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THE EFFECTS OF WATER HARDNESS AND pH UPON THE TOXICITY OF ZINC TO THE BROWN TROUT SALMO TRUTTA L.

A Dissertation Submitted

to

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by

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TO MY PARENTS

ABSTRACT

THE EFFECTS OF WATER HARDNESS AND pH UPON THE TOXICITY OF ZINC TO THE BROWN TROUT SALMO TRUTTA L.

In continuous-flow toxicity tests on brown trout, the 96 h LC_{50} for zinc was substantially reduced in artificial soft water (1.07, 2.31, 1.41, 0.60, 0.05, and 0.22 mg Zn l^{-1}) compared with mains hard water (2.02, 3.20, 2.69, 0.64, 1.00 and 0.46 mg Zn l^{-1}) at nominal pH values of 4,5,6,7,8 and 9 respectively. The reduced zinc toxicity in hard water was attributed to an ameliorative effect by calcium at high hardness. pH influenced metal toxicity in the pH range 5 - 7 with increased toxicity of dissolved zinc correlated with rising pH and the highest toxicities of zinc were recorded in the pH range 8 - 9 in the presence of metal complexes. With acute exposure to zinc over the pH range 4 - 9, fish survival was dependent upon the deleterious effects of the metal on branchial respiratory and ionoregulatory mechanisms.

In radioisotopic studies, fish were exposed in hard and soft water to 0.05 mg Zn l^{-1} with 10 μCi $^{65}Zn^{2+}$ added and zinc influx and efflux was monitored over 48 h. In both hard and soft water an initial fast uptake component over some 0 - 5 h was followed by a second slower component of 5 - 48 h. In hard water both the

fast and slow influx rates of total zinc (52 and 1.2 μg h^{-1} kg^{-1}) were significantly less ($P < 0.01$) than the corresponding fluxes in soft water (121 and 2.3 μg h^{-1} kg^{-1}). Tissue zinc concentrations reflected both the uptake and excretion rates of the metal in hard and soft water fish.

The ameliorative effects of water hardness appear to act by reducing zinc uptake in high calcium waters due to reduced branchial surface permeability and by enhancing excretion. In contrast pH modifies zinc toxicity by altering the chemical form of the metal.

N.C. Everall.

DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degree.

We certify that the work submitted was carried out by the author. Due acknowledgement has been made of any assistance received.

Signed ..N.C. Everall.....

(Candidate)

Signed ..Nathan Jarlam.....

(Director of Studies)

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N.C.E.

ABBREVIATIONS

The majority of symbols used to identify physical quantities in SI units are defined in : The Symbols Committee of The Royal Society (1975). Quantities, units, and symbols. 54 pp. The Royal Society. Any other abbreviations not fully defined in this work are given below.

C. min. $^{-1}$ g.$^{-1}$	Counts (radioactivity) per minute per gramme.
C. min.$^{-1}$ µg.$^{-1}$	Counts per minute per microgramme.
C. min.$^{-1}$ ml.$^{-1}$	Counts per minute per millilitre.
HE	Haematoxylin and eosin stain.
LC_{50}	Median lethal concentration, the concentration of a toxicant lethal to one half of a test population of fish in a given time.
LT_{50}	Median lethal time, the survival time of one half of a population of fish in a given concentration of toxicant.
µg. h.$^{-1}$ kg.$^{-1}$	Microgrammes per hour per killogramme.
µg. l^{-1}	Microgrammes per litre.
mg. l^{-1}	Milligrammes per litre.
P.	Probability.
SD	Standard deviation about the mean.

SE	Standard error of the mean.
SEM	Scanning electron microscope.
^{65}Zn	Radioisotope of stable zinc.

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INTRODUCTION

Zinc is a naturally occurring and abundant metal which has been estimated to represent 0.004% of the earth's crust (Rice, 1961). ''Background levels`` of zinc in inland surface waters are produced by the processes of geological weathering and the metal may occur in high levels in areas characterized by metal-bearing substrata. For such areas where metal-polluted precipitation does not occur a typical range of values for the zinc levels of surface waters in the U.S.A, Canada and N. Europe is from 0.001 to 0.3 mg Zn l^{-1} (O'Connor, 1968; Beamish, 1976; Henriksen and Wright, 1978).

However, human activities release far greater levels of zinc and other trace metals into the environment (Lantzy and Mackenzie, 1979; Jeffries and Snyder, 1981). Global anthropogenic emmissions of zinc, cadmium, copper, lead and nickel were estimated to have been 14 x 10^9kg, 0.32 x 10^9kg, 2.2 x 10^9kg, 20 x 10^9kg and 1.0 x 10^9kg by 1980 (Weatherley et al., 1979). During the industrial processing of ores and metals the major source of zinc input into surface waters is by way of the atmosphere (Hem, 1972; Leland et al., 1978).

Atmospheric pollution appears to be the major source of zinc and other trace metals in surface waters of North America and Southern Scandinavia (Henriksen and

Wright, 1978). Rainfall and runoff have been found to contain high levels of zinc (Vymazal, 1985) and could be classified as polluted precipitation. Zinc concentrations ranging from 0.11 - 0.32 mg l^{-1} have been reported in precipitation from parts of the United States, southern Norway and north-west England (Lazarus et al., 1970; Dale et al., 1974; Harrison and Williams, 1982). Rainfall collected in the vicinity of a metal smelter at Flin Flon, Manitoba, Canada, contained from 1.5 - 42.9 mg Zn l^{-1} (Franzin and McFarlane, 1981) with high concentrations of zinc in nearby surface waters (Van Loon and Beamish, 1977). Similarly, snow and snowmelt waters have also been reported to possess high levels of zinc (Franzin et al., 1979; Snekuik et al., 1973) and thaw melts can lead to episodic release of toxic levels of trace metals. Loss of fisheries from zinc polluted waters caused by metal deposition in rainfall and runoff have been documented (Van Loon and Beamish, 1977; Spry et al., 1981; Solbe, 1977).

The highest concentrations of zinc have been observed in surface waters receiving acid mine drainage where metal levels in the range of 7-3840 mg Zn l^{-1} were reported (Vymazal, 1985). Acidification of surface waters has frequently been observed to be accompanied by raised concentrations of many trace metals (reviews : Forstner and Wittman, 1981; Haines, 1981; I.E.R.E., 1981; Spry et al., 1981) as a consequence both of

atmospheric deposition and of mobilisation by runoff at low pH. Fish in acid waters show increased absorption of metals e.g. Cd, Hg, Mn and Zn (Moreau et al., 1983; Tomlinson et al., 1980) and mortalities are often due to a combination of low calcium concentration, low pH and elevated metal levels (The Watt Committee on Energy, 1984). Indeed, the biomass and population density of Salmonidae in upland streams of the U.K. were found to be positively correlated with pH, calcium concentration and alkalinity but negatively with the zinc, aluminium, copper and lead concentrations (Turnpenny, 1985). Turnpenny concluded that it was the level of trace metals, rather than pH per se, which was at the root of these population trends. Similarly, the significance of the observation that fish from acid waters contain elevated levels of zinc and other trace metals had not previously been understood (The Watt Committee on Energy, 1984).

The decline of fish populations as a result of elevated zinc and other metal levels appears to be ameliorated by increased calcium concentrations in the water (Judy and Davies, 1979; Brown, 1983; Turnpenny, 1985). Calcium concentrations in the range of 1-10 mg l^{-1} are typical of many upland oligotrophic streams (with near circumneutral pH) in the U.K. which support salmonid and other fisheries (Sadler and Lynam, 1984). Similarly, in the U.K., Scandinavia and North America

acid waters which are soft and poorly buffered have calcium levels in the range 0.5 - 2.5 mg l^{-1} (Reader, 1986) and are characteristically of low conductivity. Soft waters are low in concentration of the hardness metals (calcium and magnesium) but calcium is the more important of the two, its concentration is typically 2-3 times that of magnesium (NRCC, 1981). The reported deleterious effects of zinc and other trace metals upon inland fisheries may therefore often occur in soft surface waters (c. 1-2 mg Ca l^{-1}) over a wide pH range from 4-7.

The boundary between hard and soft waters is particularly arbitrary and undefined (McDonald et al., 1986) but generally considered to be at a hardness of approximatley 50 mg l^{-1} as $CaCO_3$ (Marrier et al., 1979). Problems with metal toxicity from industrial sources of zinc pollution can occur in fish in natural waters of much higher hardness (504 mg l^{-1} $CaCO_3$) (Solbe, 1973). Surface waters of high hardness are usually associated with areas of soft geology and are often derived from limestone or chalk run-off. Hard waters are generally of alkaline pH and although hardness is often expressed as the $CaCO_3$ concentration, the soluble product of $CaCO_3$ dissolution is predominently $Ca(HCO_3)_2$ at or below pH 8 (Stumm and Morgan, 1981). The alkalinity of natural surface waters is usually expressed as the acid neutralising capacity due to the bicarbonate

concentration. Thus hardness and alkalinity are usually present in a 1:2 molar ratio in waters lacking acid or base input (Stumm and Morgan, 1981). The ameliorative effects of water hardness upon the toxicity of zinc to fisheries (EIFAC, 1973) may be dependent upon a range of environmentally relevant calcium and other metal concentrations which will determine both short-term (acute) impact and long-term (chronic) effects of pollution. However, the mechanisms by which hardness may exert its protective effects upon metal toxicity to fish are not fully understood (Pagenkoff, 1983; Bradley and Sprague, 1985b).

The effects of surface water pH upon fisheries may be direct through the impact of increased hydrogen-ion concentration, which may cause mortality (Leivestad and Muniz, 1976; Henriksen et al., 1984; Skogheim et al., 1984) and reproductive failure (reviews : Haines, 1981; I.E.R.E., 1981) at pH below 5. However, recent experimental investigations with carefully controlled media have attributed the effects of low pH to associated elevated metal levels at pH > 4.3, rather than pH effects per se (Brown and Lynam, 1983; Dalziel, 1986; Reader, 1986).

The effects of pH upon metal toxicity are not fully understood (Alabaster and Lloyd, 1980; Bradley and Sprague, 1985b) but pH is thought to affect both the solubility and speciation of many metals (Campbell and

Stokes, 1985). Zinc ion concentration in surface waters increases as pH decreases (NRCC, 1981) with dissolved zinc dominant over the pH range 7-4 and particulate complex species of the metal (.e.g. hydroxides and carbonate) prevelant from pH 7-9 (Vymazal, 1985). In surface waters of alkaline pH co-precipitation of heavy metals with hydroxides and carbonates appears to be an important mechanism controlling the metal concentrations in the aquatic environment (Jenne, 1968; Lee, 1976). However, the solubilities of zinc carbonate and hydroxide seem to be too high to exert a very effective control on the metal concentration of natural waters unless there is an unusually high activity of dissolved carbon dioxide species (Vymazal, 1985). It is possible that relatively small concentrations of zinc complexes may be highly toxic to fish (Pagenkoff, 1983). However, at present, there is little general agreement in the metal toxicology literature as to what proportions of which complex species of zinc were present in previous studies and whether or not these particulate forms were toxic to fish (Bradley and Sprague, 1985b).

From the available evidence it seemed that there is a need for a detailed study of metal toxicity to fish with careful control and monitoring of water content and precaution against other stresses. The effects of water hardness on metal toxicity to brown trout, Salmo trutta, were to be explained using zinc as a trace metal of

environmental relevance. Similarly, the roles of pH in determining zinc toxicity and the toxicity of the [H^+] per se were also to be examined. In addition, experiments are described, which investigate the interactions of water hardness (calcium concentration) with zinc uptake, accumulation and excretion. The rates of zinc influx and efflux with varying external water hardness were to be examined as a means of determining the mechanisms of acute and chronic toxicity of the metal. Therefore, the present investigation explores various effects of hardness and pH upon the toxicity of zinc to brown trout with particular emphasis on population survival, physiological causes of death and underlying mechanisms of metal toxicity.

2.1 INTRODUCTION

For this study zinc levels were selected that represent the ranges of elevated metal concentrations found in surface waters of industrially polluted and acid-mine drainage areas (see Chapter 1). Similarly, water hardness and pH conditions were chosen to produce environmentally relevant water qualities from a range of metal contaminated waters.

The main effect of hardness on zinc toxicity to fish is the reduction of toxicity in hard waters (Jones, 1938; Lloyd, 1960; Mount, 1966; EIFAC, 1973; Sinley et al., 1974; Farmer et al., 1979; Alabaster and Lloyd, 1980; Bradley and Sprague, 1985b). Of the hardness metals calcium appears to be a more effective protecting agent than magnesium (Potts and Fleming, 1971; Judy and Davies, 1979).

Lloyd (1965) concluded that the influence of hardness on zinc toxicity was through a biological mechanism, since Salmo gairdneri acclimated to hard water, but exposed to zinc in soft water, had a lower mortality than had soft water-acclimated fish exposed to zinc in soft water. Similar observations were made for this species when exposed to cadmium in the same manner (Calamari et al., 1980). The process of hardness acclimation must have provided the fish with some

resistance to zinc in hard waters and fish were acclimated to both hardness and pH in the present studies for these reasons. Indeed, other water quality conditions were also closely controlled or monitored because variations in temperature (Lloyd, 1960; Hodson and Sprague, 1975; Smith and Heath, 1979), dissolved oxygen (Lloyd and Swift, 1976; Hughes and Flos, 1978), alkalinity (Holcombe and Andrew, 1978; Bradley and Sprague, 1985b), ammonia concentration (EIFAC, 1970), and organic (EIFAC, 1973) or inorganic matter (Ministry of Technology, 1971) may affect the toxicity of zinc.

Water pH *per se* may be lethal to fish below pH 4.3 (see Chapter 1) and above at pH 9 (Daye and Garside, 1976) but the pH of the water also determines the major chemical species of zinc present. The pH dependent concentrations of dissolved or particulate zinc at low and high pH have been reported to have differing toxicities to fish (Mount, 1966; Farmer *et al.*, 1979; Pagenkopf, 1983; Bradley and Sprague, 1985b; Cusimano *et al.*, 1986). To aid in distinguishing between the effects of pH *per se* and chemical speciation of the metal upon the toxicity of zinc to the fish, brown trout were acclimated to pH for 14 days before metal exposure. While previous studies have considered hardness acclimation to be important, few metal toxicity studies have acclimated test species to pH.

The present study includes an investigation of the interactions of pH and metal toxicity with water pH over the range 4-9, for hard and soft water acclimated brown trout exposed to environmentally relevant zinc, pH and hardness levels typical of polluted surface waters. Brown trout are thought to be more resistant to zinc than other salmonid species (EIFAC, 1973) but there is a general lack of toxicological data on this species (Alabaster and Lloyd, 1980) despite its rise in commercial and conservational importance in the U.K. and Scandinavia. It seemed logical that this species warranted further investigation with the aim of protecting fisheries from zinc pollution (Jones, 1940; Jones, 1958) since concentrations of zinc that are lethal to fish in the field are also generally comparable to those found in the laboratory (EIFAC, 1973).

2.2 MATERIALS AND METHODS

2.2.1 Stock fish

Brown trout of suitable age and size ranges were obtained from two sources. The first, The Trent Fish Culture Co., Brailsford, Derbyshire, experienced some problems with disease so, subsequently, fish were obtained from Leadmill Trout Farm, Hathersage, Derbyshire. Fish from Hathersage were progeny of the brood stock at Trent Fish Culture so all the fish came from the same genetic stock.

At the Polytechnic, fish were put into circular 3000 l GRP tanks with a continuous flow of mains water at c.200 l h^{-1}. The fish were fed twice daily at c.0900 and 1600 h on Mainstream Fingerling No.1 trout pellets. Such fish received the natural prevailing photoperiod of c.16 h light and 8 h darkness. Having established a feeding cycle, a prophylactic treatment of antibiotic (Tribrissen) medicated food pellets was given over 5 days. Following treatment the fish were kept another four weeks before any experimental work. If more than 10% of any batch of fish became diseased the entire group was destroyed.

2.2.2. Effects of water hardness on zinc toxicity

2.2.2.1. Acclimation to hard and soft waters

At intervals of 3 weeks 80 0+ group, 8-10 g fish were transferred from the holding tanks into two 550 l polythene tanks in temperature/photoperiod controlled rooms. The tanks were aerated and received, via a header tank, 0.5 l min^{-1} of mains hard water thermostatically controlled at 15± 0.05 °C. A 7 day holding period allowed partial temperature acclimation and any fish showing signs of disease were discarded. Over a further 14 days temperature acclimation was completed and adaptation to soft and hard water conditions took place. These acclimation times were considered adequate for full acclimation to varied water hardness and to temperature (Lloyd, 1965; McDonald et al., 1980). Throughout the total three weeks of acclimation fish received an artificial photoperiod of 12 h light and 12 h darkness which was maintained during toxicity testing. The fish were not fed for the last 5 days of acclimation before being used for toxicity testing.

The mains water proved to be a suitable supply of hard water and this was softened to provide an artificial soft water. The mains supply was passed through an ion exchange column (ELA3 water softener, ELGA Ltd.) before entering the acclimation tanks. The

ion exchange column contained a sodium-based cation resin which preferentially binds calcium or magnesium ions in exchange for sodium ions. This resin was recharged by backflushing with brine every 12 hours.

Both hard and soft water supplies were analysed for a range of water quality parameters at 3-4 month intervals by Severn-Trent Water Authority (STWA). Samples of acclimation water were taken daily and the concentrations of zinc, calcium and magnesium were determined by standard methods of atomic absorption spectrophotometry (AAS) using a Perkin-Elmer 103 spectrophotometer. Total hardness was measured using Schwarzenbach water hardness reagents `CVS'.

Table 1 shows the major water quality factors during acclimation (see also Appendix). Water quality remained reasonably constant for the independent hard or artificial softened water supplies though more subtle changes did occur in the process of softening. These changes included elevated and depressed levels of sodium and chloride respectively in softened mains water plus decreased sulphate levels in soft waters. There was also a smaller but marked decrease in the levels of some background trace metals of the softened water supply compared with the hard mains water.

Table 1 The major water quality factors during acclimation

Water Quality Factor		Mains hard water	Artificial soft water
Temperature °C		15 ± 0,5	15 ± 0,5
pH		7,80 ± 0,05	8,00 ± 0,05
Dissolved oxygen	mg l^{-1}	10,7 ± 0,3	10,2 ± 0,4
Total water hardness as	mg l^{-1} $CaCO_3$	180 ± 3	9 ± 1
Alkalinity as	mg l^{-1} $CaCO_3$	94	66
Calcium	mg l^{-1} Ca^{2+}	60 ± 2	< 1
Magnesium	mg l^{-1} Mg^{2+}	10 ± 2	< 1
Sodium	mg l^{-1} Na^{+}	71 ± 6	95 ± 9
Chloride	mg l^{-1} Cl^{-}	99 ± 8	54 ± 8
Sulphate	mg l-l SO_4^{2-}	122 ± 11	64 ± 9
Zinc	mg l^{-1} Zn^{2+}	< 0,02	< 0,01
Aluminium	mg l^{-1} Al^{3+}	< 0,01	< 0,01
Cadmium	µg l^{-1} Cd^{2+}	0,1	< 0,1
Chromium	µg l^{-1} Cr^{2+}	1	< 1
Copper	µg l^{-1} Cu^{2+}	8	9
Iron	mg l^{-1} Fe^{3+}	0,01	0,01
Lead	µg l^{-1} Pb^{2+}	< 1	< 1
Manganese	mg l^{-1} Mn^{2+}	< 0,01	< 0,01
Nickel	µg l^{-1} Ni^{2+}	0,6	7
Free chlorine	mg l^{-1}	< 0,01	< 0,01

The data are expressed as the mean with the maximum and minimum range (±) of values so the majority of results-recorded fall within 10% of the mean or target during the acclimation period.

2.2.2.2 The automatic - dosing apparatus

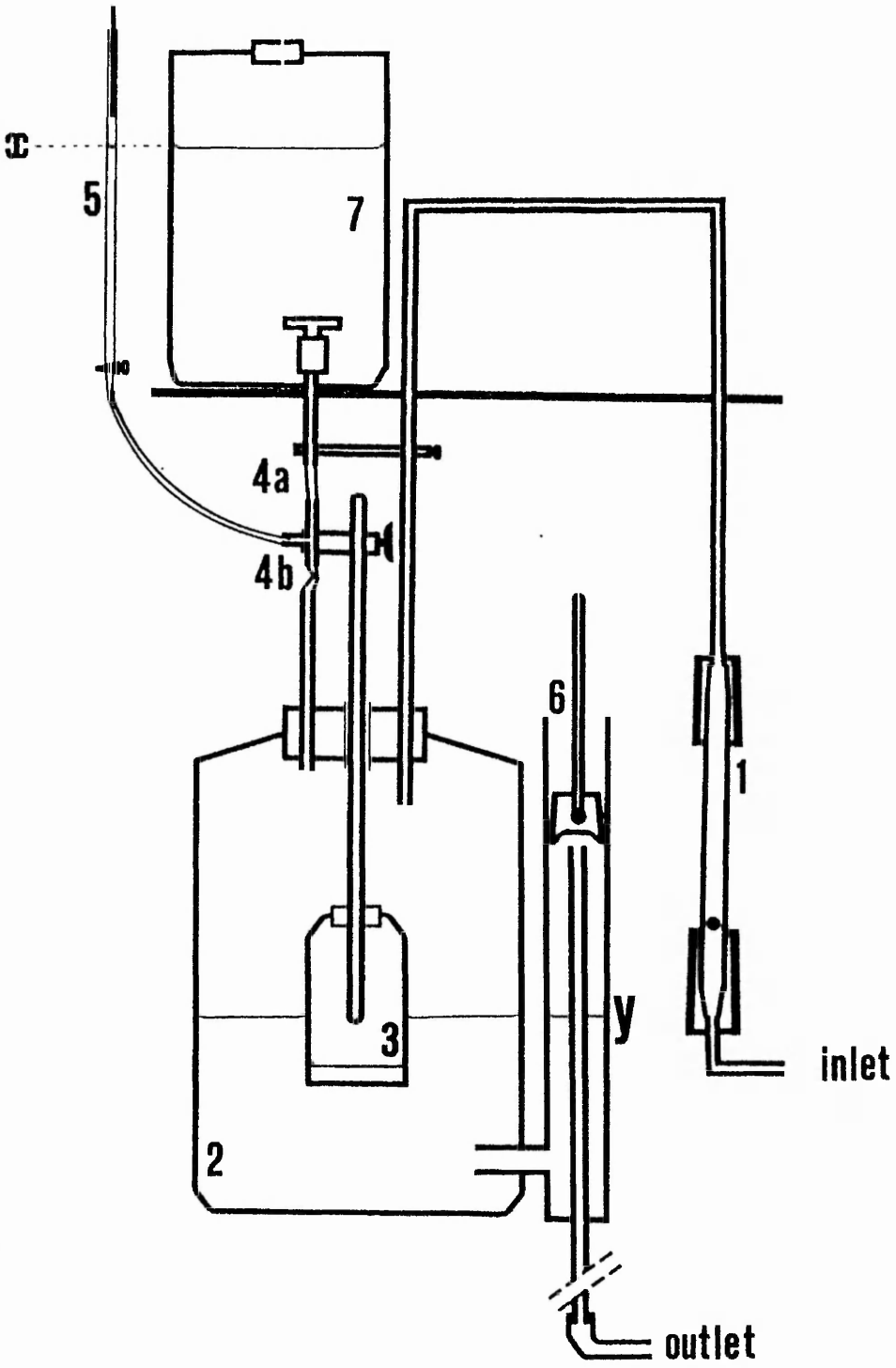
To determine the acute toxicity of zinc to brown trout, both static and through flow test procedures were considered. Due to the high adsorptive properties of zinc, the speciation of the metal with pH and the requirement of trout for high water quality, a continuous-flow apparatus was regarded as most suitable.

Zinc concentrations and water hardness regimes were set and controlled by a kinking-tube and syphon type of dosing apparatus (Fig. 1). This automatic system was based on models designed by Abrams (1960) and Stark (1967) with modifications by O'Neill (1981).

The doser comprised a wide necked glass 2 litre dilution chamber (2), a siphon tube (6), a float and valve assembly (3,4) and a measuring burette (5). A continuous flow of hard or soft water entered the dilution chamber through a 0-1.5 l min^{-1} flow meter (1). The head of water (Y) was controlled by the stoppered plunger (6). At an inlet flow of 0.4 l min^{-1} the doser provided an outflow of 1.4 l each time the syphon emptied the dilution chamber. Over a period of 96 hours there was an error of ± 4% in the volume of water delivered from the syphon.

The toxicant solution was made up in 25 l polythene vats from zinc sulphate $ZnSO_4 . 7H_2O$. (Reagent grade) dissolved in hard or soft water. The zinc reservoirs (7) were replenished once every 24 h or as required and

Figure 1 Automatic - dosing apparatus


x
5
7
4a
4b
6
1
y
3
2
inlet
outlet

positioned above the level of the dosing apparatus to facilitate gravity feed (Fig. 1). The zinc solution reaches the doser via the ``kinking T-valve'' incorporating valves of `Microwall' tubing connected to a solid glass T-piece. The rise and fall of the 0.1 l polythene float (3) during the dilution chamber filling and emptying cycles, opened or closed the T-valves (4a and b). When T-valve 4a was open with 4b closed the zinc solution entered the burette to a set level $\propto$ and the opening of valve 4b with 4a closed during the filling cycle enabled the appropriate volume of zinc solution to empty into the dilution chamber. The incoming dilution water and zinc solution which mixed in the dilution chamber were delivered to a 120 l polythene test tank as hard or soft water at the desired zinc concentration.

Six replicate dosing systems and six corresponding 120 l test tanks were used in the toxicity tests. The six dosing systems were soaked for 24 hours in 0.1 M H_2SO_4 and washed by allowing clean water to flush through for 24 hours on through flow between tests.

2.2.2.3 Toxicity tests

Preliminary 96 h zinc toxicity sighting tests were carried out on fish adapted to hard and soft water. These sighting tests determined the approximate range of

zinc concentrations to be used in the definitive 96 h toxicity tests.

The same water quality conditions experienced during acclimation (Table 1) were maintained in the tests and the values and ranges of these factors remained constant over the test period. The logarithmic series of five zinc concentrations tested in the 96 h toxicity studies are given in Table 2. They are shown as nominal and actual values in the hard or soft water series. Control test waters contained only background levels of zinc.

Each of the six dosing systems was run for 24 hours before starting a test in either hard or soft water. This allowed appropriate zinc concentrations to become established, adsorption of the metal to occur and formation of zinc complexes at pH 8 to reach a stable equilibrium (Hahne and Kroontje, 1973; Patterson et al., 1975).

The flow rate of 400 ml min^{-1} ensured a 90% molecular replacement time in the test tanks of 11-12 hours. Constant mixing and rapid turnover times of zinc in test waters helped to expose the fish to a homogeneous zinc solution. With fish exposed to zinc in alkaline test waters visual checks were made to see that precipitated complexes (e.g. $Zn(OH)_{2}$, $ZnCO_{3}$) were kept in suspension. In all tests at alkaline pH the 'milky white'

Table 2 Nominal and measured zinc concentrations in test waters

Tank No.	Total zinc concentration mg l-1			
	Hard water		Soft water	
	Nominal	Actual	Nominal	Actual
1 (Control)	0	<0.02	0	<0.01
2	0.80	0.86 ± 0.02	0.10	0.05 ± 0.01
3	1.60	1.68 ± 0.04	0.20	0.10 ± 0.01
4	3.20	3.66 ± 0.05	0.40	0.31 ± 0.04
5	6.40	6.75 ± 0.12	0.80	0.70 ± 0.04
6	12.80	12.55 ± 0.26	1.60	1.65 ± 0.10

Measured zinc (actual) concentrations are presented as the mean with the maximum and minimum range (±) of values. All values recorded fall within 10% of the mean or target level during a 96h test.

precipitated complexes of zinc appeared to have a uniform distribution in test waters i.e. both at the surface and at the bottom of the solutions in the tanks.

A toxicity test used 60 fish in groups of 10 randomly chosen individuals which were placed abruptly in the five zinc equilibrated tanks and a control tank. The fish were exposed to the range of zinc concentrations for 96 hours and their condition was checked every 1-2 hours by day and 3-4 hours at night. Times of death for individual fish were noted during the test and at 96 hours the number of fish alive, overturned and dead in each tank was recorded. The criterion for death was the absence of opercular or body movements for two minutes following gentle prodding. Dead fish were removed at the time of death and if more than one control fish died the test was invalidated.

Water samples were collected in 500 ml polythene bottles at the start of each test and at 12 h intervals subsequently for the duration of the test. The samples were always obtained from the central mid-water level in a test tank, sealed and the stored at 4 °C for later analyses. Prior to storage samples of test media were acidified with 3·5 M HNO_3 (A.R.) in a ratio of 1:100, HNO_3: sample volume and metal standards for A.A.S. were also acidified in this manner.

2.2.2.4 The median lethal concentration LC_{50}

Transformed toxicity curves were constructed by plotting % mortality against zinc concentration and LC_{50} values estimated by interpolation. 95% confidence limits were calculated using the standard techniques described by Lichfield and Wilcoxon (1949). The graphical estimates of LC_{50} were compared with calculations of log-dose effects using probit analysis (Finney, 1971). Probit analyses were done by linear regression using a generalised linear interactive modelling package (GLIM II) on a DEC SYSTEM 20 computer.

2.2.3 Effects of pH and water hardness on zinc toxicity

2.2.3.1 Acclimation to pH in hard and soft water

0^{+} group, 12-25 g fish were initially acclimated to temperature and photoperiod during a holding period as established in the previous experiments. However, these fish were then acclimated not only to hard and soft water as previously described but also to a range of pH values.

12 fish were placed in each test tank and maintained on a through-flow of 250 ml min^{-1} of hard or soft water by means of the dosing apparatus. The natural pH of the acclimation water (mains hard water or artificial soft water of pH 8) was gradually adjusted to the desired value during the following 24 hours. A calculated dose

of diluted sulphuric acid or sodium hydroxide solution was added to the tanks from 25 l reservoirs connected to the dosers. The concentrations of stock acid or alkali were selected to give the required exposure pH following dilution. No adjustment was needed at pH 8, the normal mains water supplies, but these fish were also allowed the same period to adapt to the test tanks. After 24 hours in which pH levels stabilized, fish were allowed to acclimate for 14 days to water hardness over the pH range 4-9. This period was considered suitable for acclimation to pH on the basis of previous work (McWilliams and Potts, 1978; McWilliams, 1980 a and b; McDonald et al., 1980). This period was also sufficient for direct toxic acidotic effects (Leivestad and Muniz, 1976) or toxic alkalotic effects (Lloyd, 1961) of pH to become apparent.

Fish were not fed for the final 5 days during pH acclimation before their use in zinc toxicity tests. Water quality conditions during this acclimation period are shown in Table 3. The pH of acclimation waters was recorded every 12 hours but it was checked more frequently. Other water quality factors were determined using the techniques previously described.

