoraal onderwijs in de vorm van begeleiding aan 'eerste fase' studenten en doctora: udenten.

STELLINGEN

Hoewel de vrije-ion theorie (Neville & Campbell, 1988. Water Air Soil Pollut. 42: 311-321) en de aluminium-speciatie theorie (Smith & Hem, 1972. Geol. Surv. Water-Supply Paper 1827-D) uitgaan van verschillende vooronderstellingen zijn ze beide in staat om de pH-afhankelijke aluminium toxiciteit op de vroege ontwikkelingsstadia van karper te verklaren (dit proefschrift).

Gezien het feit dat onder sommige omstandigheden de vroege levensstadia van karper gevoeliger zijn dan die van salmoniden, betekent dat deze laatsten niet zondermeer als meest gevoelige bioindicator voor vissen gekozen kunnen worden (dit proefschrift).

Wanneer toxiciteitstesten met visseëieren, zoals uitgevoerd in dit proefschrift, een algemene toepassing vinden zullen zowel de concentratie-normen van deze stoffen in het aquatisch milieu als het proefdiergebruik dalen.

Beter dan allerlei specifieke assays, is lichaamsgroei een algemeen bruikbare parameter om aan te tonen of onbekende stoffen de fysiologie van vissen verstoren. Contra: Woltering, Aquatic Toxicol. 5: 1-21 (1984).

Om een lang verhaal kort te maken: aluminium is onder bepaalde omstandigheden giftig.

Hoewel het natuurkundige wereldbeeld in toenemende mate benaderd wordt vanuit een wiskundig-energetische achtergrond, blijft het biologische wereldbeeld nog sterk bepaald door een naïef-realistische visie.

Indien het met menselijke activiteit geassocieerde massale uitsterven van soorten wordt geplaatst in het licht van de periodiek terugkerende grote perioden van uitsterven in de evolutie, kan geconcludeerd worden dat soortgerichte natuurbescherming een conservatieve bezigheid is.

Naar verwachting zal "het idee" voor zover het tot uiting komt in het menselijk handelen in de toekomst een steeds belangrijkere determinant worden voor de evolutie van het materiële; voor evolutionaire voorspellingen zal een samenwerking tussen de biologie en de geesteswetenschappen vruchtbaar kunnen blijken.

De huidige vrijheidsverslaving kan uiteindelijk wel eens een inperkende factor blijken te zijn, aangezien vele mogelijkheden slechts gecreëerd kunnen worden binnen een beperkende structuur.

De spiegel die de gedragingen van voetbalsupporters ons voorhoudt wijst meer op een kudde-model dan Veronica ons wil doen geloven.

Gezien de grenzen die de natuur *weten*-schap zichzelf oplegt is het de vraag of ze haar claim dat ze *weet* nog waar kan maken (het weten op het schap?).

Indien men de huidige waterkwaliteit in ogenschouw neemt, kan "als een vis in het water" de lading "zich als een vis in het water voelen" niet langer meer dekken.

Waar in de biologie BIO en LOOG elkaar ontmoeten, is het van belang te constateren dat ook tegenwoordig de grens tussen leugen en waarheid over het leven niet altijd te handhaven is.

Ferd Oyen

Nijmegen, 28 april 1993

Hij die denkt veel te weten, weet slechts veel over het denken.
Hij die niet denkt kan veel aan de weet komen.
Denk hier dus niet over na, opdat u gaat weten (B.T. Bartholomeus).