In contrast with the previous experiments these fish were acclimated to a slightly harder water, and to slightly lower background zinc levels in both hard and

Table 3 The major water quality factors during acclimation

Water Quality Factor		Mains hard water	Artificial soft water
Temperature °C		15 ± 0,5	15 ± 0,5
Nominal pH	(4)	4,10 ± 0,1	3,98 ± 0,1
	(5)	5,02 ± 0,1	5,06 ± 0,1
	(6)	5,89 ± 0,1	6,01 ± 0,1
	(7)	7,03 ± 0,1	7,07 ± 0,1
	(8)	7,89 ± 0,1	8,03 ± 0,1
	(9)	9,11 ± 0,11	9,06 ± 0,1
Dissolved oxygen mg l^{-1}		10,1 ± 0,2	10,3 ± 0,3
Total water hardness as mg l^{-1} $CaCO_3$		204 ± 4	10 ± 1
Alkalinity as mg l^{-1} $CaCO_3$		103	62
Calcium	mg l^{-1} Ca^{2+}	66 ± 2	< 1
Magnesium	mg l^{-1} Mg^{2+}	10 ± 2	< 1
Sodium	mg l^{-1} Na^{+}	63 ± 5	84 ± 7
Chloride	mg l^{-1} Cl^{-}	100 ± 10	58 ± 9
Sulphate	mg l^{-1} SO_4^{2-}	134 ± 15	69 ± 7
Zinc	µg l^{-1} Zn^{2+}	7	< 5
Aluminium	mg l^{-1} Al^{3+}	< 0,001	< 0,001
Cadmium	µg l^{-1} Cd^{2+}	0,1	< 0,1
Chromium	µg l^{-1} Cr^{2+}	1	< 1
Copper	µg l^{-1} Cu^{2+}	8	9
Iron	mg l^{-1} Fe^{3+}	0,01	0,01
Lead	µg l^{-1} Pb^{2+}	< 1	< 1
Manganese	mg l^{-1} Mn^{2+}	< 0,01	< 0,001
Nickel	µg l^{-1} Ni^{2+}	6	7
Free chlorine	mg l^{-1}	< 0,01	< 0,01

The data are expressed as the mean with the maximum and minimum range (±) of values indicated. All values recorded fall within 10% of the mean or target during the acclimation period. Bracketed pH figures represent nominal values with the measured results tabulated.

soft water. The concentrations of other trace metals was similar to earlier experiments.

2.2.3.2 Toxicity tests

Preliminary 96h tests in hard and soft water over the pH range 4-9 were done before a final toxicity testing regime was designed.

Using a range of six pH levels and six zinc concentrations all possible combinations were tested for the two water hardnesses using a random sequence of 96 h toxicity tests. The matrix of combinations and the test design in hard and soft water are shown in Tables 4a and 4b respectively. A logarithmic series of zinc concentrations, pH and appropriate controls were randomly assigned to test tanks and tests. Due to the nature of the water supply only one hardness could be maintained per test with six test tanks.

The toxicity tests were done using the established methods with the following modifications :

1. The zinc solutions and sulphuric acid or sodium hydroxide solutions were mixed in the same reservoirs before dosing. pH ranged from 3-10 over the six toxicant reservoirs and problems of precipitation of zinc complexes in alkaline stock solutions were overcome by fitting recirculating pumps.

Tables 4a & 4b

A, C, E, G, I and K represent nominal zinc concentrations to be tested in hard water for any six test tanks at nominal pH.

B, D, F, H, J and L similarly represent their equivalents in soft water.

The progression A to L represents the order in which the toxicity tests were performed with alternating hard and soft water studies e.g. series A followed by series B tests.

Table 4a Possible combinations of pH and zinc concentration in the hard water toxicity-test designs

Nominal pH	Nominal zinc concentrations per test tank in mg l^{-1}					
	0 (Control)	0.40	0.80	1.60	3.20	6.40
4	A	C	E	G	I	K
5	K	A	C	E	G	I
6	I	K	A	C	E	G
7	G	I	K	A	C	E
8	E	G	I	K	A	C
9	C	E	G	I	K	A

Table 4b Possible combinations of pH and zinc concentration in the soft water toxicity-test designs

Nominal pH	Nominal zinc concentrations per test tank in mg l^{-1}					
	0 (Control)	0.10	0.40	0.80	1.60	3.20
4	B	D	F	H	J	L
5	L	B	D	F	H	J
6	J	L	B	D	F	H
7	H	J	L	B	D	F
8	F	H	J	L	B	D
9	D	F	H	J	L	B

2. In contrast with the earlier experiments the fish were placed in the test tanks for acclimation to pH. While final zinc concentrations were being established the fish from control and test tanks were moved to other tanks. Fish were held in 20-30 l of aerated acclimation water of the required pH, temperature and water hardness under static conditions for approximately 10-12 hours. To start the tests the fish were returned to the test tanks and so were introduced abruptly to stable zinc concentrations.

During the tests water hardness, pH and other water quality parameters remained similar to those maintained during acclimation (Table 3).

The zinc concentrations and pH of the test waters which are shown in Table 5a and 5b were a good representation of the variation and extremes of pH and zinc concentration encountered by the fish during the 96 h toxicity tests.

The mean zinc concentrations and the cumulative percentage mortality at 96 hours were used to calculate the 96 h LC_{50} values in the pH range 4-9 using computer assisted probit transformation (GLIM). Confidence limits were again fitted by the method of Lichfield and Wilcoxon (1949) and were similar to values estimated by GLIM.

Table 5a - Matrix design of actual pH and zinc combinations tested in hard water over 96 hours

	Nominal zinc concentration in mg l^{-1}					
	0(Control)	0.40	0.80	1.60	3.20	6.40
Measured pH		Measured zinc concentration in mg l^{-1}				
4,09 ± 0,09	0,007	0,45 ±0.03	0,82 ±0.05	1,68 ±0.06	3,11 ±0.08	6,64 ±0.12
5,08 ± 0,07	0,007	0,40 ±0.02	0,89 ±0.08	1,67 ±0.07	3,18 ±0.09	6,48 ±0.11
6,06 ± 0,13	0,007	0,46 ±0.03	0,86 ±0.05	1,61 ±0.06	3,14 ±0.08	6,45 ±0.09
7,04 ± 0,06	0,007	0,41 ±0.03	0,88 ±0.06	1,70 ±0.08	3,20 ±0.07	6,68 ±0.12
7,93 ± 0,10	0,007	0,40 ±0.03	0,91 ±0.07	1,60 ±0.05	3,15 ±0.10	6,52 ±0.10
9,10 ± 0,08	0,007	0,43 ±0.04	0,83 ±0.04	1,62 ±0.08	3,10 ±0.09	6,44 ±0.09

Table 5b - Matrix design of actual pH and zinc combinations tested in soft water over 96 hours

	Nominal zinc concentration in mg l^{-1}					
	0(Control)	0.10	0.40	0.80	1.60	3.20
Measured pH		Measured zinc concentration in mg l^{-1}				
4,05 ± 0,05	0,005	0,09 ±0.02	0,42 ±0.03	0,86 ±0.05	1,54 ±0.06	3,21 ±0.08
5,02 ± 0,09	0,005	0,14 ±0.01	0,37 ±0.03	0,91 ±0.06	1,61 ±0.07	3,18 ±0.06
6,01 ± 0,07	0,005	0,11 ±0.01	0,46 ±0.03	0,82 ±0.06	1,68 ±0.08	3,28 ±0.07
7,05 ± 0,06	0,005	0,08 ±0.01	0,43 ±0.03	0,80 ±0.07	1,59 ±0.06	3,30 ±0.06
8,03 ± 0,10	0,005	0,14 ±0.02	0,48 ±0.04	0,82 ±0.07	1,63 ±0.08	3,17 ±0.08
9,04 ± 0,09	0,005	0,13 ±0.01	0,44 ±0.03	0,89 ±0.08	1,63 ±0.08	3,15 ±0.05

The pH values are expressed as the mean for six test tanks taken from six toxicity tests and the zinc concentration is given as the mean for a given test tank over 96 hours. Both sets of data are represented with maximum and minimum ranges such that all recorded results fall within 10% of the mean or target level during the test periods.

The concentrations of various zinc ion species at equilibrium were estimated using the computer program 'COMICS' (concentrations of metal ion and complexing species) from Perrin and Sayce (1967). The program was adapted for use with the Applesoft II System. This program calculates equilibrium concentrations for all species in multi-metal multi-ligand mixtures from the pH of the solution, the total concentration of each metal plus each complexing agent and the relevant equilibruim constants (pKa values and stability constants). The chemical equilibria reactions involving zinc and the free metal-free ligand components present in the hard or soft test waters are listed in Table 6. The alkalinity was assumed to be equal to the bicarbonate ion concentration. There were few data for CO_2 concentration in aqueous solution and although this can be estimated from the bicarbonate levels and the pH, the likely error involved necessitates cautious interpretation of these results.

Table 6 - Equilibria regulating zinc speciation in hard or soft water at a given pH

Reaction	Log K	Reference
CO_2 aq = H^+ HCO_3^-	-6.34	1 Sillen and Martel (1964)
HCO_3^- = H^+ CO_3^{2-}	-10.17	1 " " " "
Ca^{2+} + CO_3^{2-} = $CaCO_3$.aq	2.21	2 Pytkowicz and Hawley (1974)
Ca^{2+} + SO_4^{2-} = $CaSO_4$.aq	2.05	3 Garrels and Thompson (1962)
Mg^{2+} + CO_3^{2+} = $MgCO_3$.aq	2.05	2 " " " "
Mg^{2+} + SO_4^{2-} = $MgSO_4$.aq	2.10	3 " " " "
Zn^{2+} + OH^- = $Zn(OH)^+$	6.31	4 Gubeli and Ste-Marie (1967)
Zn^{2+} + $2OH^-$ = $Zn(OH)_2$.aq	11.19	4 " " " "
Zn^{2+} + $3OH^-$ = $Zn(OH)^-_3$	14.31	4 " " " "
Zn^{2+} + SO_4^{2-} = $ZnSO_4$.aq	2.05	5 Owen and Gurry (1938)
Zn^{2+} + CO_3^{2-} $ZnCO_3$.aq	5.0	6 Garrels and Christ (1965)
$ZnCO_3$(s) = Zn^{2+} + CO_3^{2-}	-10.0	7 Smith and Martel (1976)
Zn $(OH)_2$(s) = Zn^{2+} + $2OH^-$	-16.8	4 " " " "

2.3 RESULTS

2.3.1 Zinc toxicity in hard and soft water

Graphical estimations of 96 hour LC_{50} (as mg Zn l^{-1}) were derived from time mortality curves for fish exposed to zinc in hard and soft waters. These are shown in Figs. 2a, b, c and 3a, b, c respectively. The lines were fitted by eye and where applicable greater weight was given to those points lying between 25% and 75% mortality (Lichfield and Wilcoxon, 1949).

In Fig. 2b the time at which the hard water LC_{50} of zinc became independent of exposure was close to the reported 96 hour value. In contrast the soft water LC_{50} curve shown in Fig. 3b was more linear and there was no evidence of a break to an asymptotic value in 96 hours.

Different methods of LC_{50} evaluation were used but the results were similar and are compared in Table 7. Estimates of 96 hour LC_{50} values by probit analysis using the GLIM computer package did not differ by greater than 0.03 mg Zn l^{-1} from graphical methods. Both computed probit analysis and graphical Lichfield-Wilcoxon (1949) plots were used to estimate the final LC_{50} values but confidence limits were determined by the method of Lichfield and Wilcoxon (1949).

The 96 hour LC_{50} values with 95% confidence limits were 1.00 ± 0.17 mg Zn l^{-1} in hard water (60 mg l^{-1}

Figure 2a Graphical estimation of LT_{50} values for zinc-exposed fish in hard water (●).

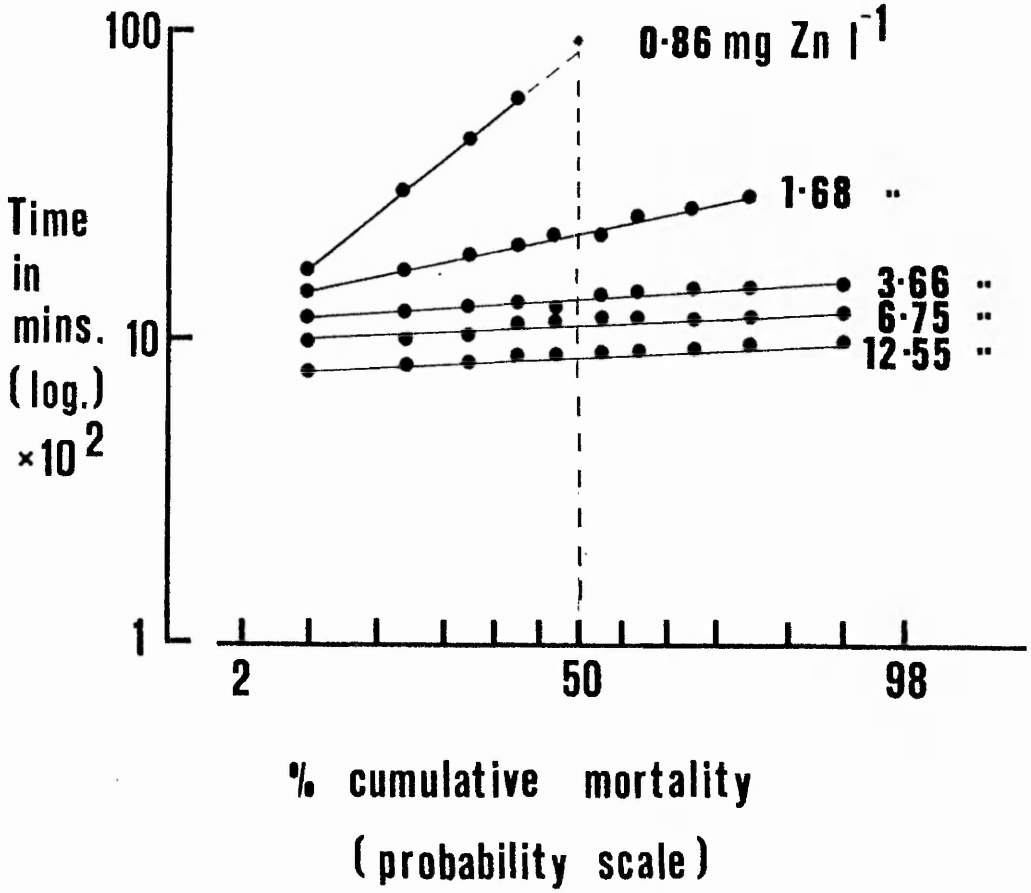

100
10
1
Time in mins. (log.) ×10 2
0·86 mg Zn l -1
1·68 "
3·66 "
6·75 "
12·55 "
2
50
98
% cumulative mortality
(probability scale)

Figure 2b Graphical estimation of LC_{50} values from LT_{50} data for zinc-exposed fish in hard water (●).

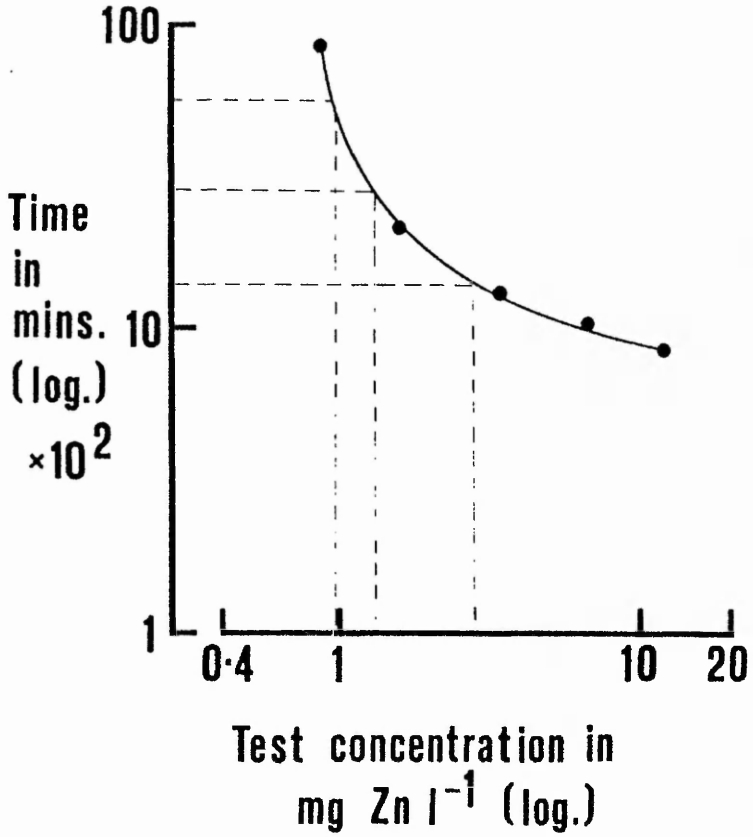

100
Time
in
mins.
(log.)
×10²
10
1
0·4
1
10
20
Test concentration in
mg Zn l⁻¹ (log.)

Figure 2c Graphical estimation of 96 h LC_{50} from percentage mortalities of zinc-exposed fish in hard water (●).

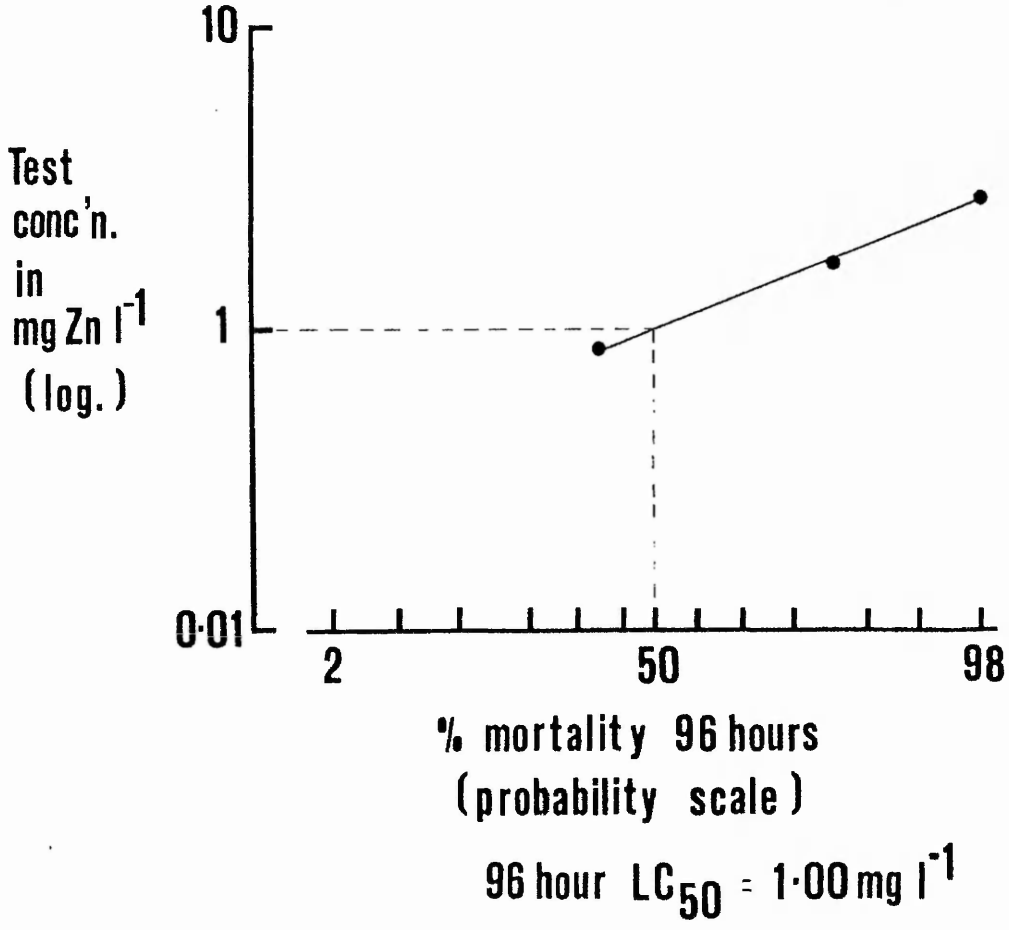

10
1
0·01
Test conc'n. in mg Zn l-1 (log.)
2
50
98
% mortality 96 hours (probability scale)
96 hour LC50 = 1·00 mg l-1

Figure 3a Graphical estimation of LT_{50} values for zinc-exposed fish in soft water (o).

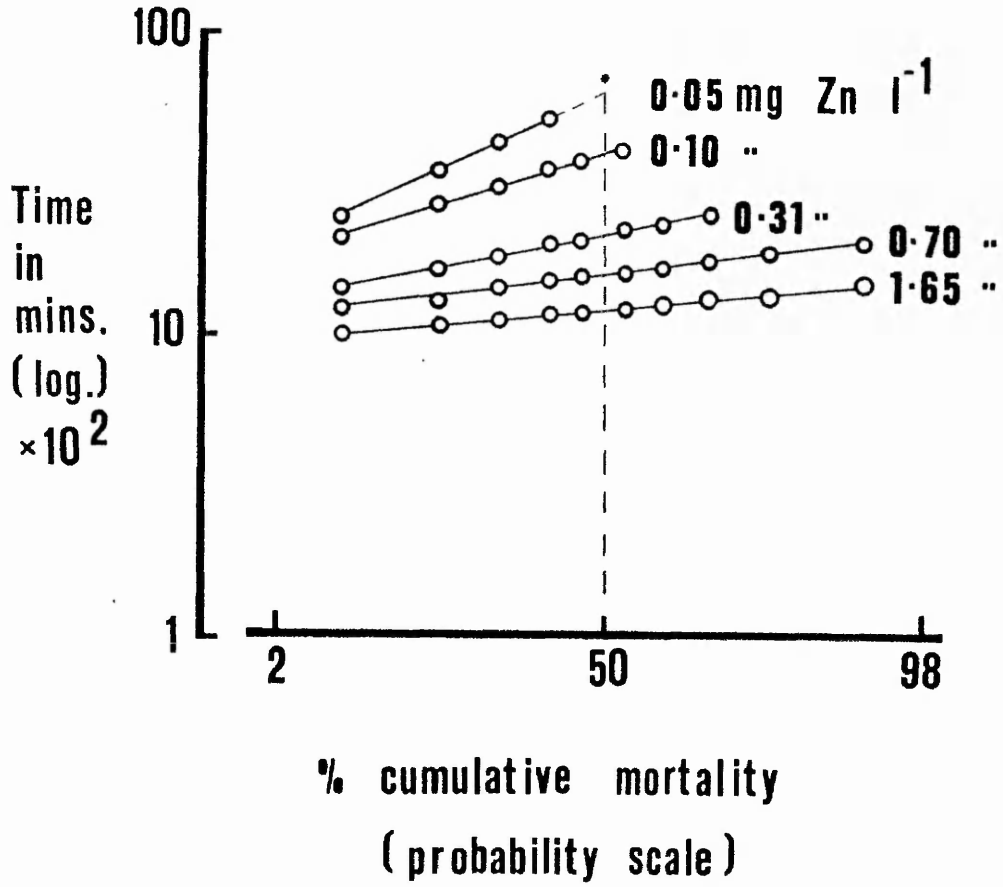

100
10
1
Time in mins. (log.) ×10 2
0·05 mg Zn l-1
0·10 ..
0·31 ..
0·70 ..
1·65 ..
2
50
98
% cumulative mortality
(probability scale)

Figure 3b Graphical estimation of LC_{50} values from LT_{50} data for zinc-exposed fish in soft water (o).

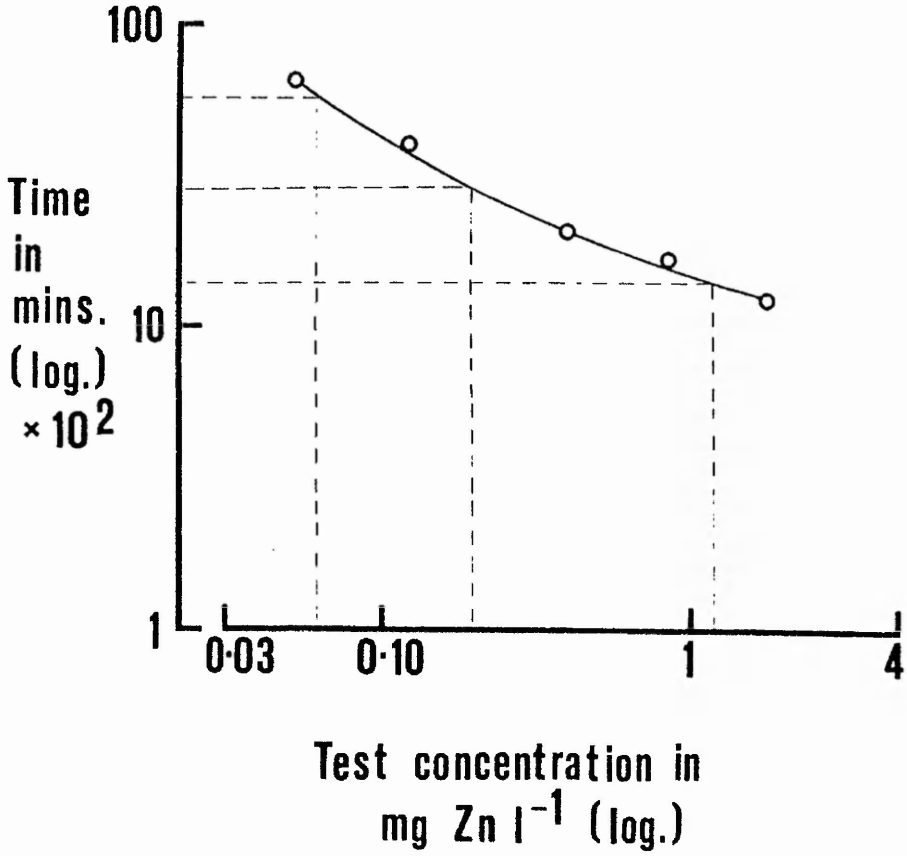
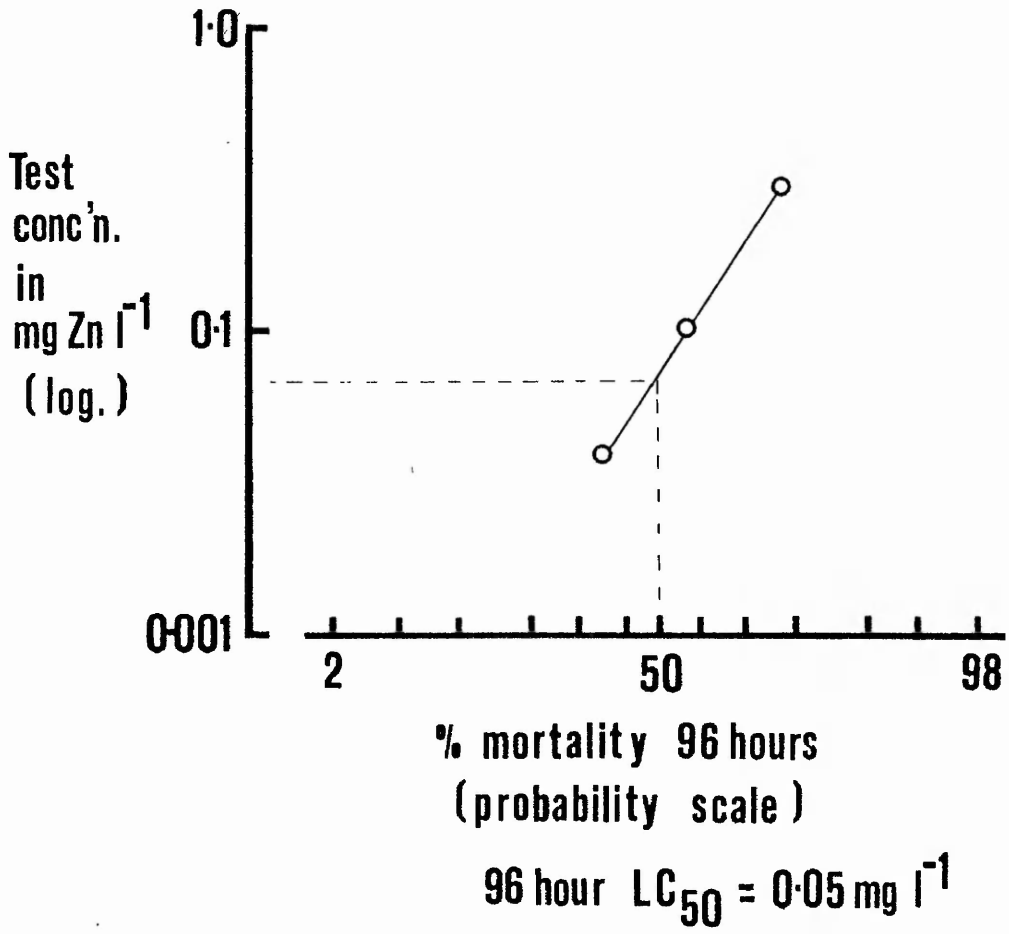

100
Time
in
mins.
(log.)
× 10 2
10
1
0·03
0·10
1
4
Test concentration in
mg Zn l −1 (log.)

Figure 3c Graphical estimation of 96 h LC_{50} from percentage mortalities of zinc-exposed fish in soft water (●).



96 hour LC_{50} = 0·05 mg l^{-1}

Table 7 Comparison of the 96 hour LC_{50} values for zinc calculated by various methods.

Method	Hard water LC_{50} mg Zn l^{-1}	Soft water LC_{50} mg Zn l^{-1}
Graphical		
LC_{50} data	0.97	0.06
% cumulative mortality data	1.00	0.06
Computer		
Probit anlaysis	1.00	0.05

Ca^{2+}, 180 mg l^{-1} as $CaCO_3$) and 0.05 ± 0.01 mg Zn l^{-1} in soft water (1 mg l^{-1} Ca^{2+}, < 10 mg l^{-1} as $CaCO_3$). The concentrations of total dissolved zinc acutely lethal to brown trout at 96 hours were therefore twenty-fold higher in hard water than soft water at 15 °C and pH 8.

2.3.2 Zinc toxicity in hard and soft water over the pH range 4-9

96 hour LC_{50} valves and 95% confidence limits were calculated graphically and the results are summarised in Table 8. In Figure 4 the values for 96 h LC_{50} given in Table 8 are plotted against pH for hard and soft waters. This plot produced a three dimensional toxicity surface. The LC_{50} for total zinc varied in a complex manner with pH and produced marked differences between hard and soft waters across the pH range. The LC_{50} of zinc ranged from 0.05 mg l^{-1} in soft alkaline waters to 3.20 mg l^{-1} in hard acid waters.

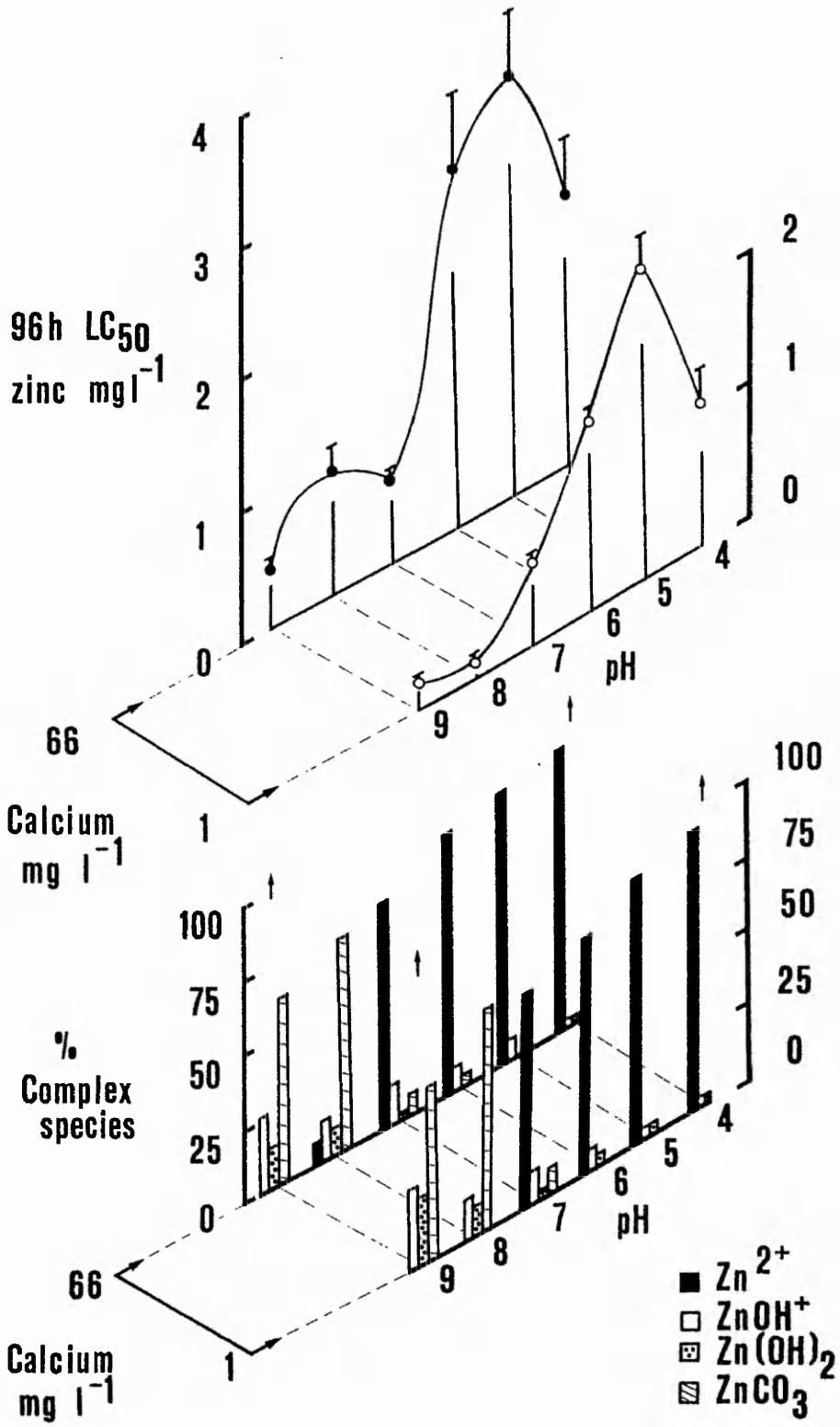
Interactions between water hardness and pH were complex but generally high hardness reduced zinc toxicity over the pH range. However, the toxicity of zinc increased at pH 7 in hard waters but not in soft waters. This reversal of the trend at pH 7 was confirmed by two sets of tests.

Also in Figure 4 are computed estimates (COMICS) of the proportions of the main ionic species of zinc

Table 8 96 hour LC_{50s} and 95% confidence limits for total dissolved zinc

Nominal pH	Hard water LC_{50} mg Zn l^{-1}	Soft water LC_{50} mg Zn l^{-1}
4	2.02 ± 0.42	1.07 ± 0.26
5	3.20 ± 0.52	2.31 ± 0.25
6	2.69 ± 0.56	1.41 ± 0.12
7	0.64 ± 0.12	0.60 ± 0.11
8	1.00 ± 0.17	0.05 ± 0.01
9	0.46 ± 0.10	0.22 ± 0.06

Figure 4 96 h acute toxicity surface for fish exposed to zinc in hard (●) and soft water (○) over the pH range 4 - 9. 96 h LC_{50} values are shown with 95% confidence limits. pH dependent metal speciation for test waters are shown as an insert ↑ below the toxicity surface.


96h LC50
zinc mgl-1
4
3
2
1
0
2
1
0
4
5
6
7
8
9
pH
66
Calcium
mg l-1
1
100
75
50
25
0
%
Complex
species
100
75
50
25
0
4
5
6
7
8
9
pH
66
Calcium
mg l-1
1
Zn 2+
ZnOH+
Zn(OH)2
ZnCO3

present in the test waters. The zinc toxicity changed rapidly over the pH range 5 - 7 in both hard and soft water where there was a chemical transition which increased the proportion of complex species in test solutions. Indeed, the marked increase in zinc toxicity over the pH range 7 - 9 showed a strong correlation with increasing proportions of the complex species ($ZnOH^+$, $Zn(OH)_2$ and $ZnCO_3$) in both hard and soft waters. Zinc present as the free ion (Zn^{2+}) was less toxic to fish than complex species over this pH range in the hard and soft waters. It was not possible to determine which of the complex zinc species or combinations of species was responsible for the observed increase in toxicity at higher pH. Increases in zinc toxicity for both hard and soft waters below pH 5 may have been due in part to additive acidotic effects (Leivestad and Muniz, 1976; Daye and Garside, 1976) with high $[H^+]$. However, control fish at pH 4 all survived a two week acclimation period and the further four days (96 h) of the toxicity tests with zinc. At pH 9, alkalotic effects (Daye and Garside, 1976) may have contributed to the overall toxicity following zinc exposure. Comparing effects on zinc toxicity, pH was as important as hardness over the pH range 6 - 8 and particularly noticeable for hard water over this pH range.

2.4 DISCUSSION

The values for the 96 h LC_{50} of 0.05 mg Zn l^{-1} in artificial soft water (pH 8) and 1.00 mg Zn l^{-1} in mains hard water (pH 7.8) obtained for brown trout indicated a twenty-fold increase in sensitivity to the metal in soft waters. Throughout this study it has been assumed that the ameliorating influence of hard water was a function of increased calcium concentration, rather than of increased magnesium concentration, but experimental evidence has not been presented in support of this assumption. Nevertheless, some related studies do indicate that the calcium ion is of prime importance. The acute toxicity of zinc to fathead minnows was reduced at higher concentrations of calcium, as distinct from other cations or anions (Judy and Davies, 1979). Similarly, the calcium ion provides the main protection against cadmium toxicity to the brook trout (Salvelinus fontinalis) (Carrol et al., 1979) and aluminium to brown trout (Brown, 1981). In hard water where the toxicity of zinc to brown trout was substantially reduced, the calcium ion concentration was six times that of magnesium and was sixty times the calcium concentration present in soft waters compared with only a ten-fold difference in magnesium concentration. It would seem logical that, of the large changes in zinc toxicity

occurring by the alteration in concentration of these ions, the greater increase in calcium concentration should be responsible for the reduction in metal toxicity. However, extension of the present study, to distinguish possible magnesium induced effects at higher concentrations from those of calcium, would be helpful.

The 96h LC_{50} values for zinc obtained with brown trout in hard and soft water at c.pH 8 were higher than equivalent data for rainbow trout in some of the literature (Alabaster and Lloyd, 1980). 4 day LC_{50} values for rainbow trout at comparable water hardness were in the range 0.2 - 0.3 mg Zn l^{-1} in soft water and 2-3 mg Zn l^{-1} in hard water. However, much of the work reviewed by Alabaster and Lloyd (1980) was conducted at circumneutral pH and unfortunately some details of acclimation and test conditions were lacking. The present studies on brown trout indicated that the magnitude of pH changes over the range 6 - 8 substantially influenced the LC_{50} and was equivalent to differences in hardness in changing the toxicity of zinc. For such reasons, it was decided that a realistic comparison of the toxicity data obtained for brown trout could only be made with that determined for other salmonids (Alabaster and Lloyd, 1980; Alderdice and McLean, 1982) when pH, water hardness and other water quality conditions were similar. The only comparable data with similar conditions produced a 96h LC_{50} for

rainbow trout in soft water (12 mg l^{-1} $CaCO_3$) at pH 6.7 of 0.56 mg Zn l^{-1} (Lloyd, 1960) compared with 0.60 mg Zn l^{-1} for brown trout in soft water (10 mg l^{-1} $CaCO_3$) at pH 7 in the present study. On the basis of such limited comparable data it would be unwise to attempt to draw conclusions about the relative toxicity of zinc to these two species of salmonid. In considering this problem, it became evident that there is a need to standardise toxicity testing procedures in carefully controlled media of known composition and to ensure detailed accounts of acclimation and test conditions are given by workers in this field.

Differences in osmolality and alkalinity between artificial soft waters (high osmolality, low alkalinity) and mains hard water (low osmolality, high alkalinity) may have contributed to changes in the overall toxicity of zinc to brown trout. However, as determined in other studies on the toxicity of trace metals, small differences in sodium and chloride concentrations (osmolality) between test waters would have little bearing on the toxicity or toxic effects of the metal (Carrol et al., 1979; Wright et al., 1985; Reader, 1986). Indeed, any implicated effects have been reported to be of secondary importance to the effects of pH and calcium concentration in test waters (Leivestad and Muniz, 1976; McWilliams, 1980). It was not possible to distinguish between the effects of alkalinity and

water hardness in the present study. Alkalinity has been shown to be of some importance in ameliorating the toxicity of zinc at high levels (Holcombe and Andrew, 1978) but increased water hardness as opposed to alkalinity has been shown to have the major protective role (Bradley and Sprague, 1985b).

The toxicity of low pH per se was observed for brown trout acclimated to pH5 and pH4 for two weeks in both hard (high calcium) and soft (low calcium) waters. Under the most severe conditions of acclimation, in artificial soft water of low calcium concentration (1mg l^{-1}) at pH 4, brown trout survived without apparent deleterious consequences at a level well below the upper limit (c.pH 5.6) for fish population loss in the field. Similar findings have also been reported for brown trout using artificial media of low trace metal content (Brown and Lynam, 1983; Dalziel et al., 1985; Sadler and Lynam, 1985b; Reader, 1986). The lack of toxic levels of trace metals in test waters of low pH may account for the survival of fish in acid waters of pH 4 - 5.5. Indeed, in an investigation of upland streams in England and Wales, the biomass and population density of Salmonidae were positively correlated with pH, calcium concentraton and alkalinity, and negatively with aluminium, copper, lead and zinc concentrations (Turnpenny, 1985). Turnpenny concluded that it was the level of aluminium and other trace metals, rather than pH per se, which

governed the population trends. Therefore from the evidence of the present study and the literature to date it would be unwise to ascribe fish population loss to low pH *per se*.

Zinc was predicted to exist in solution as simple hydrated cations, without major speciation changes over the pH range 4 to 7 (Campbell and Stokes, 1985) although complex species may be formed above pH 7 (Pagenkopf, 1974). Results from studies on the effects of pH upon the toxicity of dissolved zinc to fish have been variable (EIFAC, 1973). It has been proposed that zinc held in suspension is more toxic to fish (Lloyd, 1960; Mount, 1966; Farmer *et al.*, 1979) but other work suggests zinc precipitates are less toxic (Tabata, 1969; Sprague, 1964). Investigation of the toxicity of zinc to rainbow trout over a narrow range of pH indicated that dissolved zinc became more toxic with increasing pH (5.5 to 9) but was substantially replaced by zinc precipitate which was less toxic to fish (Bradley and Sprague. 1985b). Often, few general conclusions can be drawn from the pH/metal toxicity literature due to the lack of comparability in data. Major differences in test conditions arise particularly from the use of static rather than continuous - flow procedures, the extent to which zinc precipitates are maintained in suspension, sufficient equilibration time for metal solutions to allow precipitate formation to be completed

and use of comparable water quality and other test conditions.

An important difference between the present study and others upon the effects of pH on trace metal toxicity was that the fish were acclimated to pH prior to metal exposure. This allowed acclimation to the effects of varied H^+ concentrations, before determining the effects of zinc exposure over the pH range. At low pH this procedure may mimic environmental conditions where trout populations from acidified waters receive sudden or episodic exposure to elevated levels of potentially toxic trace metals by deposition or mobilisation (Leivestad and Muniz, 1976; Spry et al., 1981; Vymazal, 1985).

Dissolved zinc became more toxic to brown trout as pH increased in both the soft and hard waters used. Zinc toxicity increased rapidly over the pH range 5 to 7 where the majority of zinc appeared to be in solution. These observations were in accord with those of other workers (Mount, 1966; Bradley and Sprague, 1985b; Cusimano et al., 1986) and suggested that in this pH range dissolved zinc becomes increasingly toxic. No studies of zinc toxicity to fish at pH values lower than pH 5.5 were evident from the literature (Campbell and Stokes, 1985). The toxicity of zinc to brown trout at pH 4 increased substantially compared with the toxicity of the metal at pH 5.

Increased toxicity of zinc at pH 4 was probably due to synergistic effects of the high [H^+] (Leiviestad and Muniz, 1976) and the zinc. The additive effects of low pH and zinc exposure were ameliorated in the presence of high calcium levels and this was in accord with the observation that calcium concentration rather than pH was most closely correlated with the survival of fish populations (Brown, 1982a).

Below pH 7 the main reason for the general reduction in the toxicity of zinc to brown trout was probably due to increased competition from H^+ for metal binding and uptake sites on the gills. However, in the natural environment acidification tends to mobilize trace metals and consequently any ameliorating effect of H^+ on metal toxicity may be counteracted by increasing metal concentrations in the water. At and above pH 4 the toxic effects of low pH *per se* (McDonald, 1983) may often only prove to be lethal to fish when in addition to metal exposure.

A consequence of using sulphuric acid as opposed to other acids was that increased sulphate concentrations with decreasing pH might have ameliorated the toxicity of the H^+ (Graham and Wood, 1981) and possibly the metal. However, while low permeability of sulphate ions at the gill may retard H^+ entry and thus reduce the effects of acidosis (Graham and Wood, 1981), sulphuric acid solutions are highly effective at displacing

surface-bound calcium in fish (McWilliams, 1983). A calcium stripping effect of sulphuric acid solutions on the gills would suggest deleterious effects due to a possible enhanced metal and H^+ uptake rather than any ameliorative effect. However, sulphate concentration, although usually elevated at low pH, does not appear to be related to fishery status (Brown and Sadler, 1981).

Above pH 7 there was an increasing proportion of the first or second hydroxyl and carbonate species of zinc present in the test waters. In the pH range 6 - 9 the increased proportions of $ZnCO_3$, $ZnOH^+$ and $Zn(OH)_2$ correlated with the increasing toxicity of the metal. More importantly the magnitude of the pH changes over the pH range 6 - 8 substantially influenced the LC_{50} for zinc and was equivalent to the changes in hardness in influencing the toxicity of the metal. The similarity in LC_{50} of zinc at pH 7 in both hard and soft water suggested that external calcium or hardness effects may have been superceded by a specific effect of neutral pH. The combined toxicity of a mixture of transitional chemical species of the metal or pH- induced changes in the permeability of the gill surface (McWilliams, 1982a,b) may have produced this result at neutral pH. However, it is difficult to understand why this effect was only seen at pH 7 and has not previously been documented in the literature.

A large fraction of the total zinc was complexed as the carbonate species in test waters of pH 7 and above but particularly in hard waters. Formation of similar species in copper toxicity studies rendered this metal less toxic to a range of salmonids (Pagenkopf et al., 1974). However Lloyd (1960) and Solbe (1974) found that zinc precipitates were toxic to rainbow trout in water of c.pH 8 and a hardness of c.200 mg l^{-1} $CaCO_3$. Due to these conditions and the equilibration times given to test solutions, the main zinc precipitate in Lloyd's and Solbe's studies would have been $ZnCO_3$ unlike the $Zn(OH)_2$ forms thought to be present in Sprague's experiments (Bradley and Sprague, 1985b). The need to allow sufficient equilibration time for the complete formation of zinc complexes in test solutions had led Bradley and Sprague (1985b) to suggest that few conclusions could be drawn regarding the toxicity of $ZnCO_3$. These authors also suggested that $Zn(OH)_2$ at pH 9 appeared to be of low toxicity while Pagenkopf (1983) stated that a correlation existed between the sum of the concentrations of Zn^{2+} and $ZnOH^{+}$ and the observed toxicity of the metal to fish.

The present studies suggested that $ZnCO_3$ was more toxic than $Zn(OH)_2$ and may account for the difference in the toxicity of the metal at pH 9 from that observed at a similar pH by Bradley and Sprague (1985b). It is also unclear from much of the toxicity literature whether or

not particulate zinc was intended to be available to the fish or allowed to settle out in test tanks. The suggested hypothesis that at high pH (7 - 9) dissolved zinc is increasingly replaced by particulate zinc which is of low toxicity to fish (Campbell and Stokes, 1985) was not supported by the studies on brown trout. While at successively higher pH levels (5 - 7), dissolved zinc became increasingly toxic to brown trout, it was substantially replaced by complexes of the metal at high pH (7 - 9) some of which also appeared to be highly toxic to the fish. Carbonate complexes of zinc are perhaps only important in waters of relatively high alkalinity and since in general alkalinity disappears below about pH 5.0 the effect on metal availability in acid waters is likely to be negligible. Therefore at alkaline pH, both the speciation of the metal and the external calcium concentration appeared to be important factors for predicting the toxicity of zinc to brown trout.

3.1 INTRODUCTION

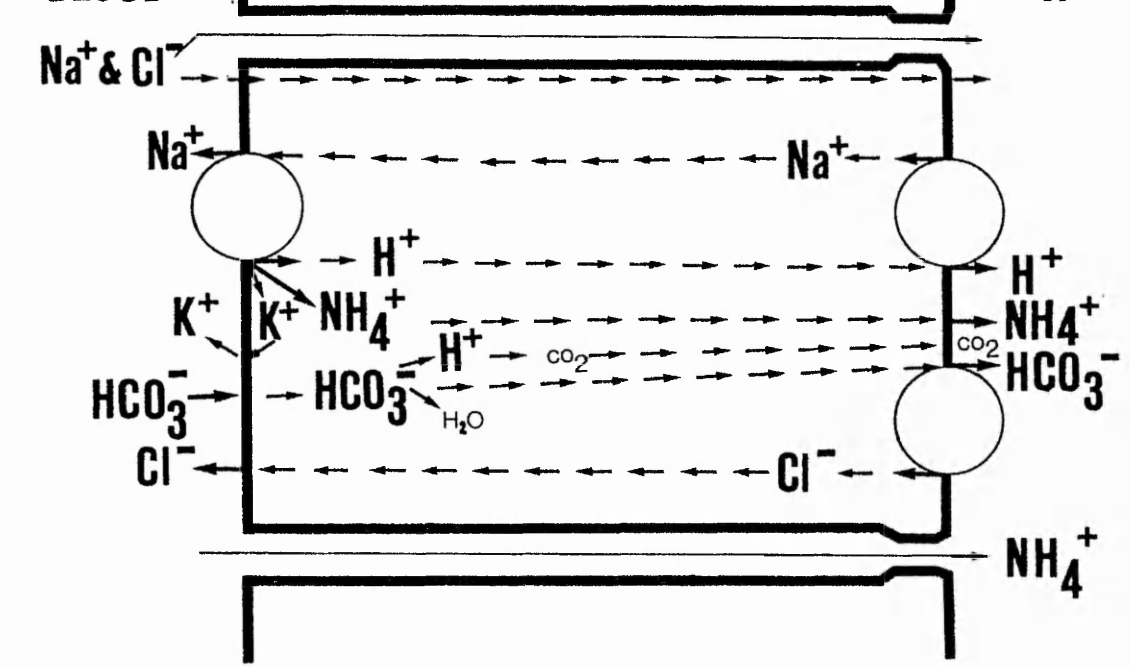
Zinc is an essential trace element required by many organisms as an important constituent of various enzyme processes and nucleic acid synthesis. In higher concentrations zinc can be directly toxic to aquatic life (Weatherley et al., 1980) and reports relating to the toxic effects of trace metals to fish are numerous (reviews : Alabaster and Lloyd, 1980; Frain, 1983; Klaverkamp et al., 1984).

An effect of zinc, at high levels, is to attack the respiratory surfaces of fish (Skidmore and Tovell, 1972) causing tissue hypoxia (Skidmore, 1970). Mechanisms of zinc toxicity in the gills of brown trout have not been previously studied and few studies have attempted to determine the effects of water hardness or pH upon the metal's toxicity (Frain, 1983; Bradley and Sprague, 1985a). Similarly, some recent studies on zinc (Spry and Wood, 1985) and other metals (Dalziel, 1985; Reader, 1986) have indicated that disturbances to branchial ion regulation might be an equally important consequence of metal exposure and dependent upon both hardness and pH. However, pH alone may have caused plasma electrolyte losses in fish examined from experimental and field studies (Leivestad and Muniz, 1976; McDonald et al., 1980; McWilliams, 1982a).

Branchial ion regulation is a likely physiological target for zinc among a variety of potential targets that are summarised in Fig. 5. The present study, aimed to investigate whether the gills of brown trout were the main structural target for zinc and whether or not ion regulation was the major physiological target. The roles of water hardness and pH in ameliorating or enhancing the toxic action of zinc in the fish gill and within other tissues were examined.

Zinc uptake may also occur through the respiratory and ionoregulatory surfaces of the fish gill and the importance of metal absorption (from ambient medium or food) in the toxic process of bioaccumulation needs to be determined (Weatherley et al., 1980; see Chapter 4). Other factors may also prove to be important in ameliorating the toxicity of zinc including increased ventilatory and coughing frequency in response to the metal (Hughes and Adeney, 1977). Long-term studies have also suggested that fish are capable of accumulating zinc in a variety of tissues e.g. gill, liver, spleen and kidney (Holcombe et al., 1979; O'Grady and Abdullah, 1985; see also Chapter 4). Fish can undergo physiological compensation to metal exposure by the production of tissue binding sites for zinc possibly as metallothioneins (Klaverkamp et al., 1984; Bradley et al., 1985) during the process of metal acclimation (Chapman, 1978). At tissue level, mechanisms of metal

Figure 5 Model of the major plasma ion diffusion and transport processes in the fresh water fish gill. In fresh water the lamellar layer of pavement cells is likely to be responsible for most of the Na^+ and Cl^- active absorption from the medium (Girard and Payan, 1980; Haswell et al., 1980). Ion - specific ATPases, responsible for transport are membrane bound to the basolateral membrane in the case of Na^+/K^+ ATPase (Payan 1978) and possibly to the apical membrane in the case of Cl^-/HCO_3^- ATPase (Kerstetter and Kirshner, 1974).


BASO-LATERAL MEMBRANE
APICAL MEMBRANE
BLOOD
FW
Na+ & Cl-
Na+
Na+
H+
H+
K+
K+
NH4+
NH4+
H+
CO2
CO2
HCO3-
HCO3-
HCO3-
H2O
Cl-
Cl-
NH4+

metabolism, production of binding sites and their possible role in detoxification have received very little attention (Kagi and Nordberg, 1979). Present investigations were done to complement metal uptake and accumulation studies described later. Any metal storage systems operating in the tissues may prove to be important, enabling safe storage of tissue bound zinc within fish and thereby ameliorating internal metal toxicity. Similarly, since zinc is an essential element, there may be pathways for metal metabolism which support the view that trace metals in fish tissue are subject to homeostatic control (Goodyear and Bond, 1972; Cross et al., 1973; Wiener and Geisy, 1979). The present study includes an investigation to determine the major structural and physiological targets for zinc with some remaining sites of tissue damage and metal accumulation examined in brown trout, in conditions appropriate to metal and acid-polluted hard and soft waters.

3.2 MATERIALS AND METHODS

3.2.1. Effects of water hardness and acute zinc exposures on fish tissues.

Following from toxicity studies, fish acclimated to hard and soft water at pH 8 were studied to determine the effects of zinc exposure on gill, blood, liver, spleen and kidney. Groups of ten fish were exposed to a range of zinc concentrations in hard and soft water as shown in Table 9. Control fish were not exposed to zinc above background levels (<0.01 mg Zn l^{-1}). Eight fish that survived a 96 h exposure to 0.86 mg Zn l^{-1} in hard water were not killed until after 3 months in a through-flow of control water. Remaining zinc-exposed fish were removed and killed at overturn while individuals surviving the test were killed at 96 hours along with the controls. All fish were netted and killed instantly by a blow to the base of the cranium and individuals were sampled for blood and tissues. The water quality was similar to that during the toxicity tests with varying hardness at pH 8 (Table 2).

Table 9 Nominal and measured zinc concentrations in test waters used for tissue studies

Total zinc concentration mg l^{-1}

Test group	Hard water		Soft water	
	Nominal	Actual	Nominal	Actual
Control	0	< 0.01	0	< 0.01
1 (exposed)	0.8	0.86 ± 0.02	0.2	0.10 ± 0.01
2	1.6	1.68 ± 0.04	0.4	0.31 ± 0.04
3	3.2	3.66 ± 0.05	0.8	0.70 ± 0.04
4	6.4	6.75 ± 0.12	1.6	1.65 ± 0.10
5	12.8	12.55 ± 0.26	3.2	3.10 ± 0.12

Measured zinc (actual) concentrations are presented as the mean with the maximum and minimum range (±) of values. All values recorded fall within 10% of the mean or target level during a 96 h test.

3.2.2 Effects of water hardness, acute zinc exposure and pH upon plasma ion balance.

Water hardness and pH acclimated fish were used to assess the effects of a range of pH from 4 - 9 and zinc concentrations on major plasma electrolyte levels. Ten fish were exposed to the range of zinc concentrations in hard and soft water over the pH range 4 - 9 as shown in Table 10. Control fish (< 7μg Zn l^{-1}) were treated as before. Water composition was similar to that of the acute toxicity tests on zinc with varying pH. The zinc exposed fish were killed at overturn followed by controls at 96 hours and both groups were immediately sampled for blood.

3.2.3. Effects of water hardness and sublethal zinc exposure on fish tissues.

Two groups of ten fish acclimated to hard and soft water at pH 6 were exposed to 0.05 mg Zn l^{-1} for 96 hours. These fish and controls (< 7 μg Zn l^{-1}) were killed at this time for blood samples. This short study was to provide background data for short-term sublethal flux studies with zinc to be performed at pH 6.

Table 10 pH and zinc combinations tested in plasma ion studies

Measured pH		Total zinc concentration mg l^{-1}				
Hard water	(Nominal)	(0)	(0,8)	(1,6)	(3,2)	(6,4)
4,09 ± 0,09	(4)	0,007	0,82 ±0,05	1,68 ±0,06	3,11 ±0,08	6,64 ±0,12
5,08 ± 0,07	(5)	0,007	0,89 ±0,08	1,67 ±0,07	3,18 ±0,09	6,48 ±0,11
6,06 ± 0,13	(6)	0,007	0,86 ±0,05	1,61 ±0,06	3,14 ±0,08	6,45 ±0,09
7,04 ± 0,06	(7)	0,007	0,88 ±0,06	1,70 ±0,08	3,20 ±0,07	6,68 ±0,12
7,93 ± 0,10	(8)	0,007	0,91 ±0,07	1,60 ±0,05	3,15 ±0,10	6,52 ±0,10
9,10 ± 0,08	(9)	0,007	0,83 ±0,04	1,62 ±0,08	3,10 ±0,09	6,44 ±0,09
Soft Water	(Nominal)	(0)	(0,8)	(1,6)	(3,2)	(6,4)
4,05 ± 0,05	(4)	0,005	0,86 ±0,05	1,54 ±0,06	3,21 ±0,08	6,52 ±0,10
5,02 ± 0,09	(5)	0,005	0,91 ±0,06	1,61 ±0,07	3,18 ±0,06	6,67 ±0,12
6,01 ± 0,07	(6)	0,005	0,82 ±0,06	1,68 ±0,08	3,28 ±0,07	6,43 ±0,09
7,05 ± 0,06	(7)	0,005	0,80 ±0,07	1,59 ±0,06	3,30 ±0,06	6,52 ±0,13
8,03 ± 0,10	(8)	0,005	0,82 ±0,07	1,63 ±0,08	3,17 ±0,08	6,61 ±0,10
9,04 ± 0,09	(9)	0,005	0,89 ±0,08	1,63 ±0,08	3,15 ±0,05	6,39 ±0,09

3.2.4. Techniques for blood and tissue analysis

A variety of analyses and histological studies were used to investigate the effects of water hardness and pH during acclimation and zinc exposure :-

3.2.4.1. Blood sampling and analyses

The blood samples were taken from fish using the two techniques of caudal severance and caudal venipucture. All blood samples were centrifuged at 1500 g for 5 minutes, the plasma decanted and stored at -20 °C.

Haematocrit was measured and serial dilutions of plasma were made with deionised water for sodium and chloride determinations. Plasma samples were analysed in triplicate using flame photometry for Na^{+} and a Corning 926 Chloride Analyser for Cl^{-} estimation.

Plasma calcium was determined by fluorimetric titration which involves the quenching of Ca^{2+}-calcein fluorescence with EGTA and subsequent colorimetric analysis. 20µl plasma samples were processed in triplicate.

3.2.4.2. Blood staining

From fresh blood samples taken by caudal severance thin blood films were prepared and air dried. The blood smears were given general staining using the standard methods of Leishman and for zinc with the dithizone method (Magor, McNary and Lionette; 1953).

Deionised water was used in the preparation of vital stains and as the rinsing medium with slides kept dust-free during air drying. These precautions were taken to avoid blood contamination with zinc.

3.2.4.3. Tissue preparations

The first, second and third hemibranchs of the right gill arch, the liver, the spleen and a 5 mm length of mid-gut were dissected from freshly killed fish.

Fish tissues were processed for histological studies using the following techniques :-

3.2.4.4 Wax sections

The fresh tissue was fixed in two changes of Bouin's solution for 5 hours and stored in 70 per cent ethanol overnight at 4° C. Tissue was then dehydrated in absolute ethanol, dosed in toluene and embedded in paraffin wax. 5 - 7μm sections were taken with a Leitz Rotary Microtome, stained by the dithizone method, counter stained with haematoxylin and then mounted in `Aquamount´.

3.2.4.5. Frozen sections

Fresh fish tissue was placed on a microtome cutting block, mounted in O.C.T. embedding fluid and frozen with liquid nitrogen at -190 °C. In a Slee Freeze Cryostat the tissue block and microtome blade were allowed to

reach -20°C before 4 - 5μm sections were taken. Sections were fixed in formal-acetic acid then stained using the dithizone and haematoxylin-eosin methods.

3.2.4.6. Resin sections

Whole gill hemibranchs were dehydrated, resin infiltrated and resin embedded using standard techniques. 1 - 2 μm sections were cut on a glass knife, floated on distilled water, mounted, air-dried and stained with 1% toluidine blue.

3.2.4.7 Glutaraldehyde specimens

The whole liver and spleen were placed on a frozen petri-dish lid (-20 °C) packed in dry ice. The lid was transfered to a tissue slicer and 1 mm transverse sections of tissue were cut. The tissue slices and whole gill hemibranchs were fixed at 4 °C for 4 hours in 25% or 2.5% glutaraldehyde. The preparations were rinsed four times, for 30 minutes each, in Sorenson buffer at pH 7.4 and dessicated at 25 °C.

Dried tissues were mounted on carbon stubs (13 mm diameter) using conductive carbon cement (Leit C). For X-ray microanalysis tissues were coated with antistatic film to prevent electron build-up. For photographic purposes many tissues were then gold coated using a SEM prep. 2 coating unit.

3.2.4.8. Freeze-dried specimens

Tissue samples were quenched by immersion for 15 minutes in liquid nitrogen before freeze drying. Whole liver, spleen and gill hemibranchs were freeze dried for 36 hours. Specimen vials were vacuum sealed and tissues not examined within the next 12 hours were stored at -20 °C. When the vacuum seal was broken the tissues were processed and examined within 6 hours to reduce the movement of ions by rehydration.

A number of the freeze-dried liver and spleen samples were sliced transversely across the central region of the organs with a razor blade. Remaining samples were gently scored at the same point and carefully fractured along a central axis to produce two halves presenting fractured surfaces. Some of the gill hemibranch samples were also treated in the same manner but the remainder were left intact. All tissue samples were mounted and coated for SEM using the standard methods.

3.2.4.9. Image analysis

The 5 μm thick dithizone and haematoxylin stained sections of liver and spleen were studied using a Zeiss photomicroscope in conjunction with a System III image analyser (40-10 Image Analyser of Analytical Measuring Systems Ltd).

It was found that the haematoxylin stained melano-macrophage aggregates were readily discriminated against

surrounding tissue. The ``detector´´ control of the System III analyser was adjusted to cover, exactly, the melano-macrophage aggregates from sections displayed on the television screen. Those aggregates to be counted were located within a previously selected variable rectangular counting frame. Operation of the ``count´´ switch would display the various computer-determined values of field area, total grain area and apparent grain count either on the screen or stored in the computer's memory.

The screen image consists of 107520 square pixels or picture points and any given frame area could be calibrated to correspond with the variety of objectives used on the microscope. The etched divisions on a 1mm stage micrometer, in which each division equalled 0.01 mm, were focussed with the chosen objective onto the television screen. By means of the adjustable frame size measured in picture points and a X1000 oil immersion objective the following calibrations were obtained :

257 pp = 0.07 mm

Therefore 1 pp = 2.724×10^{-4} mm = 0.2723 μm

and since a pp is a square, 1 pp^2 = 7.42×10^{-8} mm^2

or 0.0742 μm^{-2}

The calibration was confirmed prior to each use and 20 sections were randomly chosen from each organ for determinations of the mean melanin macrophage area.

3.2.4.10 Scanning Electron Microscopy (S.E.M.)

Carbon and gold coated tissue preparations were examined using a Cambridge S600 scanning electron microscope at magnifications of x50 - x2,000. Only gold coated tissues were used for photography with Ilford Pan F 50ASA film at 25 Kv.

3.2.4.11 Electron probe X-ray microanalysis

This analytical technique utilizes the fact that atoms, when struck by electrons from an external source, yield X-rays which are characteristic of particular atoms. The frequency of the X-ray emission is dependent upon the atomic number and so a line spectrum corresponding to various elements may be obtained.

A detector or Si(Li) electron probe was placed in the scanning electron microscope in the path of X-rays emitted from specimens undergoing electron bombardment. X-rays were collected by the electron probe and the data stored in the electron probe microanalyser (EPA-LINK 860 Series 2) for immediate interpretation.

The tissues were analysed in two ways, either a large quadrat area, or a closely defined 1 μm^2 diameter spot was used. In practice random quadrat samples incorporating X-ray spectral analysis of large areas were carried out first and features of interest warranting further investigation were studied subsequently by spot analysis.

The characteristic X-ray spectra of a specimen not only identified the elements present but they could be used to quantify the elements. The LINK 860 incorporated a calculation computer package for analysing X-ray spectra to determine the % by weight of elements based on ZAF corrections (Ref: Scott and Love, 1979) for the effects of atomic number, X-ray absorption and fluorescence.

This technique was used mainly to determine the relative amounts of zinc in different tissues. Gross differences in concentrations could be determined although smaller discrepancies could not and no importance was given to actual % weights of elements except relative to treatments (e.g. hard and soft water fish tissues).

3.3 RESULTS

3.3.1 The tissues

Fish exposed to acutely toxic concentrations of zinc in hard and soft water over the pH range 4 - 9 all showed similar external signs of poisoning. There was a consistent sequence of hyperventilation, coughing, overturn, immobilisation and death.

The results show the physiological changes that occurred in the gills, blood, spleen, liver and gut during the entry and passage of zinc through fish exposed to a range of metal concentrations. The effects of exposure to hard and soft water, pH in the range 4 - 9 and zinc were examined at tissue level in control and zinc-exposed fish.

3.3.2. Effects of water hardness and acute zinc exposures on fish tissues

3.3.2.1 Gills

The gills of control fish compared with those of overturned fish (0.70 - 3.66 mg Zn l^{-1}) from hard and soft waters at pH 8, revealed zinc-induced changes in gill morphology.

In freeze-dried gill of fish exposed to 0.84 and 0.86 mg Zn l^{-1} from hard and soft waters respectively, the

zinc content of surface mucus was examined and appeared to be different under the alternative conditions of exposure. These gills are shown in Fig. 6 and were orientated so that the afferent edge of the holobranchs were facing uppermost approximately at the tips of the primary lamellae. The primary and some secondary lamellae could be distinguished in hard water gills but only the rough shape of the holobranchs could be determined from soft water gills. It appeared that the mucus layer surrounding the lamellae of fish from soft water was thicker compared with hard water fish. X-ray electron probe microanalysis determined that the zinc content of the gill surface mucus was higher in soft water fish than hard water fish exposed to the same metal concentration.

Any zinc passing beyond the mucus barrier is placed in direct contact with the primary and secondary lamellae of the gill holobranchs. Holobranchs are derived from the paired set of five gill hemibranchs which are divided into a number of these structures. Each holobranch is composed of a primary lamella which bears two rows of secondary lamellae and these structures are shown in Fig. 7. Single holobranchs with a row of afferent-facing secondary lamellae from control and zinc-exposed gills of hard and soft water fish are shown.

Figure 6 Scanning electron micrographs of mucus covered gill holobranchs from fish in hard and soft test waters

A. Hard water fish (Control, bar = 100 μm).

B. Hard water fish exposed to 0.84 mg Zn l^{-1} (Bar = 100μm).

C. Soft water fish (Control, bar = 200 μm).

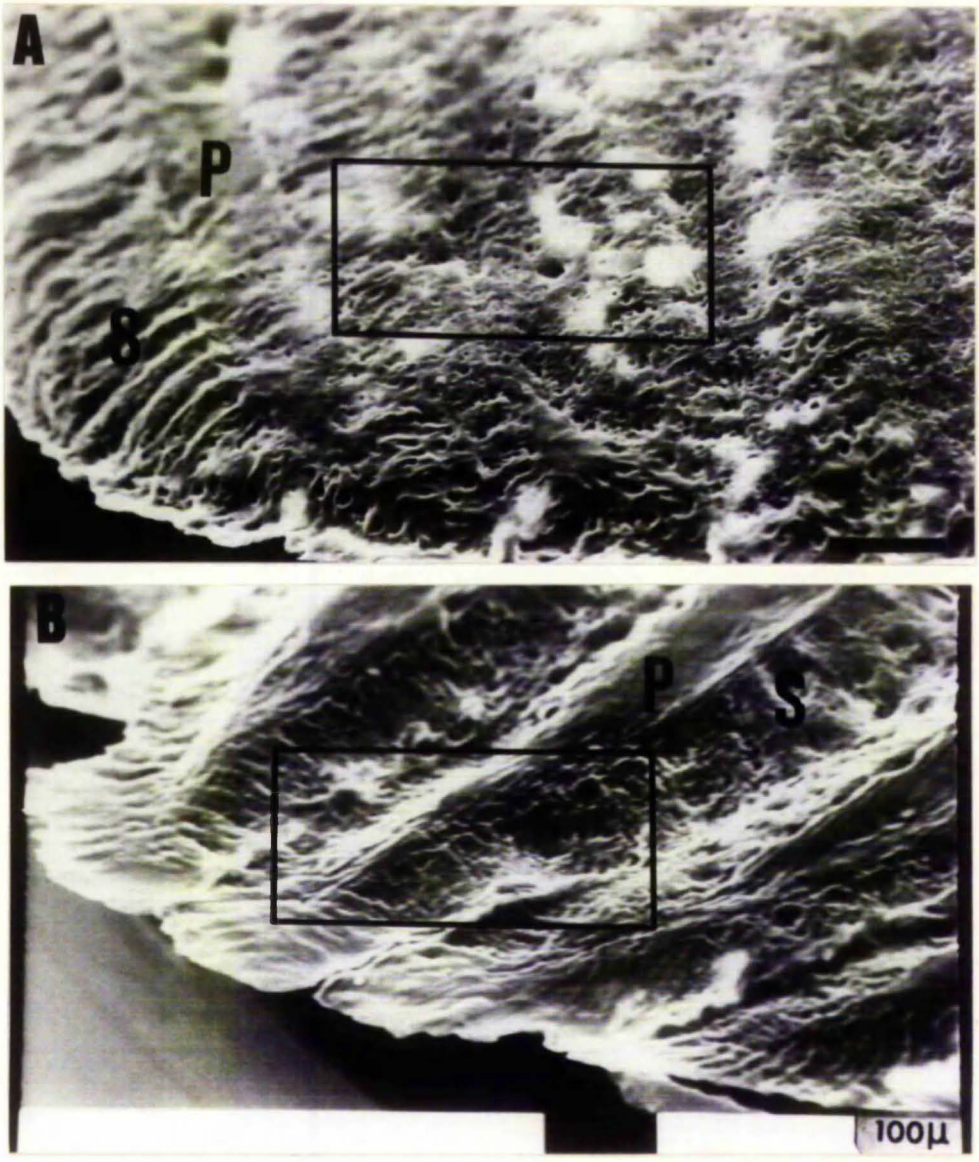
D. Soft water fish exposed to 0.86 mg Zn l^{-1} (Bar = 200 μm).

Freeze dried specimens were randomly chosen from ten fish in each treatment.

Primary lamella, P; secondary lamella, S.

Area studied with electron probe X-ray microanalysis.

X-ray probe scans of tissue composition are shown in A, B, C and D □. Element spectrum-line for zinc is marked.


A
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S
B
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S
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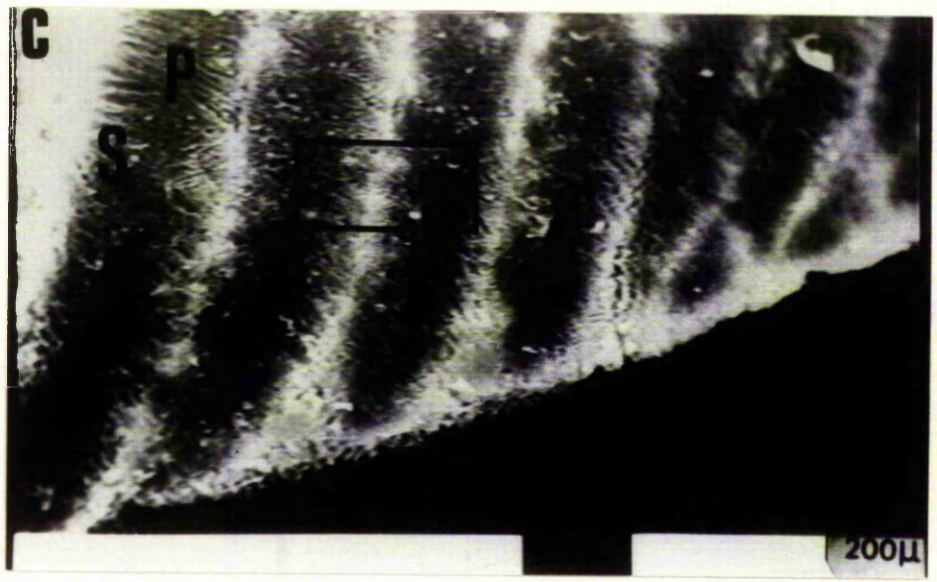
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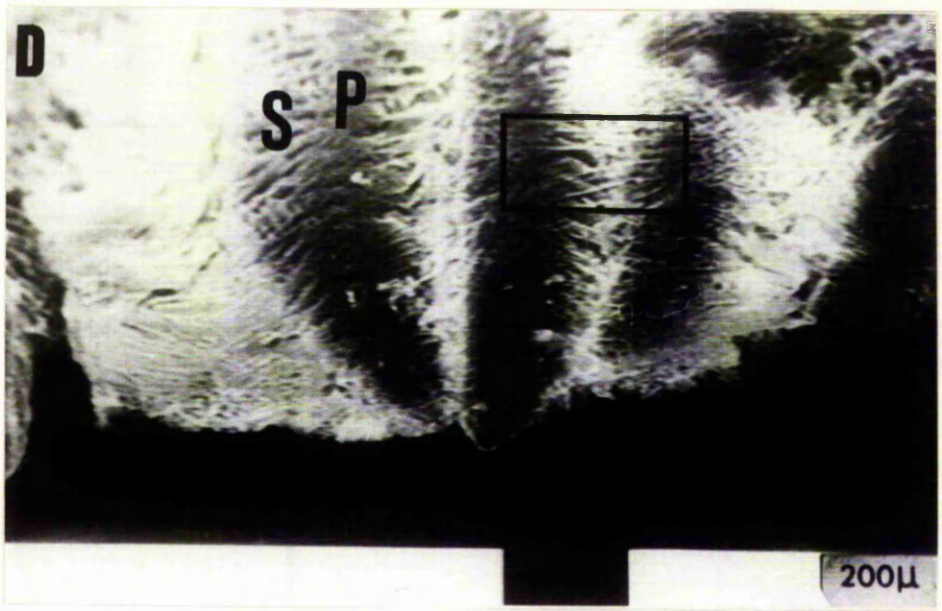
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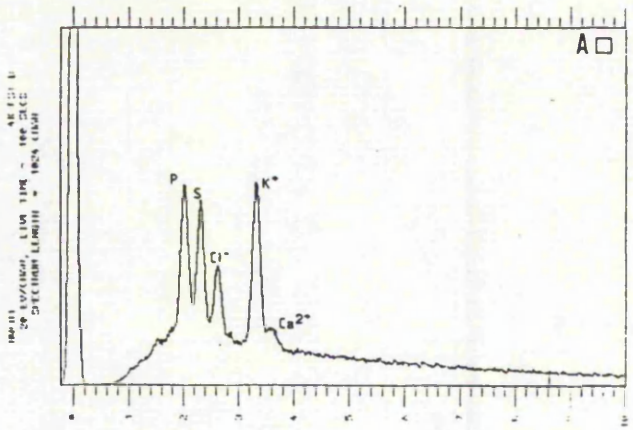
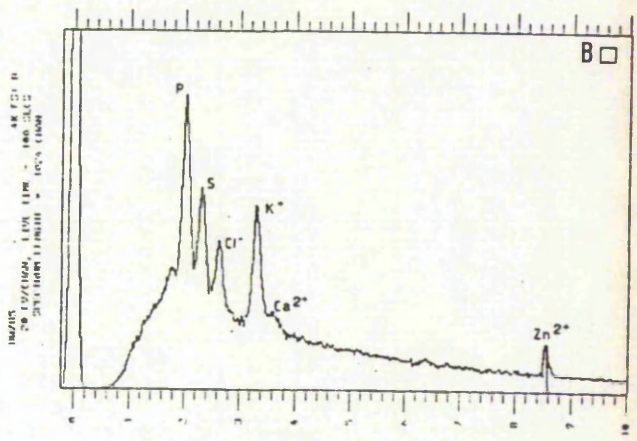
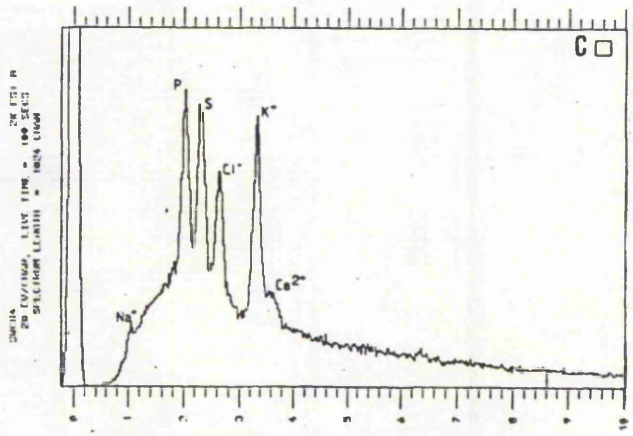


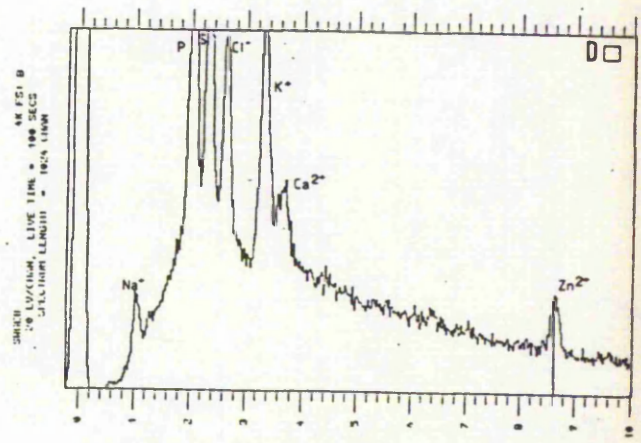
X-ray emission spectra



X-ray emission spectra



X-ray emission spectra



X-ray emission spectra

Figure 7 Scanning electron micrographs of primary and secondary gill lamellae from fish in hard and soft test waters

A. Hard water fish (Control, bar = 40 μm).

B. Hard water fish exposed to 0.84 mg Zn l^{-1} (Bar = 20 μm).

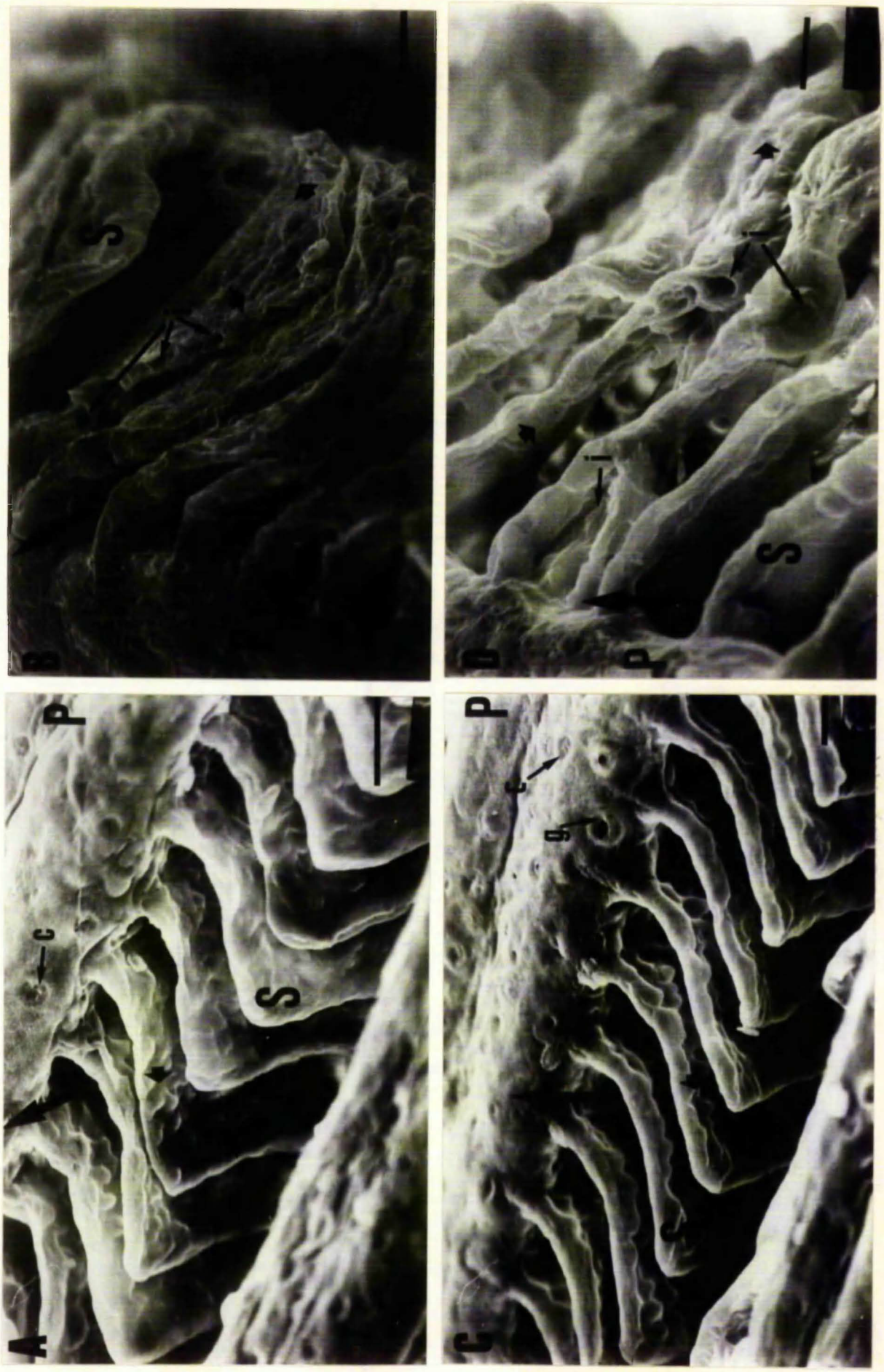
C. Soft water fish (Control, bar = 40 μm).

D. Soft water fish exposed to 0.86 mg Zn l^{-1} (Bar = 20 μm).

2.5% glutaraldehyde treated and air dried specimens are randomly chosen from eight fish in each treatment. Primary lamella (afferent side), P; secondary lamella, S; chloride cell, c; goblet cell, g; invagination, i.

Direction of water flow between secondary lamellae ➡

Direction of blood flow within secondary lamellae ➡


B
S
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P
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d

In both hard and soft water fish exposed to zinc, gross curling and collapse of the secondary lamellae had occurred with lesions and swelling of epithelial layers evident. These changes were more marked in zinc-exposed fish from soft waters than from hard waters. Arrows indicating the respiratory flow of water passing through the secondary lamellae in a counter-current exchange with the blood flow are shown for controls and zinc exposed fish. With disruption of secondary lamella structure and orientation in zinc exposed fish it was apparent that gas exchange might be impaired following metal exposure. Chloride cells and goblet cells were evident in control tissue but due to the disruption of gill epithelia from zinc exposure it proved impossible to identify these cells in metal exposed fish using this technique.

Histology of control and zinc exposed gills from hard and soft water fish is shown in Fig. 8. In controls the primary and secondary lamellae remained intact but many changes were evident in zinc-exposed fish. Epithelial damage in the gills of both hard and soft water fish was evident but more marked in soft water fish with lower external zinc exposures. Subepithelial spaces developed particularly on the concave side of curled secondary lanellae and the spaces extended to the endothelial lining of the central venous sinuses. Mucosal layers of the secondary lamellae were thus separated from the

Figure 8 Tranverse sections of secondary lamellae from fish in hard and soft test waters.

A. Hard water fish (Control, bar = 50 μm).

B. Hard water fish exposed to 3.66 mg Zn l^{-1} (Bar = 50 μm).

C. Soft water fish exposed to 1.65 mg Zn l^{-1} (Bar = 50 μm).

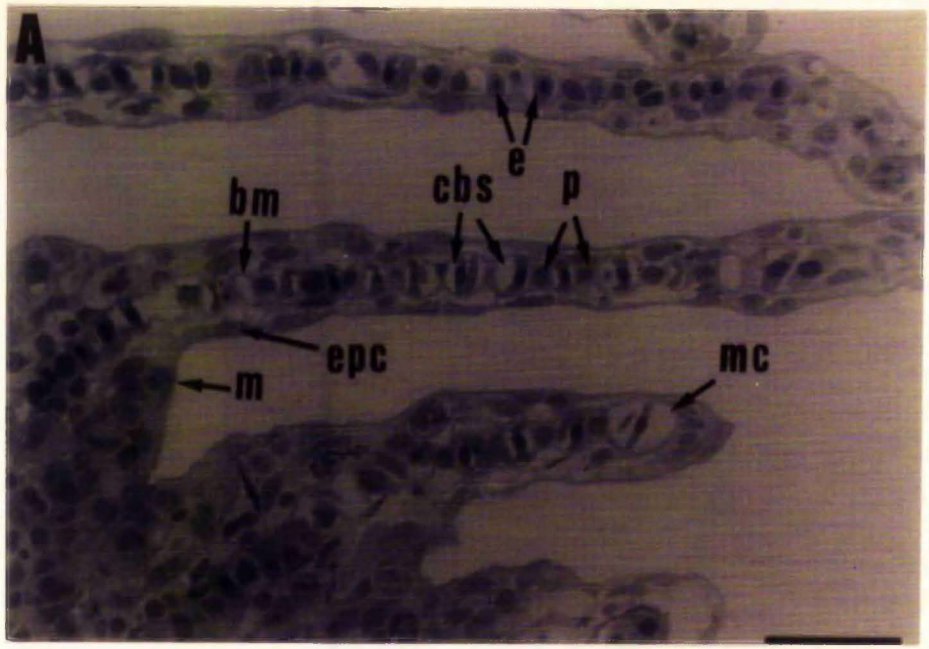
D. Hard water fish surviving an exposure to 0.86 mg Zn l^{-1} (Bar = 50 μm).

E. Soft water fish exposed to 0.70mg Zn l^{-1} (Bar = 10 μm).

F. Hard water fish 3 months after exposure to 0.86 mg Zn l^{-1} (H and E, bar = 100 μm).

Resin specimens (A-E) were randomly chosen from eight fish in each treatment. Stained in toluidene blue.

Basement membrane, bm; chloride cell, c; central blood space, cbs; central venous sinus, cvs; erythrocyte, e; epithelial pavement cell, epc; granulocyte, g; lymphocyte, l; mucous cell, m; marginal channel, mc; marginal endothelial cell, me; pillar cell, p; proximal channel, pc; subepithelial space, s; stretched interlamellar cell, si.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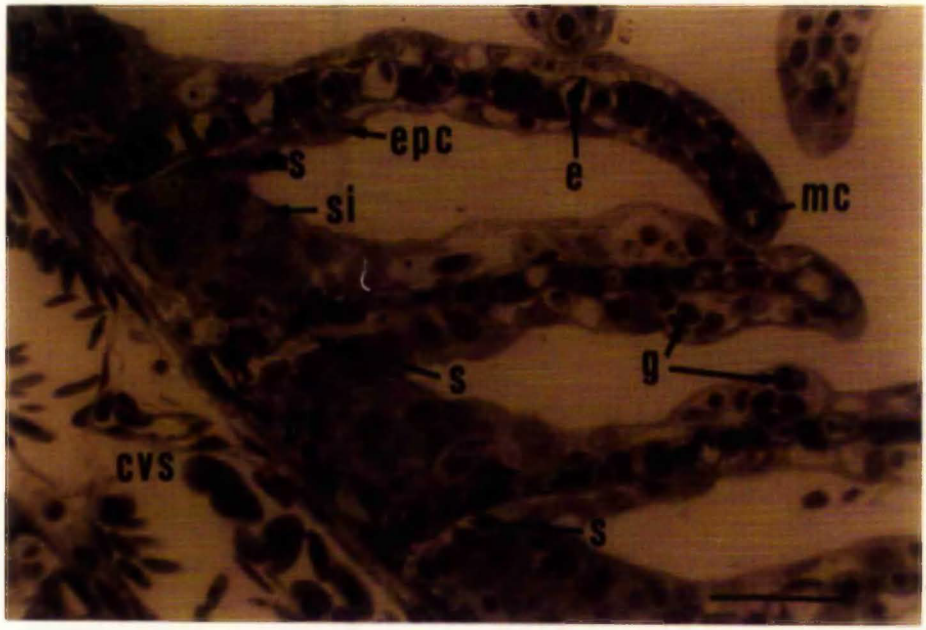
epc
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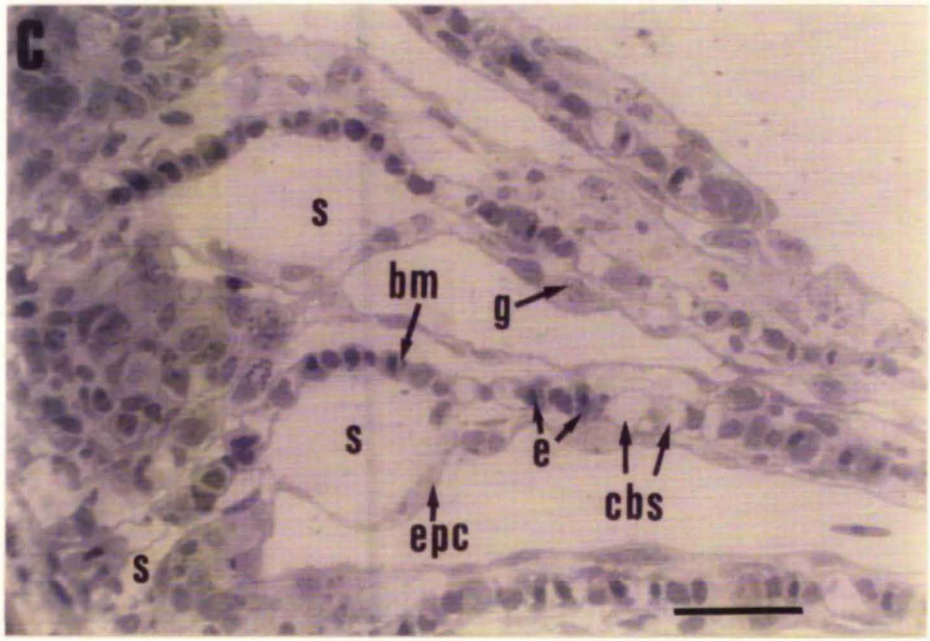
cvs
s


C
s
bm
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cbs
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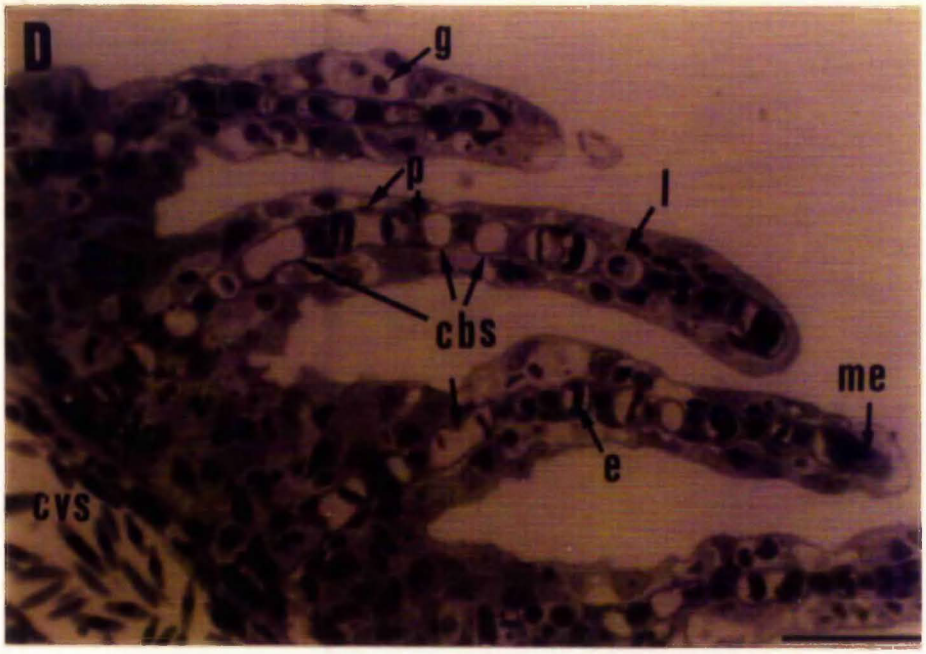
D
g
p
l
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me
e
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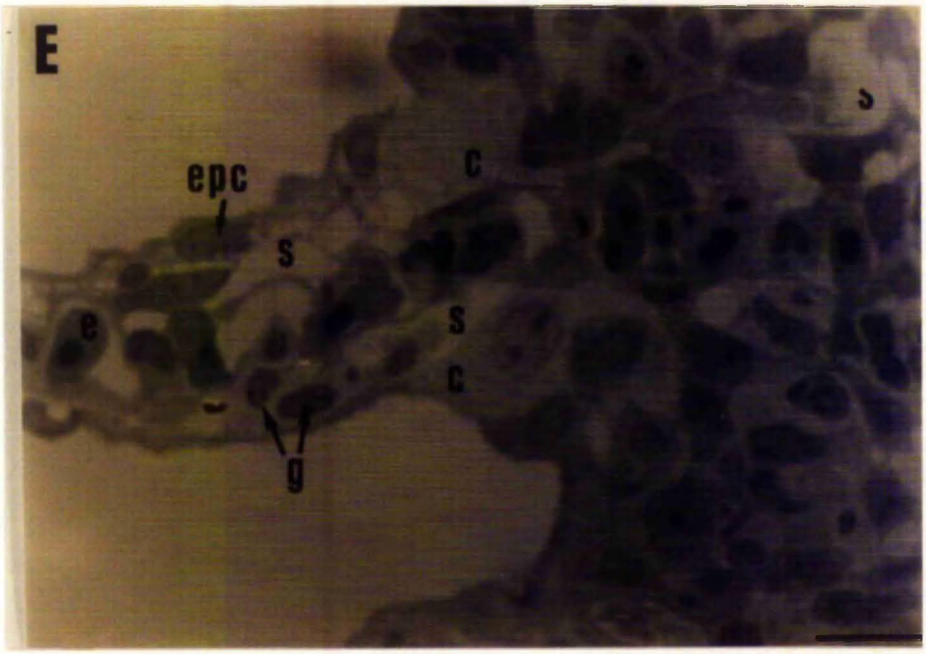
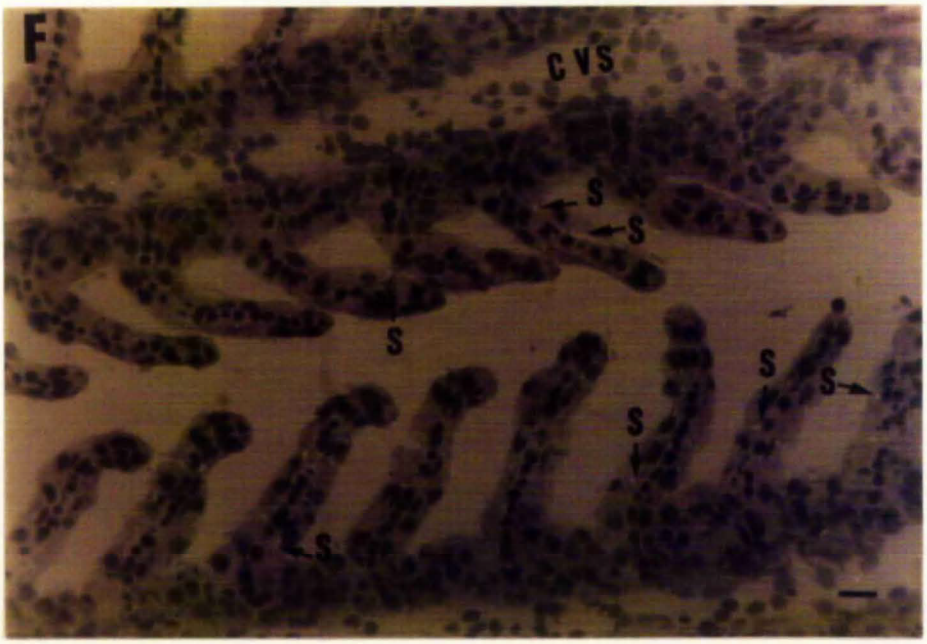

E
epc
c
s
s
e
c
g


F
cvs
s
s
s
s
s
s
s

basement membrane of the pillar cell system. Subepithelial spaces became filled with granulocytes and cell debris typical of an inflammatory response. There was also stretching of interlamellar cells, an absence of erythrocytes from central blood spaces and many pillar cells had become dislodged. In Fig. 8 E chloride cells were disrupted by the presence of subepithelial spaces thus disturbing the integrity of the outer mucosal layer.

The secondary lamellae of hard water fish recovering from zinc exposure (Fig. 8 F) gave indications of tissue repair. The subepithelial spaces caused by zinc exposure were much reduced in size and appeared to be filled by a lymph-like fluid. Replacement of damaged cells was not evident at this stage and the curling of the pillar cell system was still apparent.

3.3.2.2. Blood Cells

Zinc entering the bloodstream of fish in hard and soft waters following metal exposure produced a variety of effects on blood cells. Unstained blood smears (Fig. 9 A and B) showed distinct particles attached to the erythrocytes of zinc-exposed fish. These particles were consistently stained with the zinc-specific dithizone stain (Fig. 9 D and F). Particles attached to erythrocytes of metal-exposed fish appeared to be rich in zinc but it was not determined if these particles

Figure 9 Blood smears showing the effects of zinc exposure upon the eyrthrocyte and leucocyte of test fish.

A. Hard water fish (Control).

B. Hard water fish exposed to 1.68 mg Zn l^{-1}.

C. Soft water fish (Control).

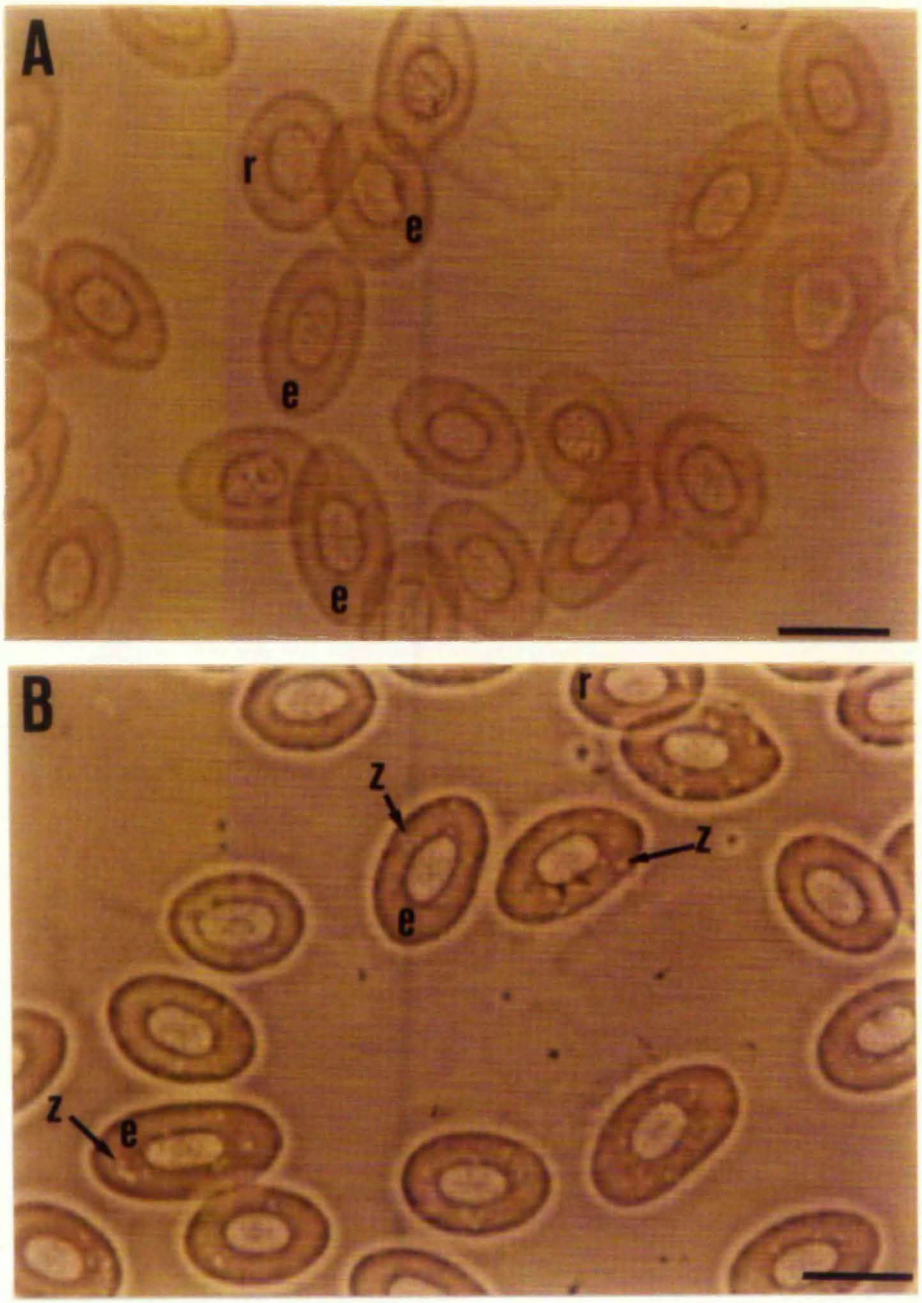
D. Soft water fish exposed to 1.65 mg Zn l^{-1}.

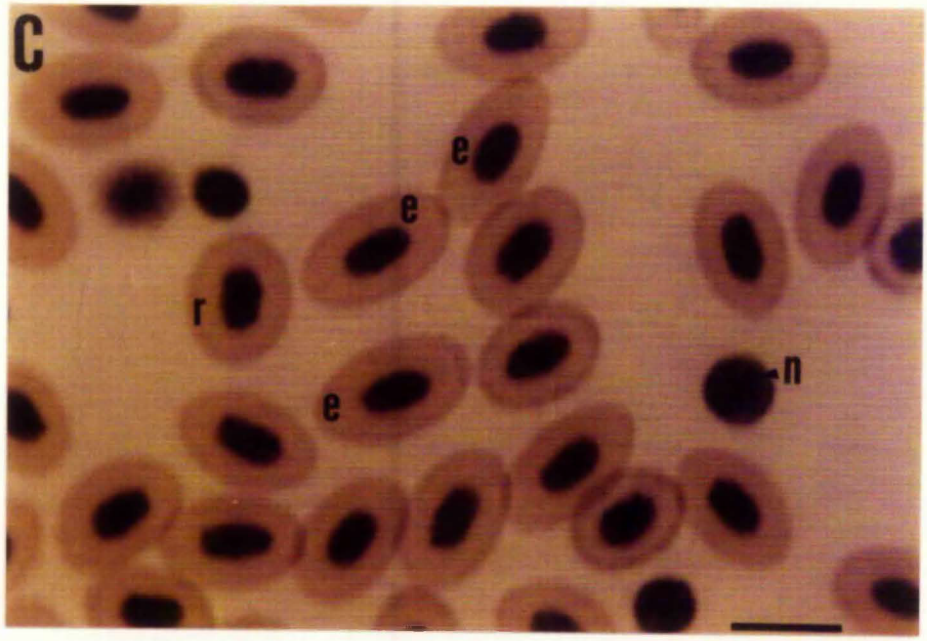
A-B No stain viewed under phase contrast

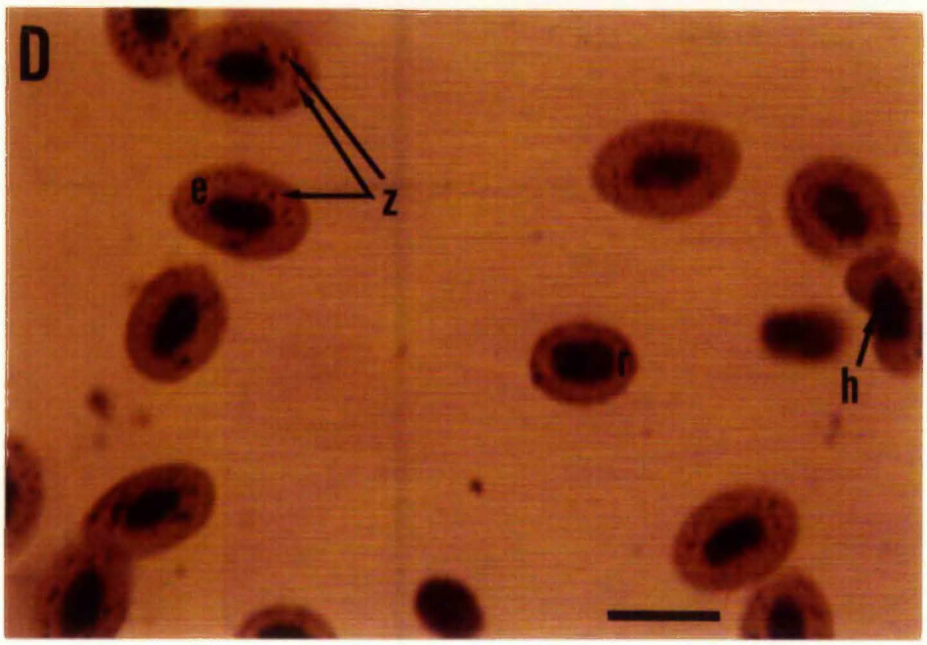
C-D Leishmans and dithizone stained.

Blood smears were randomly chosen from 42 per treatment group. All bars = 10 μm.

Erythrocyte, e; haemolysis, h; neutrophil, n; reticulocyte, r; zinc particle, z.


A
r
e
e
e
B
r
z
z
e
z
e


C
e
e
r
e
n


D
e
z
r
h

became attached during blood smear preparations. Haemolysis of erythrocytes was observed in blood smears from both groups of zinc-exposed fish. Fig. 10 shows a lymphocyte from a non zinc-exposed fish (A) and lymphocytes from zinc-exposed fish (B, C and D). Lymphocytes from the zinc-exposed fish were frequently observed phagocytising the haemolysed erythrocytes which is characteristic of a secondary inflammatory response. Such active states in zinc-exposed fish were indicated by the protrusion of leaf-like pseudopods from the lymphocytes.

Zinc exposure caused significant ($P < 0.001$) dose-dependent increases in the haematocrit of both hard and soft water fish (Fig. 11). A lower range of zinc exposures to soft water fish (> 0.31 mg Zn l^{-1}) produced comparable elevations in haematocrit with higher metal concentrations (> 6.75 mg Zn l^{-1}) administered to hard water fish. Many of the remaining plasma samples taken from both groups of zinc contaminated fish were red in colour indicating increased erythrocyte fragility.

3.3.2.3. Plasma

Levels of the major plasma electrolytes in control and zinc exposed fish from hard and soft water are shown in Fig. 12. Effects of zinc exposure on plasma sodium levels were less clear than for plasma chloride. In

Figure 10 Blood smears showing the inflammatory responses of lymphocytes to zinc exposure.

A. Hard water fish (Control).

B. Hard water fish exposed to 1.68 mg Zn l^{-1}.

C. Hard water fish exposed to 3.66 mg Zn l^{-1}.

D. Soft water fish exposed to 0.70 mg Zn l^{-1}.

Leishman stained blood smears were randomly chosen from eight fish in each treatment. Bars = 10 μm.

Erythrocyte (haemolysed), e; lymphocyte, l; neutrophil, n; perinuclear zone, p; pseudopod, ps.

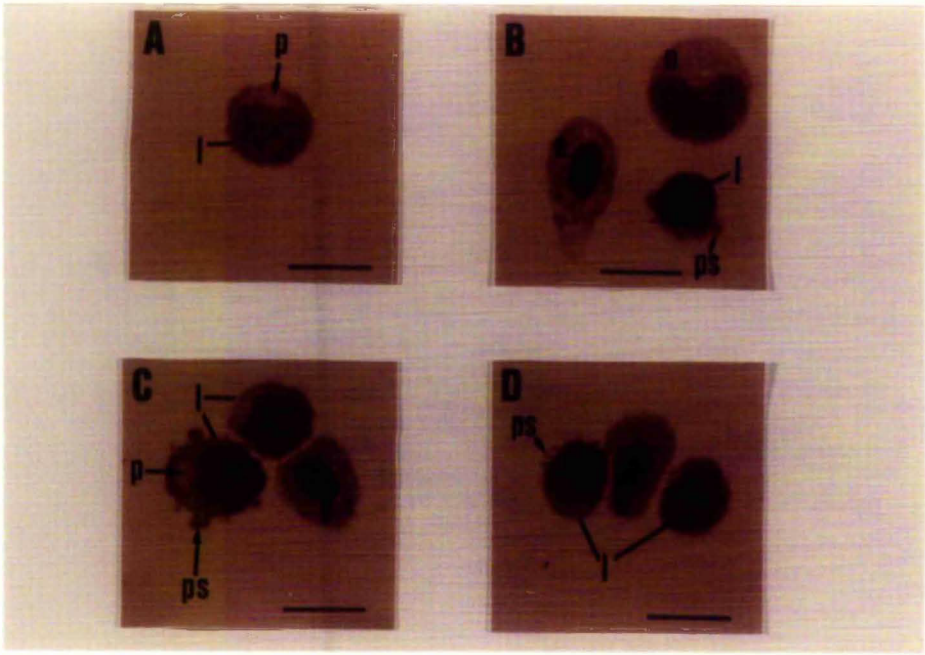

A
p
l
B
l
ps
C
l
p
ps
D
ps
l

Figure 11 Haematocrit (%) from overturned fish exposed to a range of zinc concentrations (0.10 - 12.55 mg Zn l^{-1}) in test waters at pH8. Each column represents the mean value ($n \geq 10$) and vertical bars represent the standard error. Significant differences from the control group (c) are indicated at the $P < 0.05$ (*), < 0.01 (**) and < 0.001 (***) level. (Students t-test).

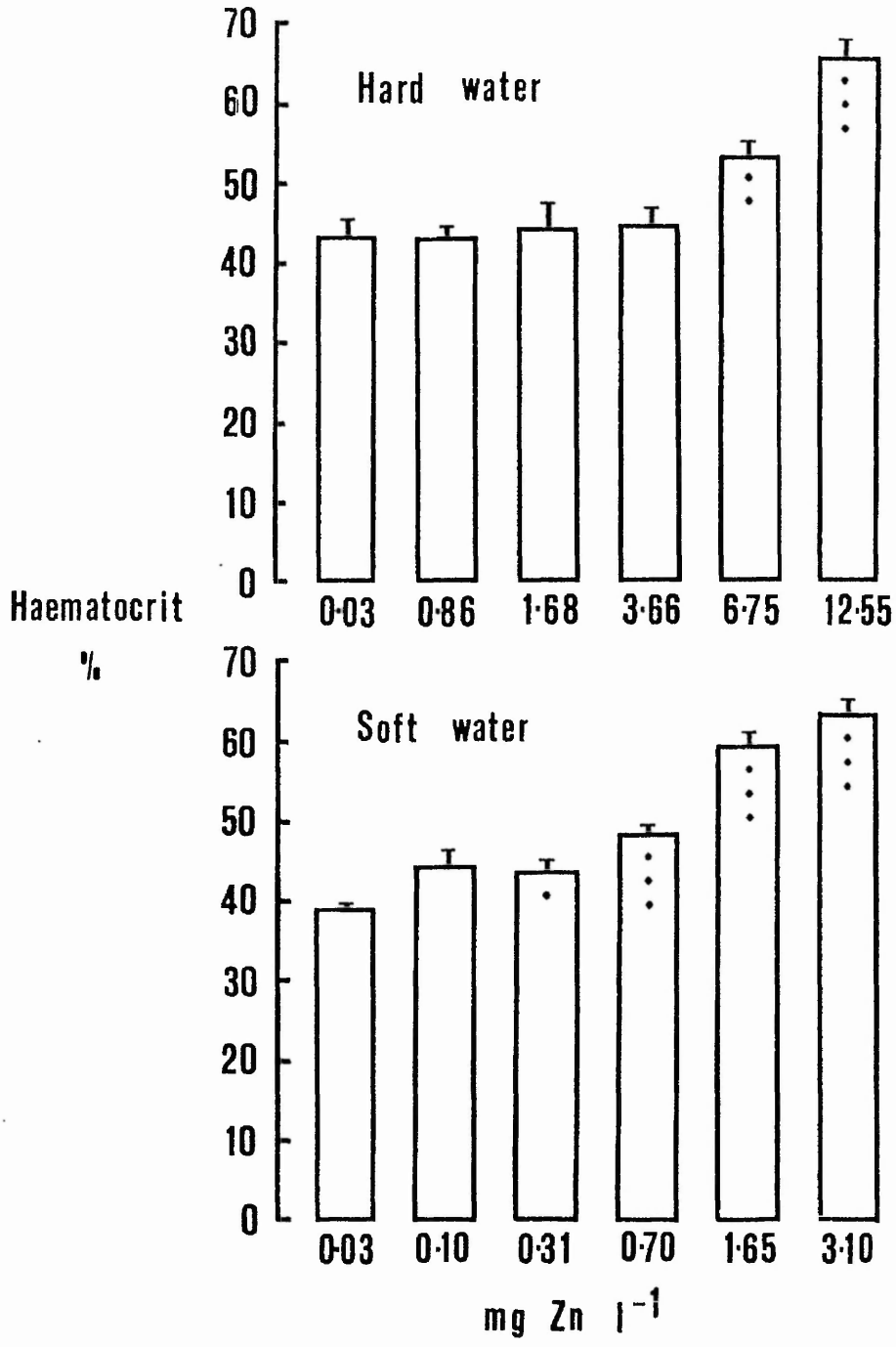
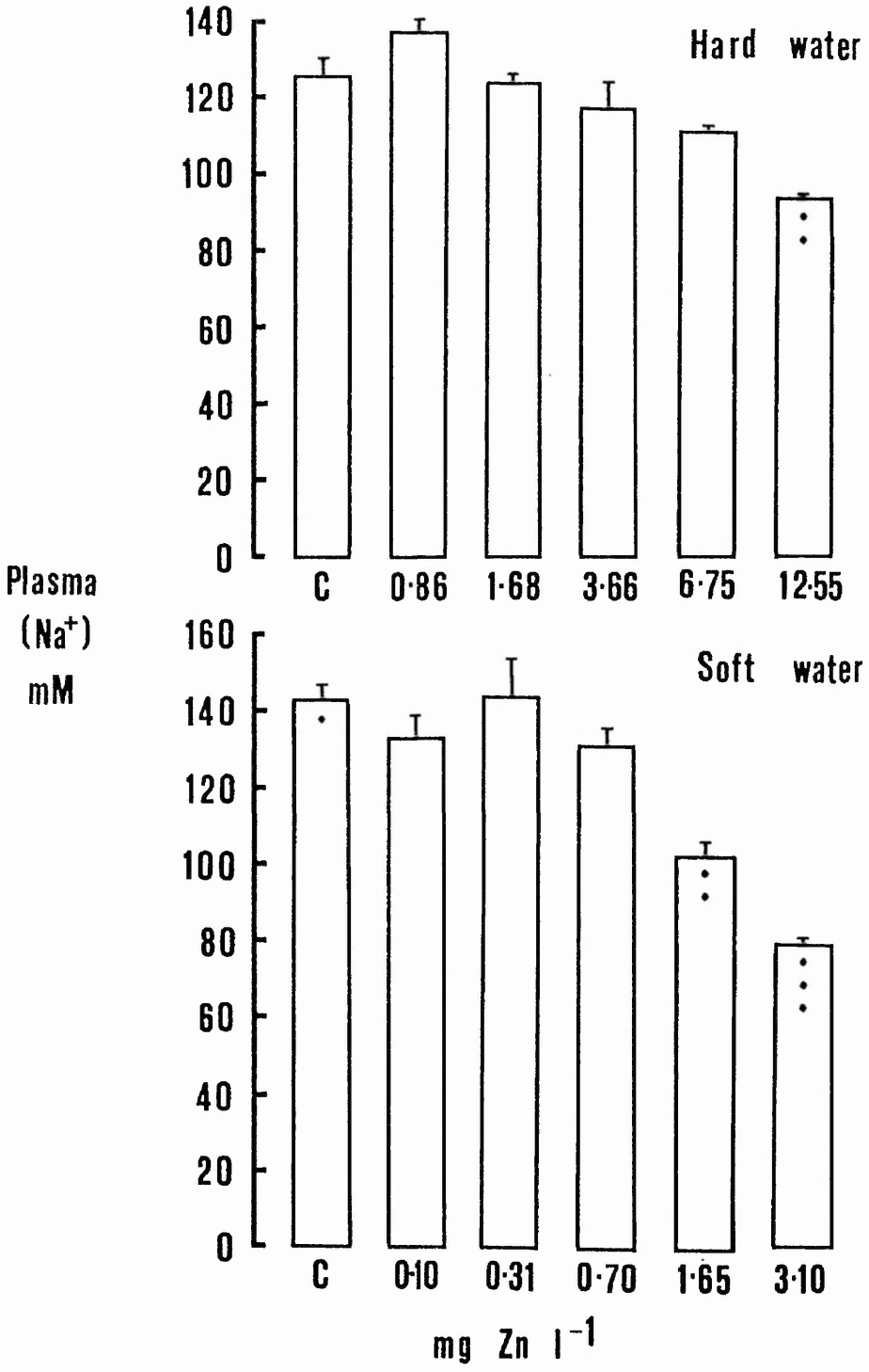
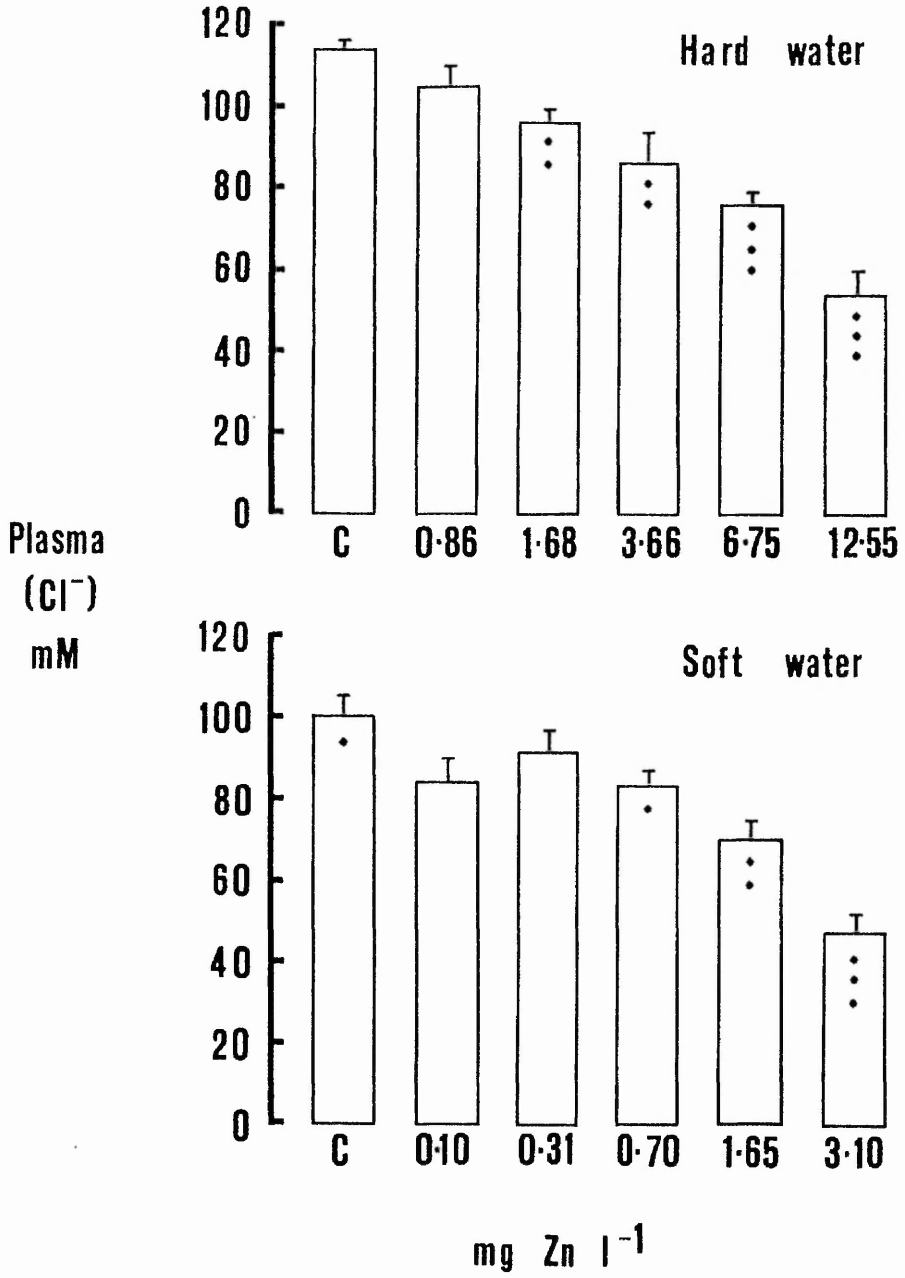

Hard water
Soft water
Haematocrit
%
70
60
50
40
30
20
10
0
0·03
0·86
1·68
3·66
6·75
12·55
0·03
0·10
0·31
0·70
1·65
3·10
mg Zn l⁻¹

Figure 12 Plasma sodium (Na^+) and chloride (Cl^-) from overturned fish exposed to a range of zinc concentrations (0.10 - 12.55mg Zn l^{-1}) in test waters at pH8. Each column represents the mean value ($n \leq 10$) and vertical bars the standard error. Significant differences between test groups are indicated at the $P < 0.05$ (*), < 0.01 (**) and < 0.001 (***) level. (Students t-test).


Hard water
Soft water
Plasma
(Na+)
mM
140
120
100
80
60
40
20
0
160
C
0·86
1·68
3·66
6·75
12·55
0·10
0·31
0·70
1·65
3·10
mg Zn l-1


Hard water
Soft water
Plasma
(Cl⁻)
mM
120
100
80
60
40
20
0
C
0·86
1·68
3·66
6·75
12·55
C
0·10
0·31
0·70
1·65
3·10
mg Zn l⁻¹

control fish the plasma sodium levels were significantly ($P < 0.05$) elevated in soft waters compared with hard waters. However, in zinc-exposed fish there was a significant ($P < 0.001$) decrease in plasma sodium levels in soft water at > 1.65 mg Zn l^{-1}. Plasma chloride levels in control fish were significantly ($P < 0.05$) depressed in soft waters compared with hard water fish and zinc exposed fish showed significant ($P < 0.001$) dose-dependent decreases in both hard and soft waters. Soft water fish exposed to > 0.70 mg Zn l^{-1} showed similar depressions in plasma electrolyte levels to hard water fish exposed to > 1.68 mg Zn l^{-1}.

3.3.2.4 Spleen

Spleen sections from control and zinc-exposed fish from hard and soft waters are shown in Fig. 13. Unstained sections A and B indicated elevated concentrations of melano-macrophages (Roberts, 1974) from zinc-exposed spleen and dithizone-stained material showed a specific positive staining of these structures. Increased levels of melano-macrophages were apparent in fish exposed to zinc in both hard and soft waters (C, D, E and F) but no significant effect of external hardness was apparent (Table 11).

Melano-macrophages were found in the axils of ellipsoid branches and observed bulging into the lumen of veins as spherical aggregates of melanin-containing

Figure 13 Transverse sections of spleen from control and zinc-exposed fish in hard and soft water

A. Hard water fish (Control).

B. Hard water fish exposed to 1.68 mg Zn l^{-1}.

C. Hard water fish (Control).

D. Hard water fish exposed to 1.68 mg Zn l^{-1}.

E. Soft water fish (Control).

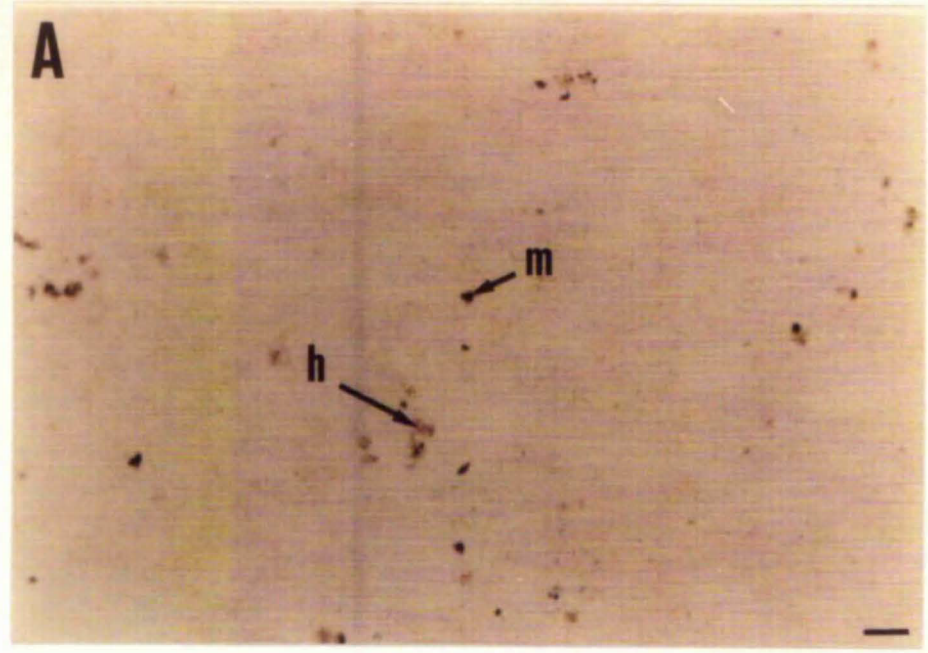
F. Soft water fish exposed to 1.65 mg Zn l^{-1}.

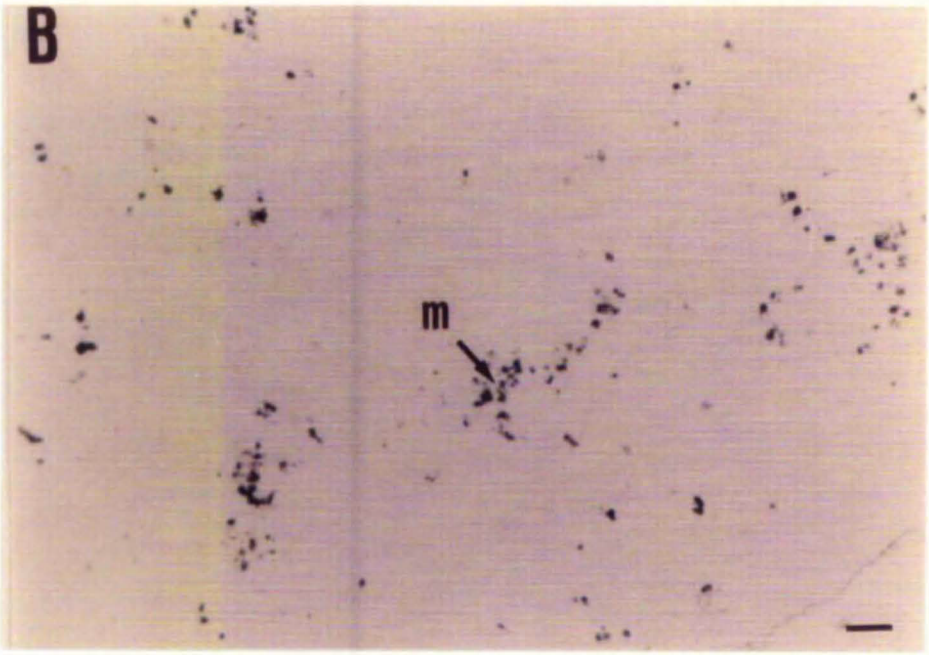
G. Melano-macrophage aggregates from D.

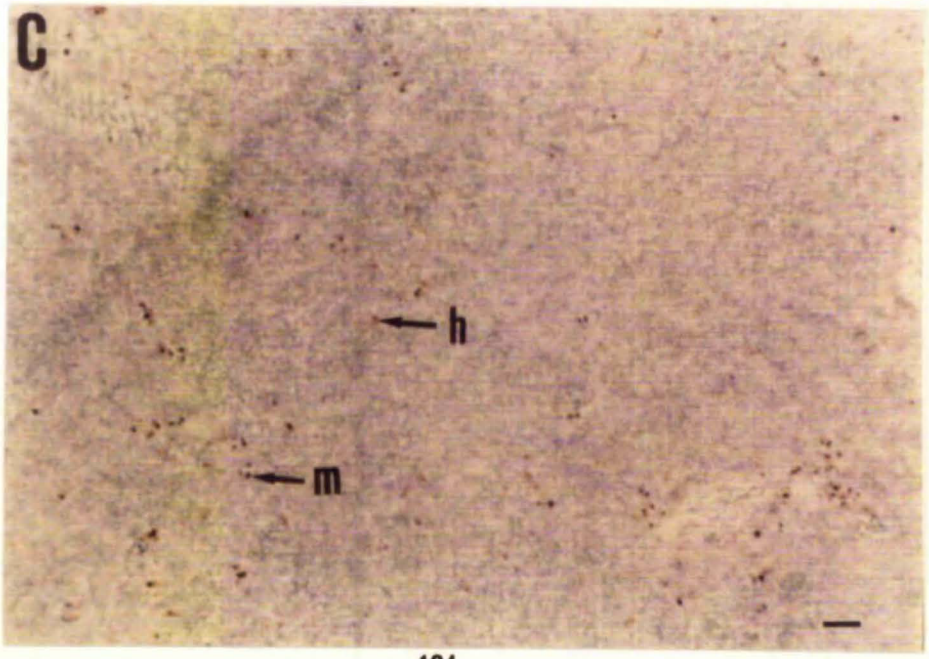
H. Melano-macrophage aggregates from F.

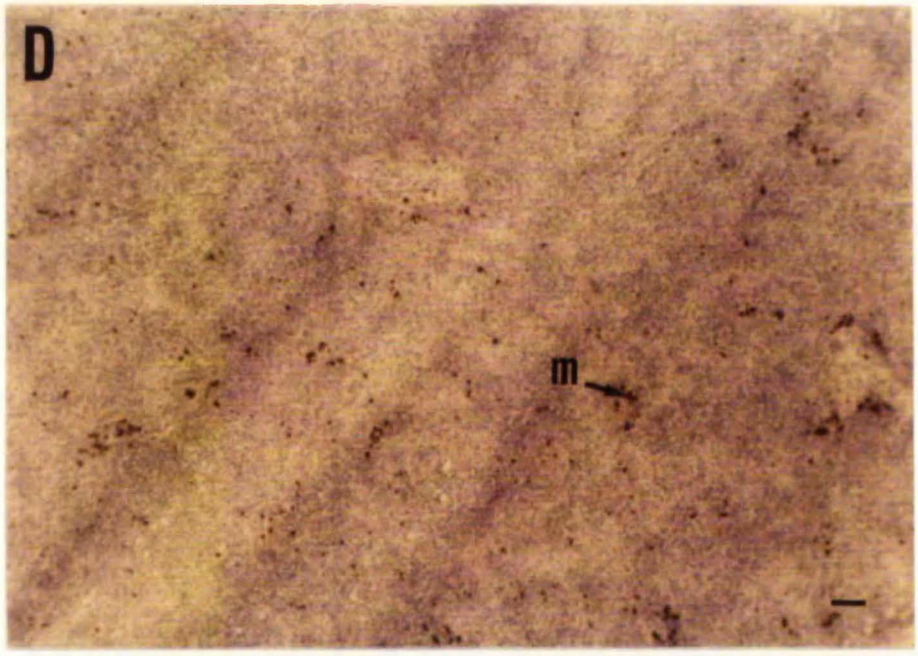
A-B are unstained and C-H are stained with HE and dithizone. Spleen sections were randomly chosen from ten fish in each treatment. Bars = 100 μm

Capillary, c; haemosidern deposits, h; melano-macrophage aggegates, m; vein, v.


A
m
h

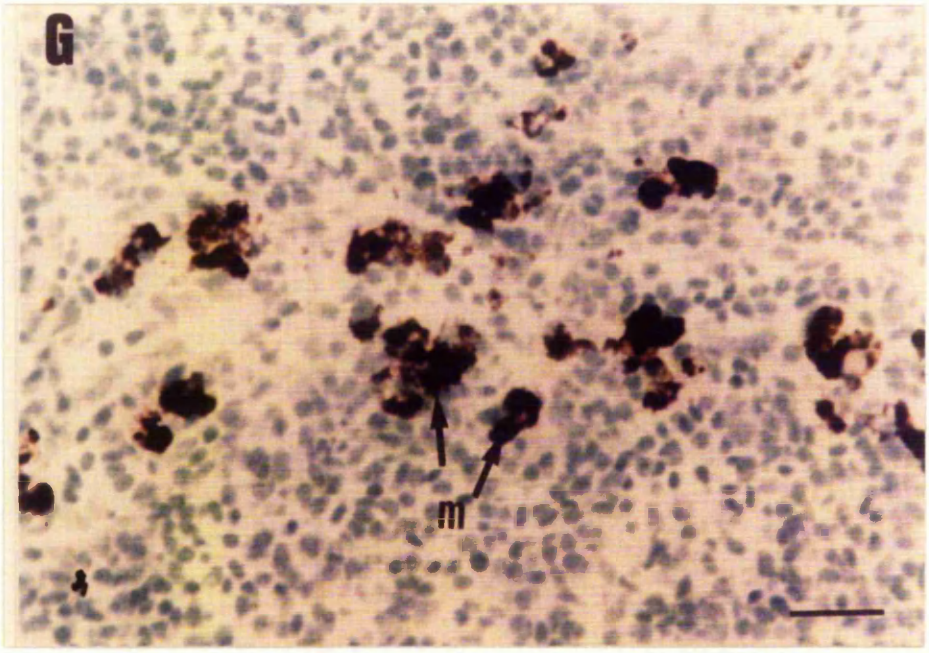

B
m


C
h
m


D
m


E
m
h


F
m


G
m

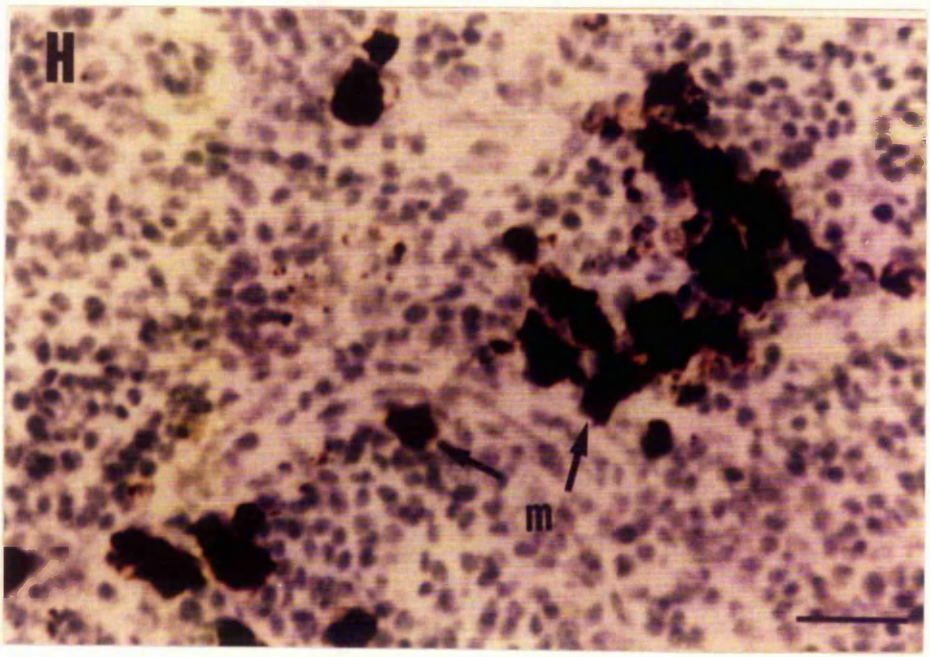

H
m

Table 11 The concentration of melano-macrophage aggregates in the spleen of control and zinc-exposed fish from hard and soft waters.

Treatment	Area (μm^2) melano-macrophages (mag.x400) (mean ± SE of 10 sections, from fish sample size shown in parentheses)	
Hard water (Control)	806.9 ± 186.5 (10)	
		***
Hard water ($0.86 mg\ Zn\ l^{-1}$)	3076.4 ± 358.0 (9)	
Soft water (Control)	1150.2 ± 172.5 (10)	
		**
Soft water ($0.70\ mg\ Zn l^{-1}$)	2980.7 ± 625.6 (9)	

Students t-test ** $P < 0.01$ *** $P < 0.001$

cells, between5and7μm in diameter. These are shown in Fig 13 G and H where melano-macrophage aggregates have an enhanced appearance as a result of specific staining for zinc. In both control and metal-exposed sections of spleen, granules stained yellowish-brown by H and E were observed. These granules are produced after the breakdown of senescent erythrocytes and are known as haemosiderin deposits (Hibiya, 1981).

Results of further studies to determine the distribution and specific sites of zinc accumulation in spleen tissue are shown in Fig. 14. Section A shows the general appearance of spleen tissue from a scanning electron micrograph. Surface details of freeze-fractured sections revealed the presence of melano-macrophage aggregates. Differences in zinc content between splenic tissue and the melano-macrophage aggregates were examined from the element scans of control and metal-exposed spleen using X-ray electron probe microanalysis (Fig. 14 B ,C , D and E). The elemental compositions of melano-macrophages aggregates were different from those of background tissue and noted for being substantially rich in sulphur (Fig. 14 scans B● to E● Vs Bo to Eo). Similarly, zinc was found in melano-macrophage aggregates from spleen sections of zinc-exposed fish but not from controls (Fig 14, C●vs B● and E●vs D●). Smaller levels of zinc were also detected in the connective pulp of spleen tissue from

Figure 14 Scanning electron micrographs and tissue analyses of freeze fractured spleen sections from test trout in hard and soft water.

A. Appearance of melano-macrophage (m) aggregates (Bar = 400 μm)

B. Hard water (Control fish, bar = 40 μm).

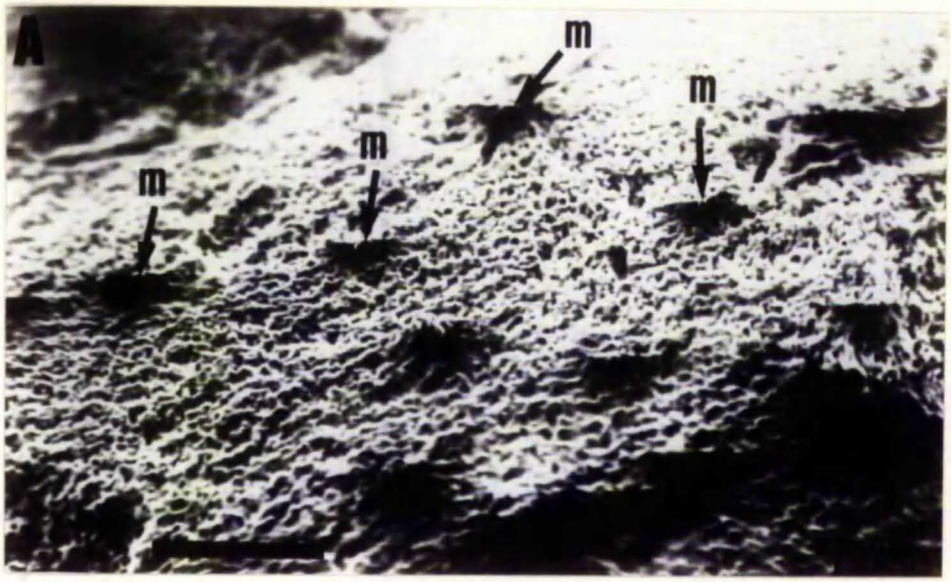
C. Hard water fish exposed to 1.68 mg Zn l^{-1}. (Bar = 40 μm).

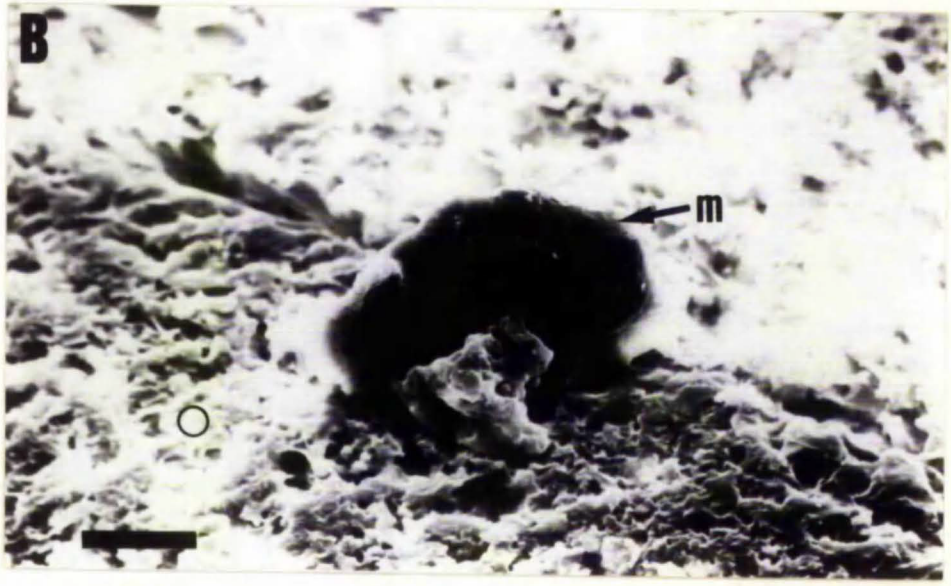
D. Soft water (Control fish, bar = 40 μm).

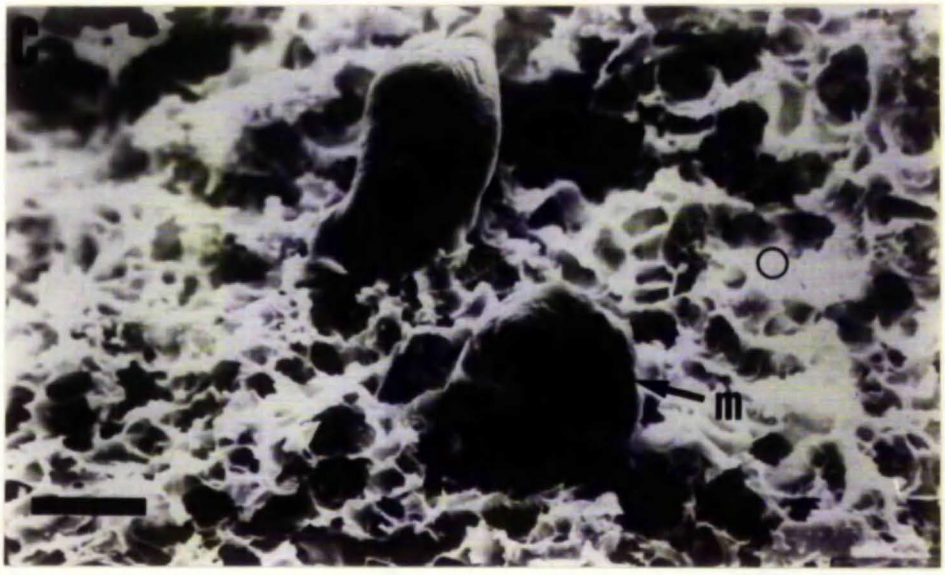
E. Soft water fish exposed to 1.65 mg Zn l^{-1}. (Bar = 20 μm).

Spleen sections were randomly chosen from eight fish in each treatment.

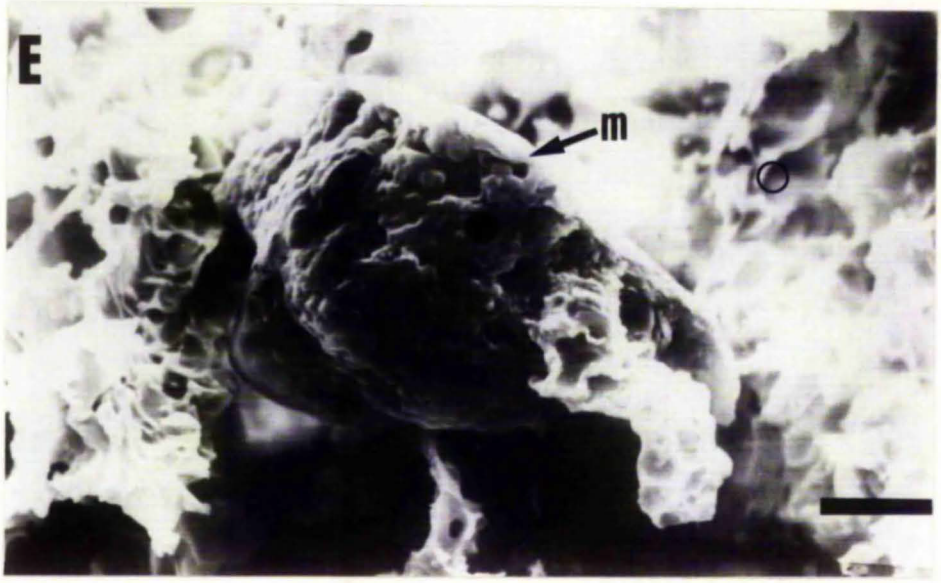
X-ray electron probe, spot (1μm^2) microanalysis ● O.

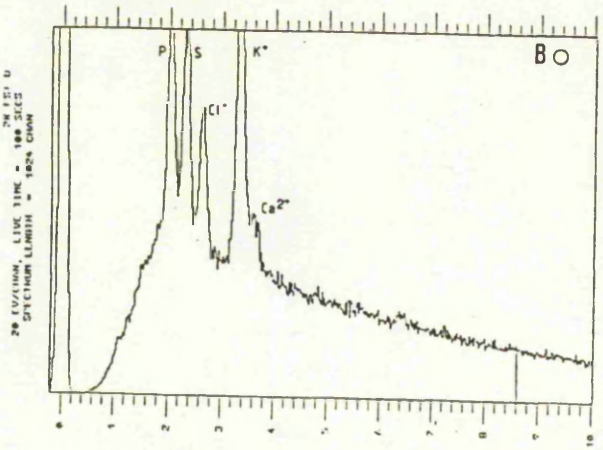

A
m
m
m
m


B
m

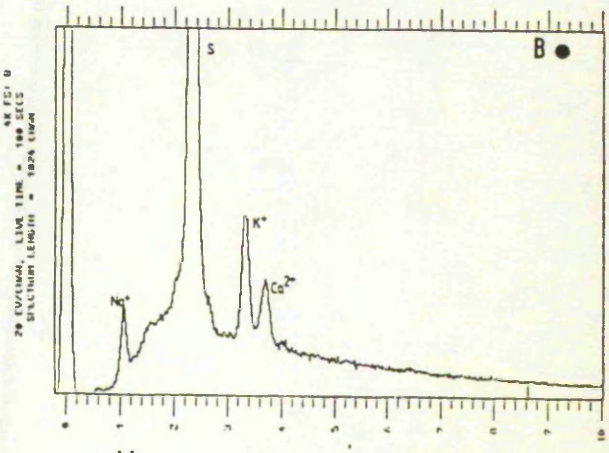

C
m


D
m

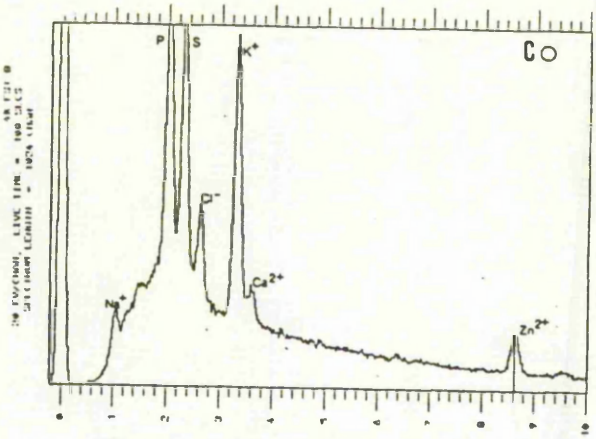

E
m



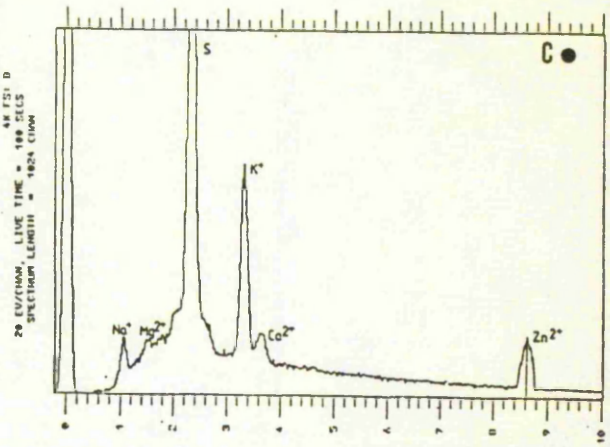
X-ray emission spectra



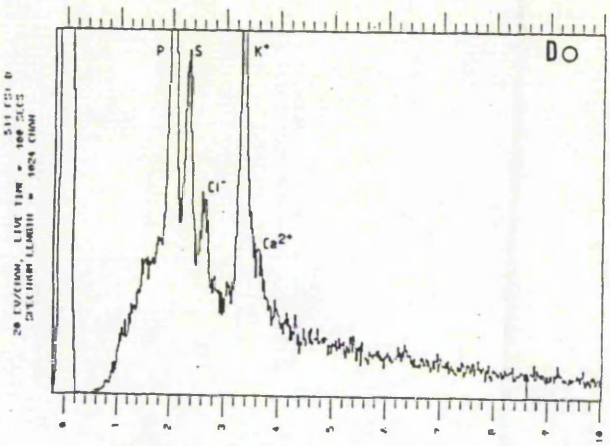
X-ray emission spectra



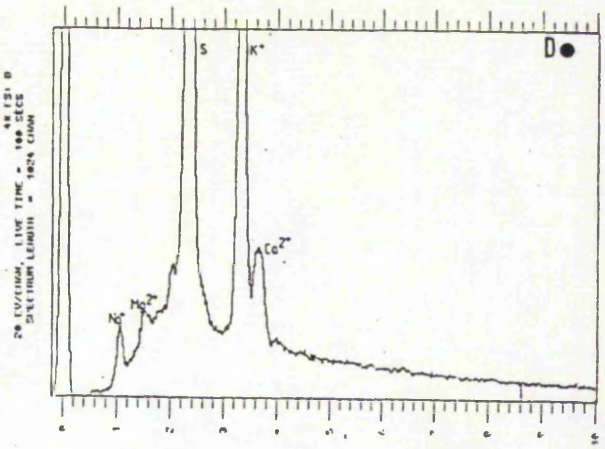
X-ray emission spectra



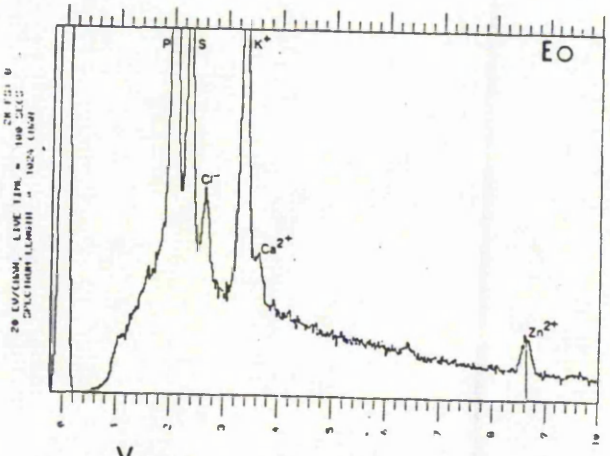
X-ray emission spectra



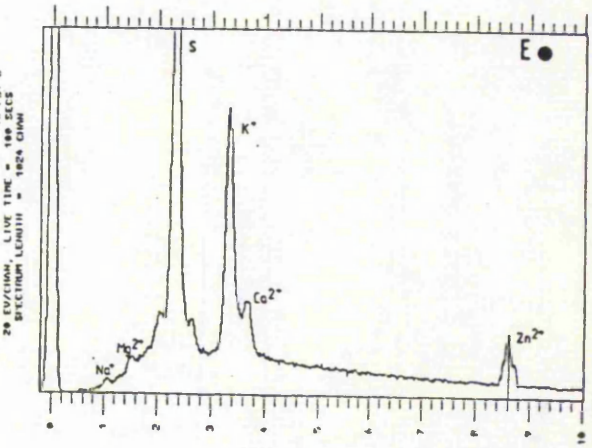
X-ray emission spectra



X-ray emission spectra



X-ray emission spectra



X-ray emission spectra

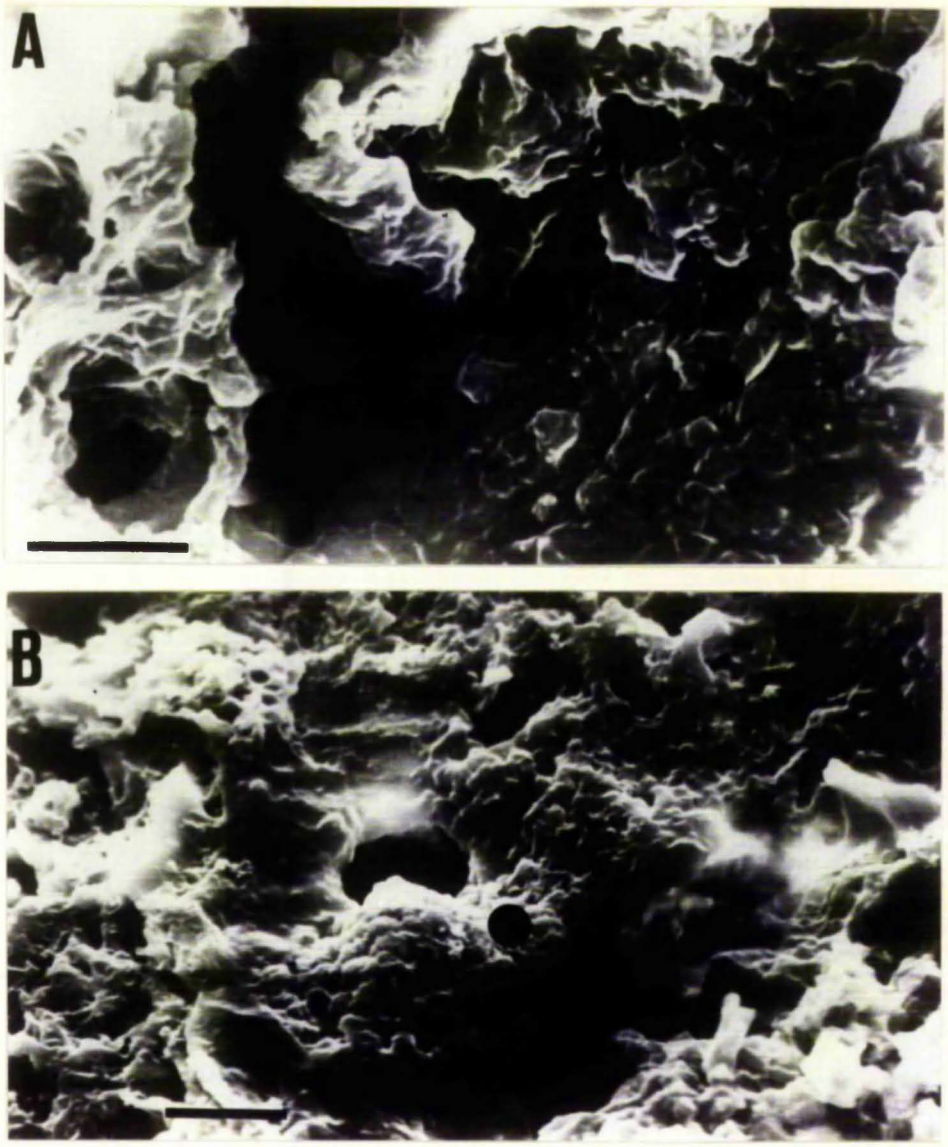
fish exposed to zinc but not for controls (Fig. 14 scan Co vs Bo and Eo vs Do).

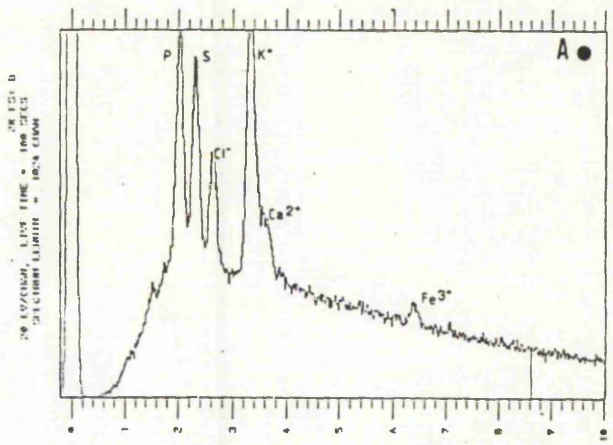
Analysis of haemosiderin deposits from spleens of control and zinc-exposed fish indicated the presence of iron deposits. These analyses are shown in Fig. 15 and also indicate the presence of zinc in haemosiderin from the spleen of metal-exposed fish.

3.3.2.5 The liver

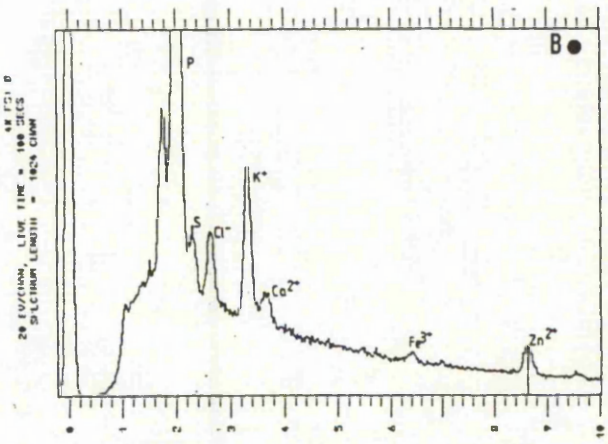
Liver sections from control and zinc-exposed fish are shown in Fig. 16. In liver from control fish (A and C) bile canaliculi are identified and hepatic cells appeared with a roundish polygonal body containing a spherical nucleus with usually one nucleolus. Melano-macrophage aggregates were present in the connective tissue of liver from control fish but noticeably deposited around the sinusoids and bile caniliculi in liver from zinc-exposed individuals (B and D). There was no evidence of increased production of melano-macrophages in response to the metal as observed for the spleen. Indeed, melano-macrophage aggregates were thinly distributed in liver sections of fish from all treatments. In zinc-exposed fish atrophy and hyperemia of the liver was evident from the development of intracellular vacuoles and blood congestion. Tissue analyses (E and F) revealed that zinc was present in the hepatic cells surrounding the bile ducts and

Figure 15 Scanning electron micrographs and tissue analyses of spleen haemosidern deposits from control (A) and zinc exposed (0.86 mg l^{-1}) fish (B) in hard water.


A
B



X-ray emission spectra



X-ray emission spectra

Figure 16 Tranverse sections of liver from control and zinc-exposed fish in hard and soft water.

A. Hard water fish (Control).

B. Hard water fish exposed to 1.68 mg Zn l^{-1}.

C. Soft water fish (Control).

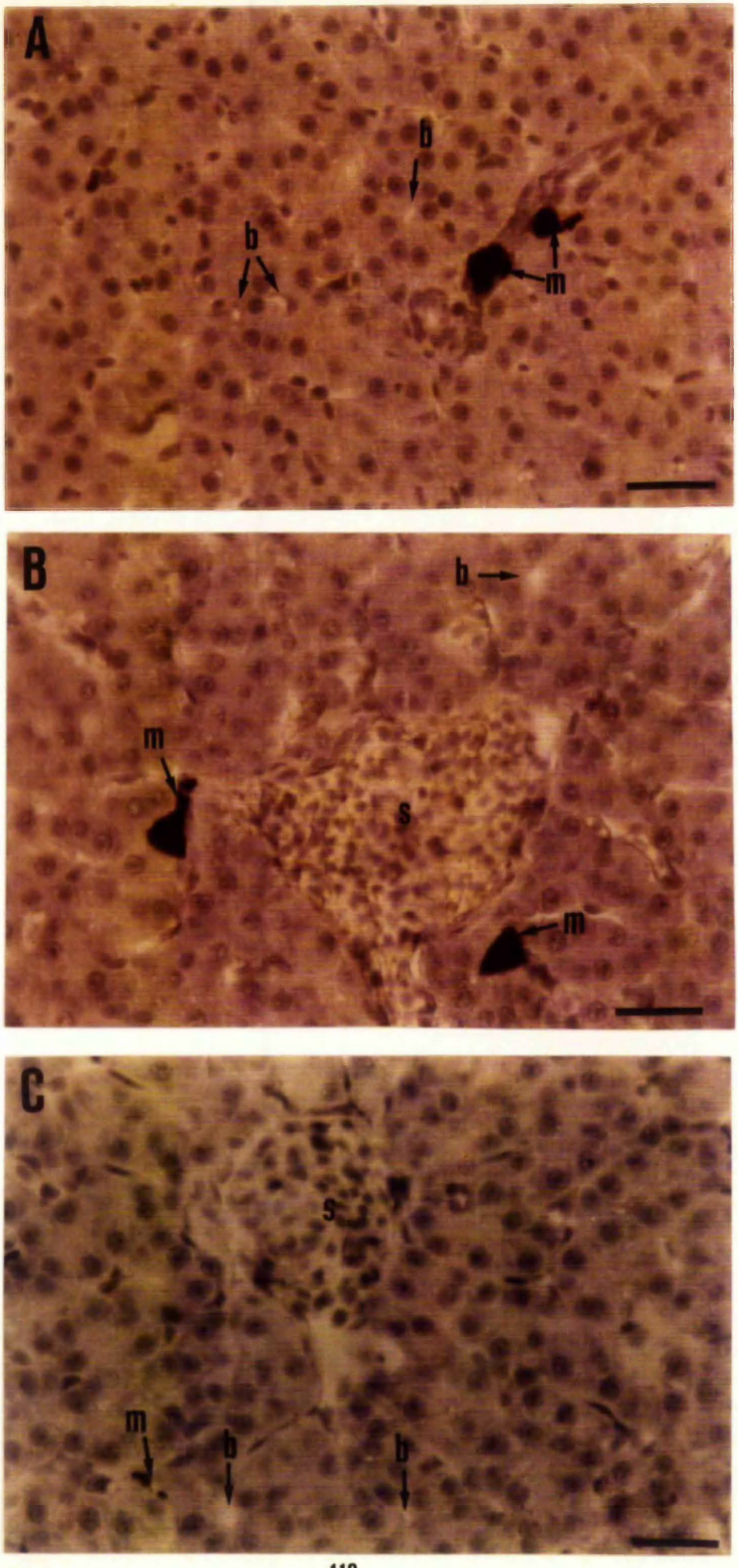
D. Soft water fish exposed to 1.65 mg Zn l^{-1}.

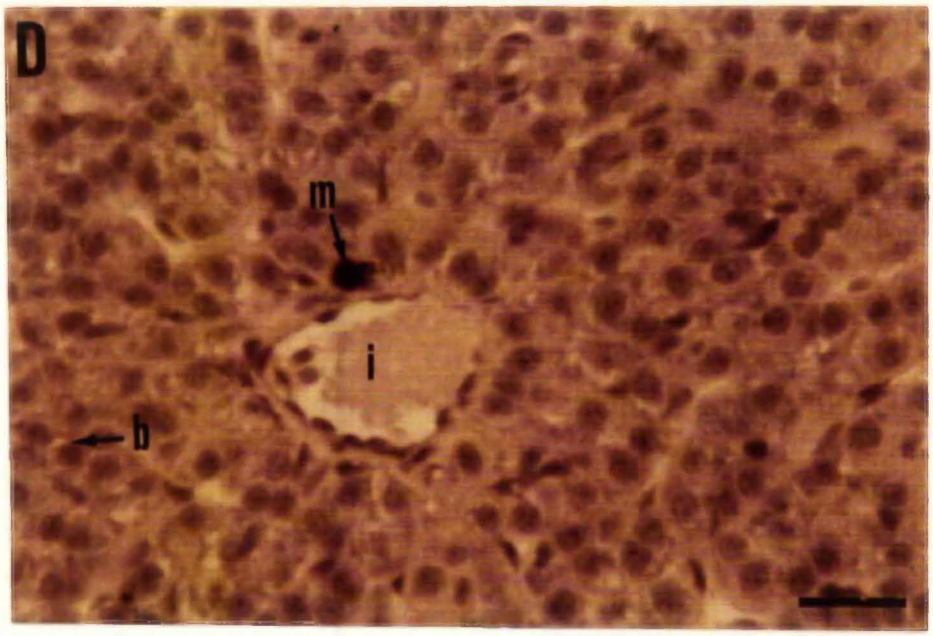
E. Hard water fish exposed to 1.68 mg Zn l^{-1}.

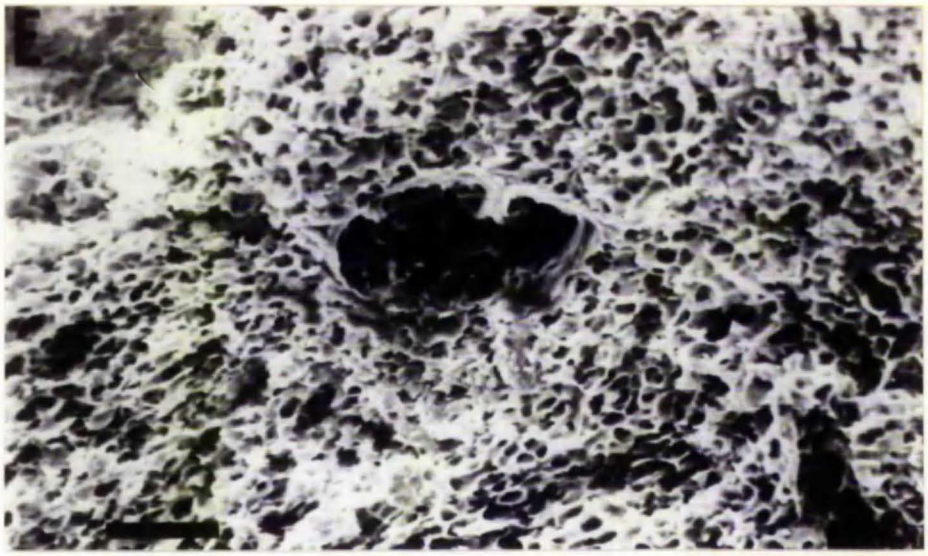
F. Soft water fish exposed to 1.65 mg Zn l^{-1}.

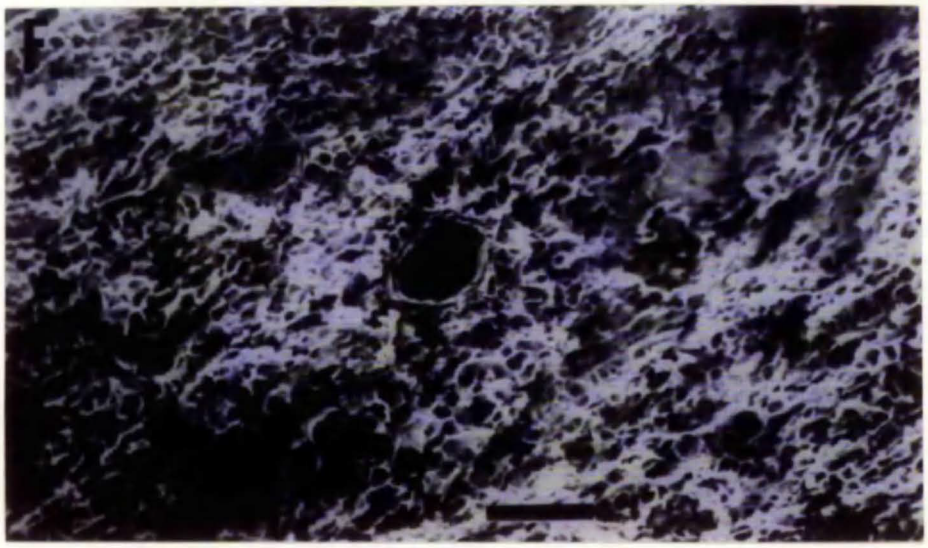
A-D were stained in haematoxylin and dithizone. Scanning electron micrographs E and F were from freeze fractured tissue, included are elemental tissue scans ●. Specimens were randomly chosen from eight fish in each treatment. Bars = 100 μm.

Bile canaliculus, b; intrahepatic bile duct, i; melano-macrophage aggregate, m; sinusoid, s.


A
b
b
m
B
b
m
s
m
C
s
m
b
b


D
m
i
b


E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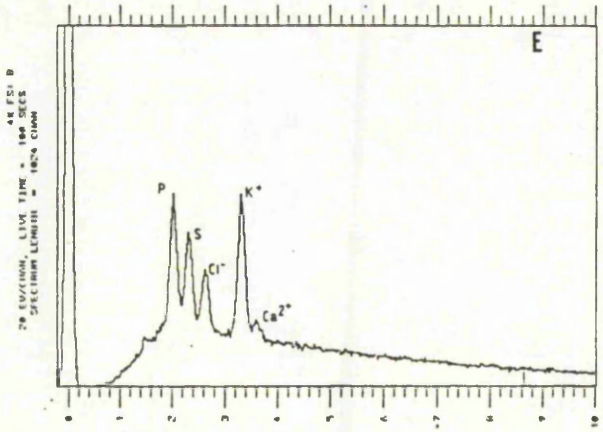
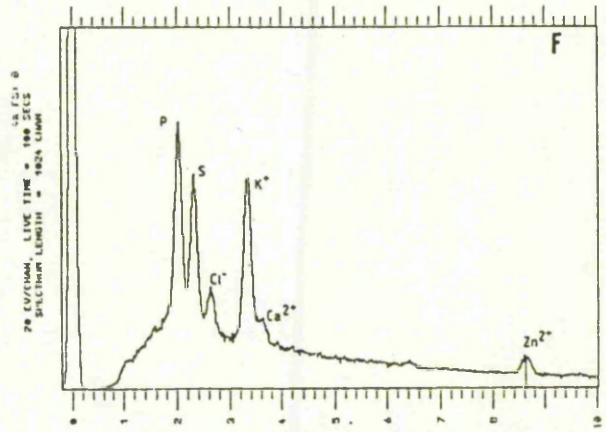


X-ray emission spectra



X-ray emission spectra

sinusoids of liver from zinc-exposed individuals but not from controls.

3.3.2.6. Other tissues

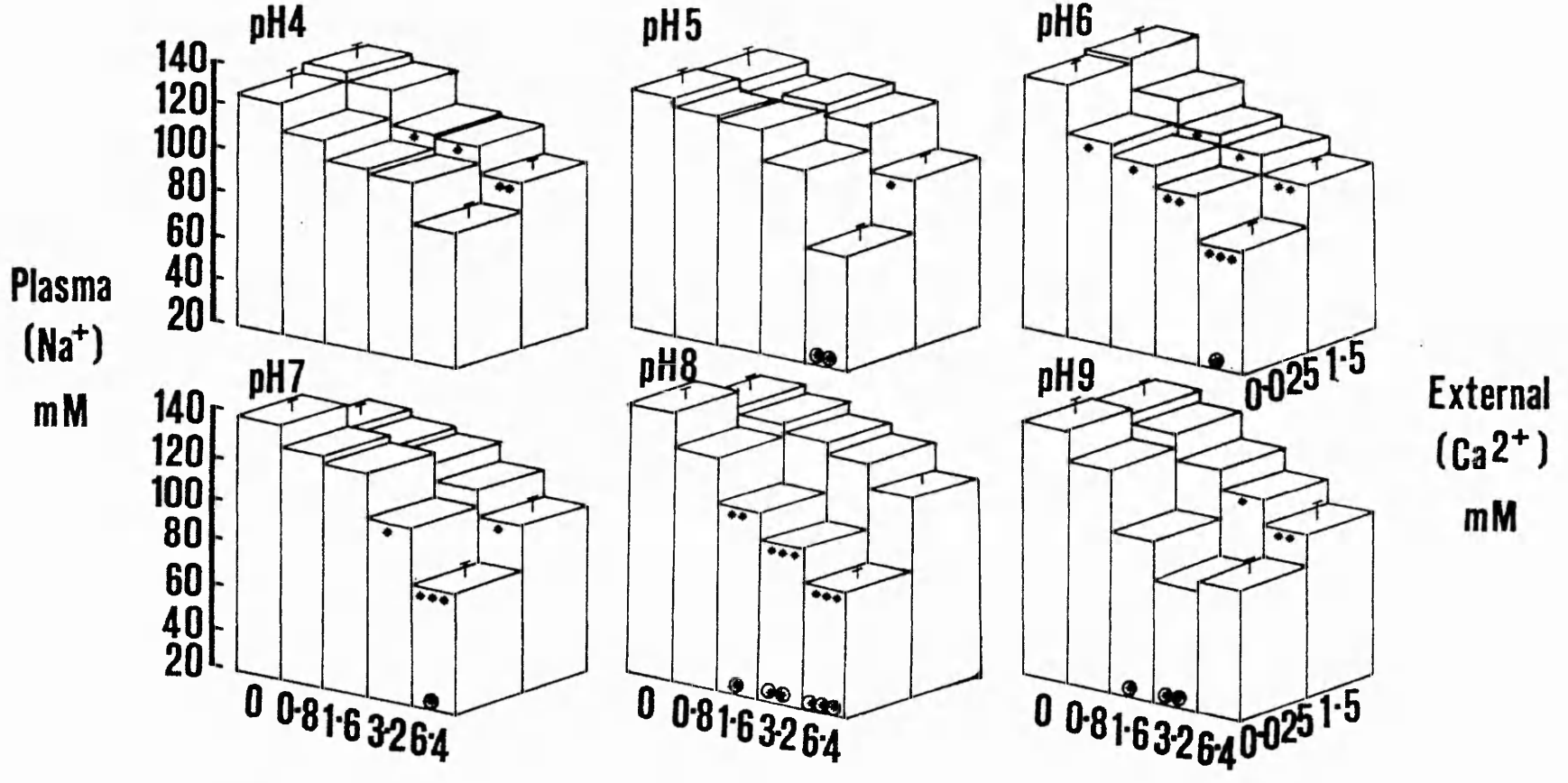
Tissue studies on the gut (of starved fish) were carried out but no difference was apparent in the histological structure of this tissue from control and metal-exposed fish. For this reason no further tissue analyses were undertaken. Tissue squashes of kidney revealed large numbers of melano-macrophage aggregates but unfortunately, due to difficulties in tissue preparation the comparative studies on the kidney were not completed.

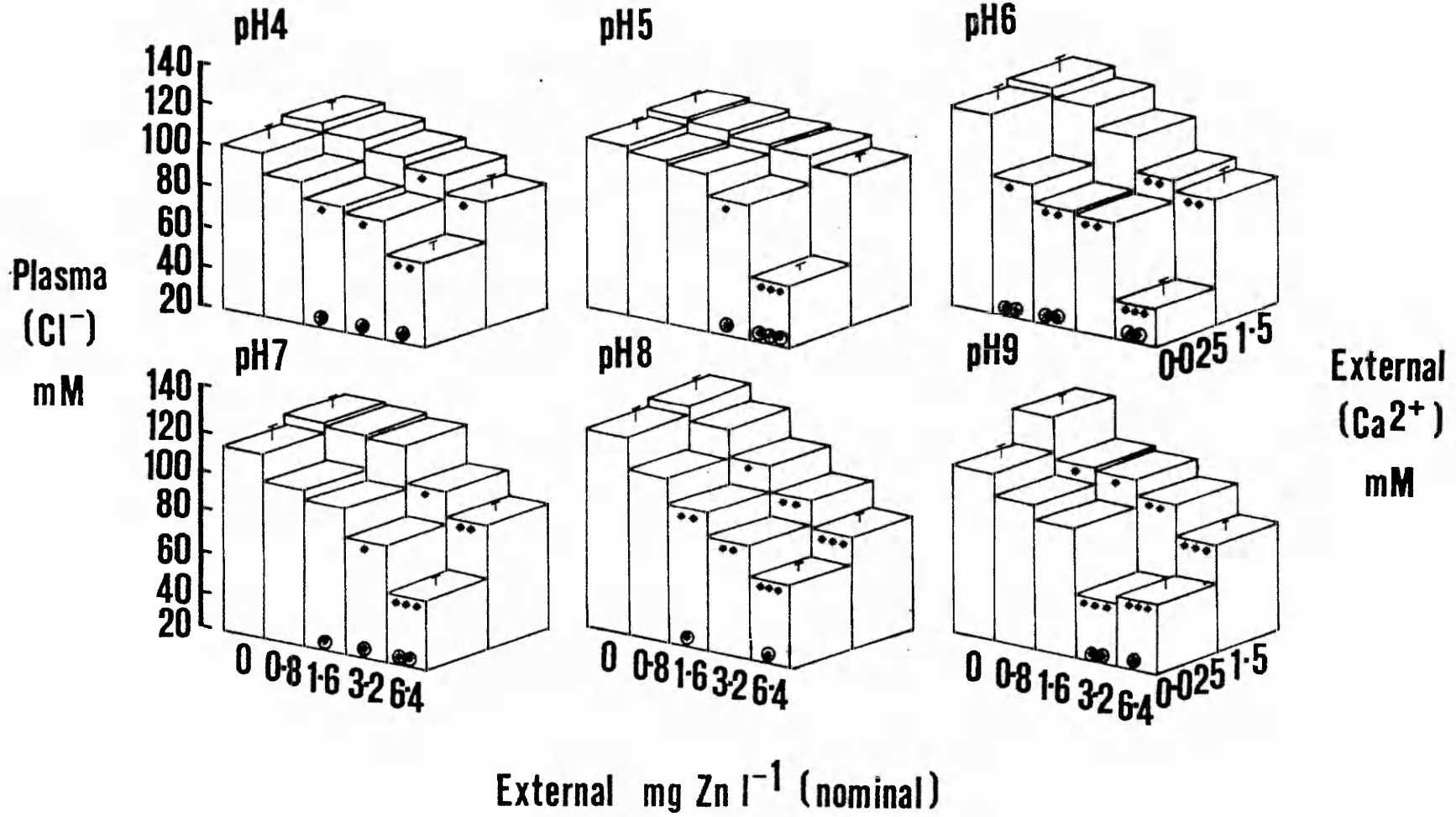
3.3.3 Effects of water hardness, pH and acute zinc exposure upon plasma ion balance

Plasma ion values are shown from each group of zinc-exposed fish at overturn, over the range of zinc concentrations tested in hard or soft water at the six pH levels (Fig. 17).

Different combinations of water hardness and pH had various effects on the plasma sodium and chloride balance of control and zinc-exposed fish. In control fish mean plasma sodium varied somewhat over the range of pHs, but not with statistical significance between hardness. Plasma sodium was substantially depleted in zinc-exposed fish at all pHs compared with controls

Figure 17 Plasma sodium (Na^+) and chloride (Cl^-) from overturned fish exposed to a range of zinc concentrations (0.80 - 6.40 mg Zn l^{-1}) in hard and soft waters over the pH range 4-9. Each column represents the mean value ($n \leq 10$) and vertical bars represent the standard error. Significant differences between hardness at a given zinc concentration are indicated at the $P < 0.05$ (*), < 0.01 (**) and < 0.001 (***) level. Significant differences between controls and a given zinc concentration are indicated at the $P < 0.05$ (●), < 0.01 (●●) and < 0.001 (●●●) level.


pH4
pH5
pH6
pH7
pH8
pH9
Plasma
(Na+)
mM
140
120
100
80
60
40
20
0 0·8 1·6 3·2 6·4
0 0·025 1·5
External
(Ca2+)
mM
External mg Zn l−1 (nominal)


pH4
pH5
pH6
pH7
pH8
pH9
Plasma (Cl⁻) mM
140
120
100
80
60
40
20
0 0·8 1·6 3·2 6·4
0 0·025 1·5
External (Ca2+) mM
External mg Zn l⁻¹ (nominal)

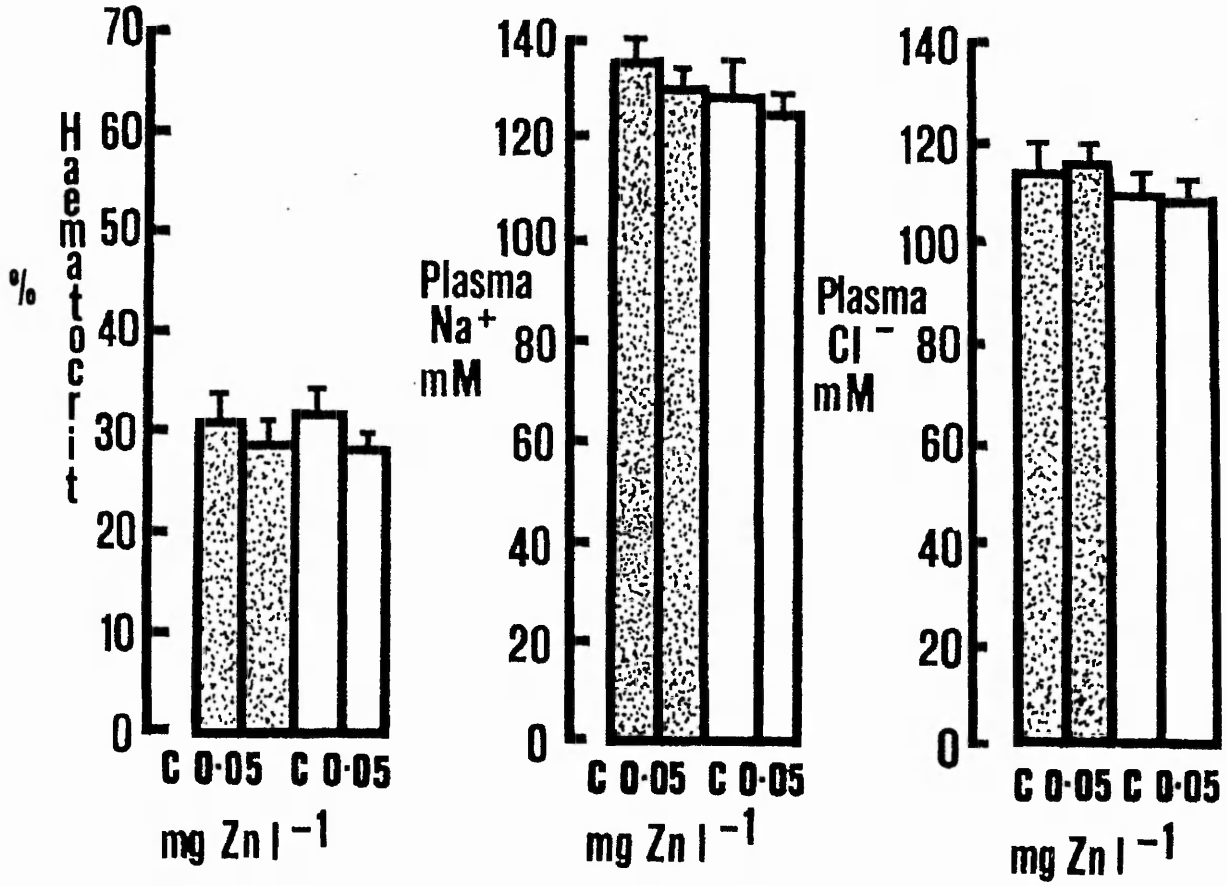
($P < 0.05$) and external high hardness was ameliorative to sodium losses at alkaline pH (see Fig. 17).

Similarly, plasma chloride varied over the range of pH for control fish with elevated levels at alkaline pH compared with acidic values. High hardness appeared to be ameliorative to plasma chloride loss over the range of pH in fish unexposed to the metal. Plasma chloride was significantly reduced ($P < 0.05$) in the majority of zinc-exposed fish at all pHs compared with controls. Increased hardness ameliorated the plasma chloride losses in zinc-exposed fish at high pH.

3.3.4 Effects of water hardness and sublethal zinc exposure on plasma ion balance

Plasma ion levels (Na^+ and Cl^-) of hard water and soft water fish were unaffected by a zinc exposure of 0.05 mg l^{-1} at pH 6 (Fig. 18). These observations were in contrast to comparable measurements with respect to fish exposed to acute concentrations of zinc at pH 6.

Figure 18 Haematocrit (%), plasma sodium and chloride from fish exposed to a sublethal zinc concentration (0.05 mg Zn l^{-1}) for 96 hours in test waters at pH6. Each column represents the mean value (n = 10) and vertical bars represent the standard error. Control groups (unexposed to metal) are indicated as C. Hard water ▩ and soft water fish □.


Haematocrit
%
70
60
50
40
30
20
10
0
C 0·05 C 0·05
mg Zn l −1
Plasma Na+ mM
140
120
100
80
60
40
20
0
C 0·05 C 0·05
mg Zn l −1
Plasma Cl− mM
140
120
100
80
60
40
20
0
C 0·05 C 0·05
mg Zn l −1

3.4 DISCUSSION

The gill is generally considered to be the site of acute zinc toxicity in fish (Jones, 1938; Lloyd, 1960; Skidmore and Tovell, 1972; Hughes and Adeney, 1977). The intrinsic toxicity of zinc has been defined as the amount of zinc present in the gills at death (Bradley and Sprague, 1985a) but differences in zinc accumulation of gills between fish from differing water hardness have remained unexplained.

In brown trout, the mucus on the gills of soft water fish consistently contained more zinc than that from hard water fish exposed to the same level of zinc. Mucus layers on the gills of soft water fish also appeared to be thicker than those of hard water fish which might have been due to increased production or to structural differences in the mucus layer. The accumulation and loss of mucus from the gills may ultimately depend upon the anchoring properties of lamellal microridges (Hughes, 1979). It remains to be determined if functional changes occur in such surface structures in response to hard or soft water acclimation and zinc exposure. However, alterations in the protein configuration of fish mucus can occur following trace metal exposure (Varanasi et al., 1975). Greater zinc content in the mucus layers of gills from soft water

fish may then be explained by an increased potential for surface adsorption of the metal at calcium-depleted sites within the mucus. The zinc content of the gill mucus may determine the initial rates of metal uptake by establishing diffusion gradients between the external medium and the mucus and then the mucus and the gill tissue/blood. Such a process may be part of the mechanisms involved with increased metal uptake and toxicity in soft waters. Mucus-mediated pathways for zinc uptake would also be affected by the mucus flow-rates across the gills in hard and soft waters, but these rates remain to be determined. However, hypersecretion of total body mucus has been shown in rainbow trout exposed to 20 mg Zn l^{-1} in soft water (Eddy and Fraser, 1982).

Tissue damage was evident from the secondary lamellae of overturned brown trout under acute exposure to zinc. With similar zinc exposures the degree of damage to gill tissue was always greater in the soft water fish. The increase in tissue damage in soft waters may be due to an increased accumulation of the metal in the gills of soft water fish compared to hard water fish. Previous work has suggested that there is a direct relationship between zinc accumulation in gill tissue and gill damage (Hodson, 1974).

The pattern of zinc-induced gill damage in brown trout was very similar to that described for rainbow

trout (Skidmore and Tovell, 1972). S.E.M. examination of the zinc-exposed secondary lamellae of brown trout suggested that the interlamellar water space had been reduced. Ultimately this would cause a reduction in the volume of water flowing across the gills and thus impair the efficiency of counter-current gas exchanges across the lamellae. This is in accord with the hypothesis that these zinc-exposed fish at overturn would eventually have died as a result of tissue hypoxia (Skidmore and Tovell, 1972; Heath and Hughes, 1973). The sloughing of epithelial cells in secondary lamellae was associated with acute zinc exposures and identified by fluid-filled sub-epithelial spaces in the tissue. The spaces may have been caused by increased production of granulocytes in the lymphoid space (Hughes and Wright, 1970). Such spaces increased the water to blood diffusion distance for oxygen and this problem was compounded when lamellar blood flow appeared to be disrupted indicating stagnation and a reduced area for effective oxygen uptake. This granulocyte response seemed similar to the inflammatory response of mammals and it has been suggested that such cells in the branchial lymphoid space may perform functions analogous to alveolar macrophages (Hughes and Gray, 1972).

The main structural target for zinc in brown trout was the gills and a primary consequence of metal exposure appeared to be respiratory failure, but a

secondary consequence of both metal and low pH exposure was a disturbance in plasma ion balance. Exposure of brown trout to a range of acute zinc concentrations caused a dose-dependent loss of plasma sodium and chloride ions over a range of pH exposures from 4 - 9.

Observed gill damage and ion losses with zinc exposure indicated the disruption of ion exchange mechanisms at the gill. The Na^{+}/K^{+} branchial exchange pumps are important in sodium uptake by fish (Maetz and Bornancein, 1975) and zinc has been seen to inhibit $Na^{+}/K+$ A.T.Pase from the gills of rainbow trout (Watson and Beamish, 1981). Inhibition of the mechanism of sodium uptake at the gill by zinc seems the likely explanation for net sodium loss in metal-exposed fish. The inhibition of chloride ion uptake may occur by similar processes to the sodium ion since injection of the carbonic anhydrase inhibitor acetozolamide prevented both sodium and chloride influx in goldfish (Maetz and Garcia Romeu, 1964) and trout (Kerstetter et al., 1970). Unlike zinc aluminium is known to inhibit carbonic anhydrase at low pH (Staurnes et al., 1984) and net sodium losses under such conditions have been attributed to inhibition of the sodium uptake mechanism (Dalziel et al., 1985 a,b).

Net plasma sodium and chloride losses in zinc-exposed fish may be due to the inhibition of the enzymes controlling the processes of sodium and chloride influx.

However, increased plasma ion losses of zinc-exposed fish from soft waters compared with hard waters may presumably also be due to changes in calcium-mediated membrane permeability (Cuthbert and Maetz, 1972; Eddy, 1975; McWilliams, 1982b; Dalziel et al., 1985a). The loss of plasma ions under such conditions may result from a stimulated efflux rather than an inhibited influx. Such a response was seen in the presence of zinc (Spry and Wood, 1985) and for cadmium and manganese (Reader, 1986). It is suggested the stimulation of plasma ion efflux by trace metals would be characteristic of the removal of calcium at epithelial cell membranes or tight junctions causing an increased passive permeability to these plasma ions.

Substantial losses of plasma sodium and chloride occurred in brown trout exposed to low pH, particularly in soft waters either in the presence or absence of zinc. With declining pH from 6 - 4 there were net losses of plasma ions during acclimation which were further increased by zinc exposure. Direct investigation of the effects of low pH have confirmed a stimulation of sodium and to a lesser extent chloride efflux (McWilliams and Potts, 1978; McDonald et al., 1983; Dalziel et al., 1985 a, b). It has also been shown that sodium influx in brown trout from soft water recovered completely after 10 days at pH 6 but recovery was only about 20% at pH 4.6 and virtually nil at pH 4

in very soft waters (McWilliams, 1980a,b). Further studies on brown trout in carefully controlled media suggested that sodium influx was stimulated in response to increased efflux at pH 4.5 but that at pH 4 stimulation of influx was absent particularly in low calcium media (Dalziel et al., 1985a, b).

The stimulation of sodium and chloride efflux, whether caused by exposure to low calcium concentrations or to low pH, would appear to be a temporary phenomenon. In long term studies of the effect of these treatments, sodium balance in brown trout has either recovered from early disturbances or has shown little or no disruption (McWilliams, 1980; McDonald et al., 1983; Perry and Wood, 1985; Dalziel, 1986). Where conditions have been such that inhibition of sodium influx occurs (pH < 4.5 and/or aluminium present) long term studies indicate high mortalities and/or severe sodium imbalance (Sadler and Lynam, 1985b; Dalziel, 1986). Symptoms of severe sodium imbalance and high mortalities were characteristic of brown trout exposed to low pH in the presence of zinc particularly in soft waters. Therefore when sodium imbalance occured it is suggested that zinc inhibited sodium influx rather than stimulating sodium efflux but the cause of the severe chloride imbalance in overturned brown trout remains largely unresolved.

Fish appear to be able to acclimate to changes in passive permeability to sodium and chloride caused by

low pH and low calcium concentrations (McWilliams, 1980a,b; McDonald et al., 1983; Reader, 1986) but not to inhibition of the carrier mechanisms by acute exposures of zinc. In prolonged exposures it may be that effects of low pH can be overcome, whereas the effects of zinc may be more permanent. Also, in the present studies where low pH acclimation was followed by low pH and zinc exposure, death was probably brought about by a rapid and substantial loss of plasma sodium and chloride resulting from the possible synergistic action of zinc and $[H^+]$. There was some evidence of structural damage to the lamellar epithelium at the extremes of low pH with extensive mucus accumulation at the gills. Such observations are important in separating the effects of low pH and zinc exposure prior to any synergistic action. Low pH per se can cause disturbances to a variety of physiological processes (Packer, 1979; Ultsch and Gros, 1979; Fromm, 1980) irrespective of zinc exposure.

Increased haematocrits were also recorded for zinc exposed fish from hard and soft waters. Haemolysis had occurred in zinc-exposed brown trout and had previously been shown with higher metal exposures (40 mg Zn l^{-1}) for rainbow trout (Kodama et al., 1982). Since changes in blood volume were not assessed in the present study it was not clear whether haemoconcentration was due to a shift in water from blood to muscle (Waiwood, 1980) or

other processes. Other possible blood changes might include a reduced uptake of sodium and chloride by the gills being compensated by a reduction in blood volume or an increased haematocrit with secondary reductions in sodium and chloride uptake being a homeostatic response by the fish to avoid an increase in blood osmolarity (Lloyd and Swift, 1976). Changes in haematocrit may also be explained by osmotic swelling of erythrocytes caused by reduced plasma osmolality as indicated by haemolysis. Further studies of cell counts, cell volumes and total blood volume would be required to resolve this problem.

Blood lymphocytes were observed phagocytosing haemolysed erythrocytes in live smears of blood from zinc-exposed brown trout. This had rarely been observed before although it had been considered that these lymphocytes had phagocytic properties. This behaviour may be analogous to that of the wandering leucocytes of molluscs which have an important role in the translocation and detoxification of metals (Bryan, 1973). However, very few leucocytes containing zinc were located in blood smears, a situation also found in plaice (Pleuronectes platessa) following intraperitoneal injection with carbon particles (Ellis et al., 1976). The absence of zinc from cellular components of the blood suggested the majority of the metal was available for exchange with the other tissues in an unbound state.

In the plaice zinc occured as a large unbound fraction in the blood of metal-exposed fish (Pentreath, 1973).

In the fish the major phagocytic sites and areas of carbon particle deposition were within the lymphoid organs of the kidney and spleen (Ellis et al., 1976) but these haemopoietic organs were also important sites of zinc accumulation in salmonids (Chipman et al., 1958; Eddy, 1975; Everall and Macfarlane, 1985). Spleen samples from hard and soft water trout following zinc exposure showed similar increases in phagocytic activity marked by increased metal concentrations in melano-macrophage centres. Zinc was found in the ellipsoid lumen of the spleen from exposed fish where this 'foreign' material may be taken up by large resident macrophages. Replete macrophages may migrate into the pulp where they may form aggregates within nodules of melano-macrophages. These aggregates were rich in zinc from all exposed fish (> 50 $\mu g\ g^{-1}$ from staining method, Mager et al., 1953) with levels greater than the surrounding splenic tissues. Melano-macrophage aggregates had large sulphur deposits characteristic of pheomelanins and trichochromes although sulphur has long been found to be present in the majority of melanins (Pearse, 1953). It was observed that these melano-macrophages possessed an abundance of melanin granules in the cytoplasm as found in plaice (Roberts, 1974). Melanosomal particles have been isolated from humans

(Seiji et al., 1963) and are rich in tyrosinase and zinc. However, high zinc levels were not found in the melano-macrophage aggregates from control fish. High sulphur levels may have been due to intracellular proteins rich in thiol groups (-SH) and associated with melanin centres. These may have been intracellular metallothioneins variously thought to be involved in zinc metabolism or to be part of a detoxification system (Kagi and Nordberg, 1979). Zinc metallothionein from the gill and liver of rainbow trout was involved in the induction of enhanced tolerance to zinc but did not appear to bind and store excess zinc (Bradley et al., 1985).

The liver of mammals has many highly phagocytic Kupffer cells, but no phagocytic activity of this type was evident in brown trout or in the plaice (Ellis et al., 1976). However, zinc was concentrated in very small numbers of melano-macrophages scattered throughout the liver of exposed trout. This does not appear to have been reported for any other teleosts but was observed in an unnamed dogfish (Hoskins and Hoskins, 1918).

Occasional melano-macrophage aggregates observed in the gills may be equivalent to the calcium-zinc granules of gills from the zinc exposed minnow, P. phoxinus (Frain, 1983). The latter granules were similar in size and shape to those found in the pigment spots of fish

skin and Frain suggested that these granules were part of a detoxification mechanism and that more granules might be produced in response to zinc exposure. The trout gills showed no evidence of this although such a response was evident in the spleen. However, Simkiss (1976) points out that structurally similar granules may have different functions in different parts of the body. If Frain's (1983) granules were within melano-macrophage aggregates, as appears to be likely, then they showed some specificity for zinc over cadmium, copper, lead and silver in the minnow gill.

Tissue squashes of kidney similar to the unstained sections of spleen showed the presence of melano-macrophage aggregates. Work on zinc and melano-macrophage distribution was unfortunately not progressed in this tissue. Phagocytosis and storage of carbon particles in the kidney of plaice (Ellis et al., 1976) may indicate the pattern of zinc distribution to be expected for metal exposed trout in the present study. Certainly melano-macrophage aggregates may play a dominant role in detoxification processes and the kidney may prove to be another major site of phagocytosis and storage.

Ellis et al., (1976) suggested that the tissues showing the most active uptake and localization of foreign substances were the lymphoid tissues which are considered to be capable of responding to foreign

antigenic matter by an adaptive defensive change, like antibody production. The melano-macrophage aggregates in association with lymphoid elements which occured in the spleen and kidney of teleosts have been likened to primitive germinal centres (Ellis and de Sousa, 1974). Positioning of these centres in relation to the vascular system with their intimate association with lymphoid tissues may be vital for the production of antibodies and hence for the efficient functioning of the immune system.

Long-term exposure to nickel and zinc in brown trout and carp (Cyprinus carpio) enhanced antibody titres increasing the immunological response (O'Neill, 1981). These findings tend to support a hypothesis that antibodies may have an immunological role in defence against zinc intoxication. Melano-macrophage aggregations of phagocytes in trout spleen and kidney may provide sites of concentrated antigen through which circulating lymphocyte percolate and where competent lymphocytes may be stimulated to respond immunogenically.

Zinc accumulation at melano-macrophages appeared to play a role in the mechanisms of detoxification for this metal in a variety of tissues. Formation of extracellular stable and insoluble zinc deposits of this nature would directly prevent toxic action at tissue and cell level. Such deposits form an important refractory

zinc pool ascribed to fish by Renfro et al (1974a) which may not be readily lost in the absence of contamination. The latter possibility may be important in helping to maintain elevated antibody levels and a potential for rapid immune response to further heavy metal challenge with chronic or sublethal exposure.

4.1 INTRODUCTION

There is general agreement in the literature that metal toxicity to fish could be influenced by water hardness (calcium concentration) and pH (see Introduction 2.1) and several theories have been proposed, suggesting that the process is biological, chemical or both. The biological hypothesis usually involves the influence of water hardness on branchial permeability, whereas chemical explanations involve changes in the speciation of the metal or competition for biological uptake sites.

Previous studies of the effects of water hardness upon zinc accumulation in fish have been inconclusive. Zinc accumulation in fathead minnows (Pimphales promelas) and carp (Cyprinus carpio) was reduced in hard water (Lipke, 1971; Lebedeva and Kuznetsova, 1967), whereas with sticklebacks (Gasterosteus aculeatus) the opposite effect was reported (Matthiessen and Brafield, 1977). However, Bradley and Sprague (1985a) found that zinc accumulation in the gills of rainbow trout was reduced in hard water and suggested that increased hardness protects fish by altering the dynamics of zinc uptake and/or excretion.

Of the various water hardness constituents, calcium provides the main protection against zinc toxicity to fathead minnows (Judy and Davies, 1979) and against

transfer of the metal across the perfused gills of rainbow trout (Spry and Wood, 1984). Therefore prior acclimation to hard water can ameliorate zinc toxicity in soft water (Lloyd, 1965) and calcium may reduce transfer of the metal across the gill (Spry and Wood, 1984). Similar findings were reported for the toxicity of cadmium to brook trout (Carrol et al., 1979) and transfer of the metal across the perfused gills of rainbow trout (Pärt et al., 1985).

The mechanisms determing both short (acute) and long-term (chronic) toxicity of zinc are not well understood. The processes of zinc uptake, accumulation and excretion were investigated in this study and the role of water hardness (calcium concentration) was examined. Studies of metal uptake in fish were carried out at c. pH 6 but with environmentally relevant (Campbell and Stokes, 1985) concentrations of zinc in carefully controlled experimental media. Therefore at the zinc and pH levels used in these studies, complex formation of the metal was relatively slight (Hem, 1972; Vymazal, 1985; Campbell and Stokes, 1985) in order to determine whether the influence of hardness on zinc uptake and excretion was likely to be biological rather than through changes in chemical speciation in the water.

4.2 MATERIALS AND METHODS

4.2.1 Radioisotopic kinetic studies using ^{65}Zn

4.2.1.1. Acclimation

Fish of age 1+ and weighing 35-45 g or 80-90 g were acclimated to hard or soft water of pH 6 at 14 °C. pH 6 was chosen for use in later radioisotopic kinetic studies with ^{65}Zn to prevent problems arising from complex species formation, associated with zinc at more alkaline pHs and subsequent losses from the test media. Essential water quality conditions for acclimation media to which both fish stock were exposed are given in Table 12.

4.2.1.2 Apparatus for flux studies

Closed-circuit individual fish systems were designed and constructed for the purpose of radioisotopic studies on zinc fluxes. The apparatus used in the study of zinc flux rates is shown in Fig. 19 and eight of these systems were used simultaneously. Each fish chamber consisted of an opaque polypropylene box (600 ml) and watertight lid.

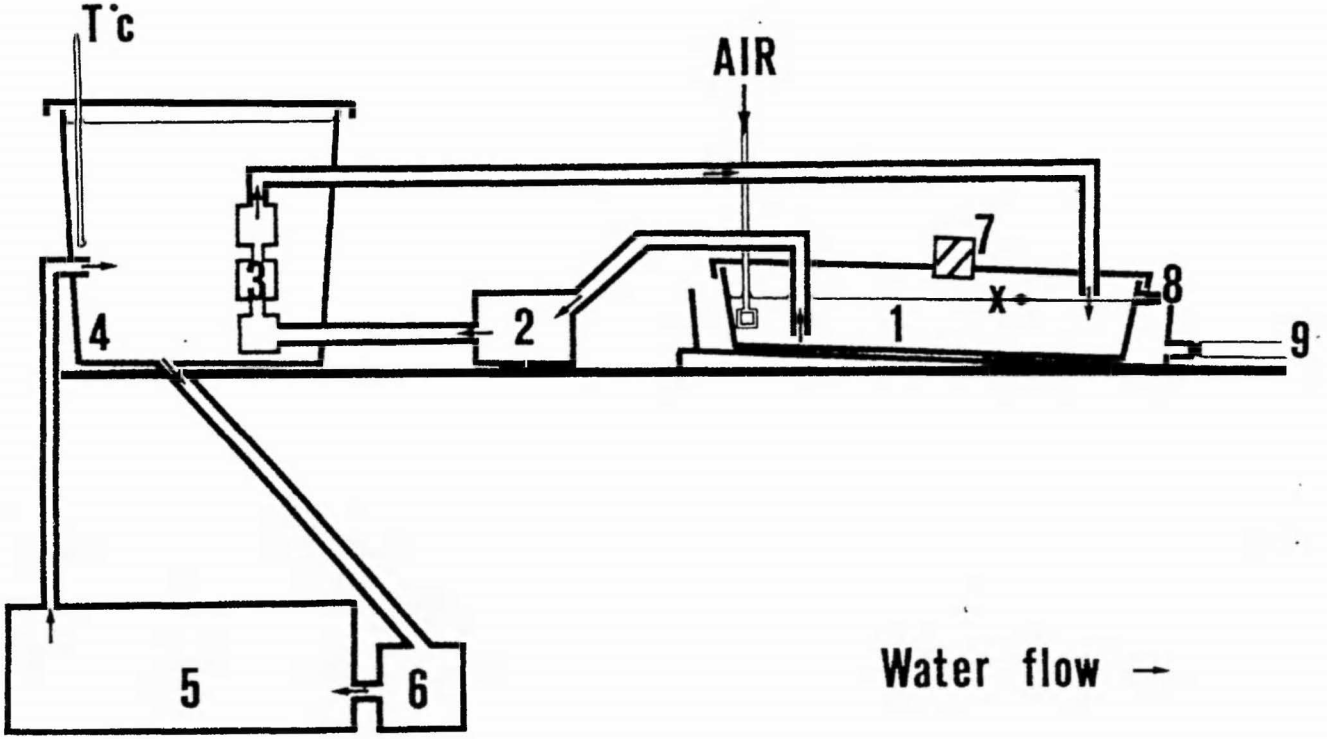
The flux apparatus was operated as a closed circuit one hour before the start of a flux experiment when the total volume of water recirculating was C. 700 ml. Under closed-circuit conditions each flux chamber (1)

Table 12 The major water quality factors during acclimation

Water Quality Factor		Mains hard water	Artificial soft water
Temperature	°C	14 ± 0,5	14 ± 0,5
pH		6,00 ± 0,10	6,00 ± 0,10
Dissolved oxygen	as mg l^{-1}	10,0 ± 0,4	9,8 ± 0,4
Total water hardness	as mg l^{-1} $CaCO_3$	220 ± 12	9 ± 2
Alkalinity	as mg l^{-1} $CaCO_3$	82	46
Calcium	mg l^{-1} Ca^{2+}	67 ± 4	< 2
Magnesium	mg l^{-1} Mg^{2+}	12 ± 3	< 1
Zinc	µg l^{-1} Zn^{2+}	< 9	< 6

Figure 19 Flux apparatus for zinc uptake and excretion studies

1. Flux chamber, water level mark x indicates c. 700 ml total volume of system.
2. 110 v recirculating pump of 1.3 l min^{-1} flow rate.
3. Glass coiled heat exchanger.
4. Reservoir of coolant water.
5. Thermostatic heat exchanger.
6. 110 v recirculating pump of 1.8 l min^{-1} flow rate.
7. Aperture for sampling test water, regulation and monitoring of pH.
8. Overflow for open circuit operation.
9. Radioactive liquid waste.


T°c
AIR
1
2
3
4
5
6
7
8
9
X
Water flow →

received a constant flow of 1.3 l min^{-1} of flux medium via the recirculation pumps (2). The pH of this recirculating water was checked at 30 minute intervals and continually adjusted to pH 6 by manual addition of a few drops of 0.1 M H_2SO_4. Water temperature was regulated by circulation through a glass coiled heat exchanger (3) situated in a reservoir of coolant water (4). Coolant water was recirculated from a thermostatically controlled heat exchanger (5) and pump (6). Aeration was through a coil of diffuser tubing fixed in the flux chamber.

The polypropylene flux chamber and the polyethylene or silicon-rubber tubing used in the construction of the system were chosen because they had low adsorption properties.

4.2.1.3 Determination of zinc uptake with an acute exposure

To assess absorption onto the apparatus a series of control experiments were performed at pH 8 and pH 6 in the absence of fish. Two sets of eight control apparatus were tested in hard and soft water over 64 hours. At the start, 0.50 mg l^{-1} stable zinc (as $ZnSO_4$) labelled with 10 µCi ^{65}Zn (as $ZnCl_2$, 370 KBq, specific activity 974 c min^{-1} μg^{-1}) was mixed in 10 ml of deionised water and added to the flux chamber. 2 ml samples of the test water were removed by autopipette at

regular intervals over the following 64 hours. The samples were dispensed into 5 ml polystyrene counting tubes for γ-spectrometry within 24-36 h of sampling.

Next, six hard water and six soft water acclimated fish (80-90 g) were exposed to 0.50 mg l^{-1} zinc labelled with 10 μCi ^{65}Zn (370 KBq, specific activity 933 c min^{-1} μg^{-1}) at pH 6 for 48 hours. After the overturn of the fish or at the end of the 48 h uptake period the fish were killed quickly by administering a lethal dose of MS222 (tricaine methanosulphate). They were then weighed and blood was withdrawn by caudal venipuncture and centrifuged. Two 100 μl plasma samples were used for γ-counting with the remaining plasma and blood cell fractions stored at -20 °C.

The gills (arches and hemibranchs), opercula, liver, spleen, kidney and gut were excised and placed in pre-weighed tubes for γ-counting. Gill arches and hemibranchs were given a gentle 10-20 second rinse in deionised water and blotted before final weighing. Tissue and residual carcass wet weight were recorded before γ-counting. Plasma and tissue radioactivities were determined within 24-36 hours of removal along with the external medium samples. The carcasses were stored at -20 °C, later homogenised for 10 minutes with 20 ml of deionised water and series of 1-2 g sub-samples counted.

All sample radioactivities were measured using an auto-gamma scintillation counter (Packard 500) fitted with a 3 x 3 inch thallium - activated sodium iodide crystal. The window - settings for analysing primary and secondary emission peaks were A1000-1214 (keV) and B900-1300 keV respectively. The Packard 500 had a counting efficiency for ^{65}Zn of 8-9% and the standard counting error for a series of 2 ml samples per 10 minutes was ± 2.24 counts per minute. Radioactivities were expressed as counts $minute^{-1}$ ml^{-1} for external media and as counts $minute^{-1}$ g wet $weight^{-1}$ for plasma and tissue samples.

It was assumed, as have other workers (Slater, 1961; Joyner, 1961; Matthiessen and Brafield, 1977; Bradley and Sprague, 1985) that the radiozinc was uniformly distributed with respect to the stable isotope in the test water. At time zero backflux in unexposed fish was considered unlikely thus the rate of zinc uptake in exposed fish could be calculated as a function of the radioactivity from the measurements of ^{65}Zn disappearance from the external medium in a given time :-

Rate of zinc uptake =

(Influx $\mu g\ h^{-1}\ kg^{-1}$)

$$\frac{\text{change in ext. medium (counts min}^{-1}) \times \text{median ext. volume (ml)} \times 1000 \times 60}{\text{sample volume (ml)} \times \text{fish weight (g)} \times \text{flux time (min)} \times \text{specific activity (c min}^{-1}\ \mu g^{-1})}$$

Specific activity =

c min^{-1} μg^{-1}

$$\frac{\text{initial counts min}^{-1}\ \text{ml}^{-1}\ \text{(ext. medium time } 0)}{\mu g\ ml^{-1}\ \text{(total zinc)}}$$

Since no measurable backflux of isotope occurred zinc influx calculations were cross-checked from the natural logarithm function given by Kirschner (1970):-

$$Jin = \frac{Q\ out.}{t.\ W} \quad (\ln Q^{*}\ out_{(0)} - \ln Q^{*}\ out_{(t)})$$

where Q out is the total amount of zinc in the medium ($\mu g\ ml^{-1}$), t is the flux time (min), W is the fish weight (g), and Q* out is the total amount of radioactivity (counts min^{-1}) at time 0 and t.

4.2.1.4 Determination of zinc uptake with a sublethal exposure

The effect of a non-lethal concentration of zinc on the pattern and rate of uptake, accumulation and excretion was examined in fish exposed to radioactive zinc solutions of total metal level 0.05 mg l^{-1}. This concentration was calculated on the basis of previous toxicity tests at pH 6. Control experiments were done at pH 6 over 48 hours with 0.05 mg l^{-1} stable zinc labelled with 10 μCi ^{65}Zn (370 kBq, specific activity 8060 c min^{-1} μg^{-1}). Then, ten hard water and ten soft water-acclimated fish (35-45g) were exposed to another series of radioactive zinc solutions identical to control concentrations and conditions. Experimental methods for these studies were as described for the acute studies of zinc uptake except that an additional 100 ml water sample was taken at the end of the experiment for determination of the stable zinc concentration.

The initial sublethal experiments had indicated the presence of a fast component of zinc uptake over some 0-5 hours the reasons for which were not clear and therefore required further investigation. In a further series of experiments ten hard water and ten soft water fish were exposed as before and ^{65}Zn uptake monitored at 15 minute intervals over the first 5 hours of uptake.

Fish were then killed at 5 hours and all samples processed.

Two further experiments were done to determine the effect of decreasing external stable and isotopic zinc on the rate and pattern of zinc uptake. Two groups of eight fish were exposed for 48 hours to 0.05 mg l^{-1} zinc labelled with 10 µCi ^{65}Zn (370 kBq, specific activity 9129 c min^{-1} $µg^{-1}$) then a second dose of zinc-65 was administered to the external media. This dose was calculated to return external medium stable and isotopic zinc concentrations to original levels. ^{65}Zn uptake was monitored for a further 12 hours before the fish were killed and samples processed.

In order to determine whether the protective effect of calcium in reducing the rate of zinc uptake was due to the level experienced by the fish in the test media during flux studies or to the result of long term exposure, further experiments were performed. Eight hard water and eight soft water fish were exposed to soft and hard waters respectively for 20 hours in open circuit flux chambers. Following this period of adaptation the systems were closed and fish were exposed to 0.05 mg L^{-1} zinc labelled with 10 µCi ^{65}Zn (370 kBq, specific activity 9011 c min^{-1} µg-1) for a 48 flux period as before.

4.2.1.5. Zinc excretion following a sublethal exposure

Six hard water and six soft water fish (35-45 g) were allowed to become 'loaded' with stable zinc and isotopic zinc by exposure to 0.05 mg l^{-1} zinc labelled with 20 µCi ^{65}Zn (740 KBq, specific activity 19,271 c min^{-1} μg^{-1}) at pH 6 for 48 hours.

Then the flux chambers (fish) and connecting systems were flushed with zinc-free hard or soft water at pH 6 at 14 °C. The volume of the fish apparatus (c.700 ml) was restored and the re-appearance of ^{65}Zn in the external medium was monitored at regular intervals for the following 48 hours. The fish were killed at the end of this period and radioactive samples processed as before.

4.3 RESULTS

4.3.1. Acute exposure to zinc

4.3.1.1. Properties of zinc in control apparatus and media.

Initial experiments gave information on the behaviour of zinc in ``fish-free`` ^{65}Zn-labelled 0.50 mg l^{-1} solutions as shown in Fig. 20. This was used to devise methods for studying ^{65}Zn uptake in fish. These were typical examples from the two pH test groups illustrating the sequestration of radiozinc and presumably stable zinc from acid or alkaline solutions. At pH 6 ^{65}Zn remained in solution but at pH 8 ^{65}Zn was lost from the aqueous phase possibly by surface adsorption or precipitation. Inset in the plot are measured concentrations of total zinc which indicated that both radiozinc and stable zinc were affected in a similar pH-dependent manner. On the basis of these experiments it was decided to study zinc uptake at pH 6.

4.3.1.2 Zinc uptake in the fish.

Results for zinc uptake in hard and soft water fish exposed to a concentration of 0.50 mg Zn l^{-1} are summarised in Fig. 21. Generally hard water fish had lower influx rates than soft water fish but some overlap

Figure 20 ^{65}Zn and stable zinc concentrations in hard (●) and soft water (ó) at pH 6 or 8 taken from control apparatus (no fish) exposed to 0.50 mg Zn l^{-1}. Typical plots selected from 10 hard and soft waters are shown and total measured zinc in mg l^{-1} is shown in parenthesis.

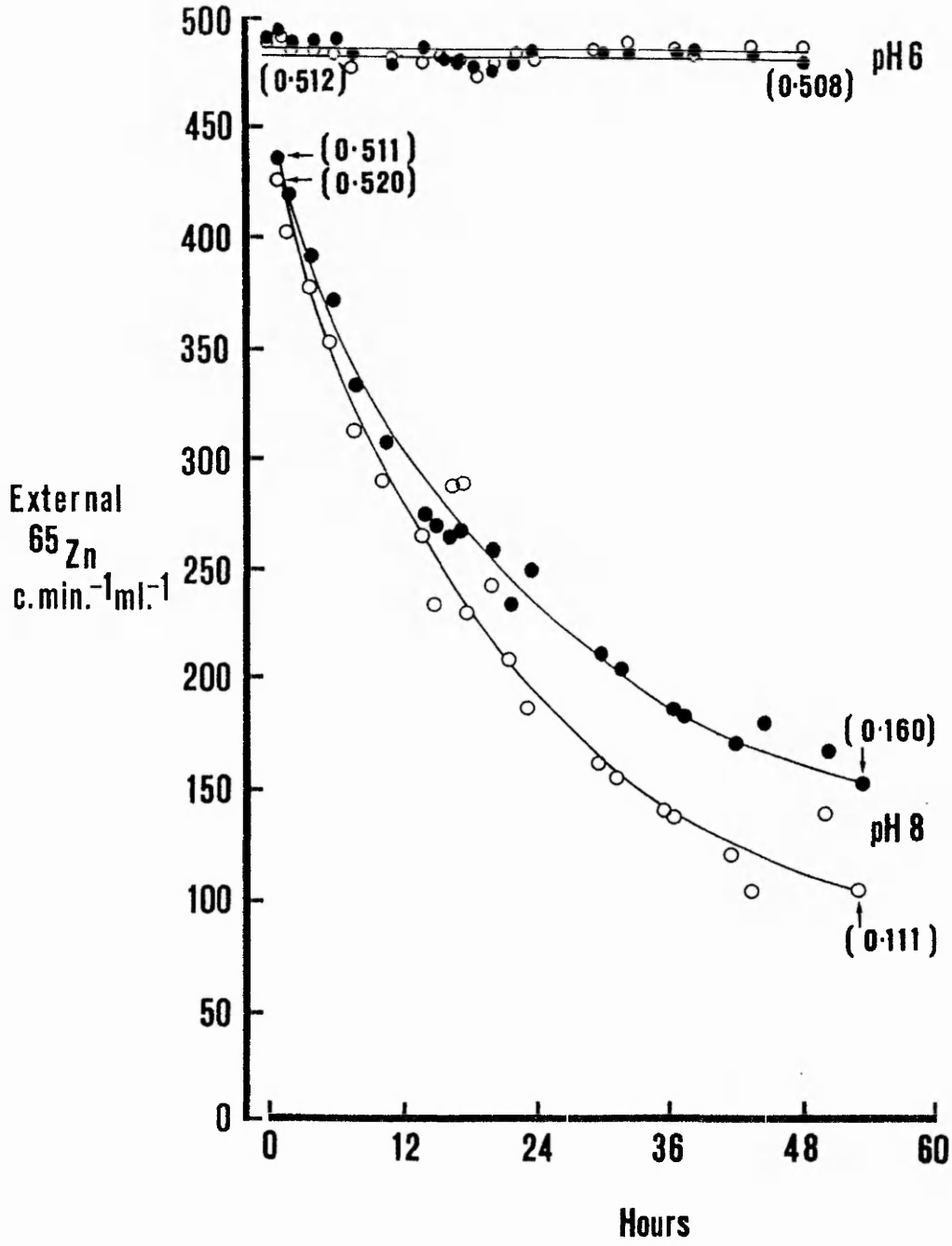
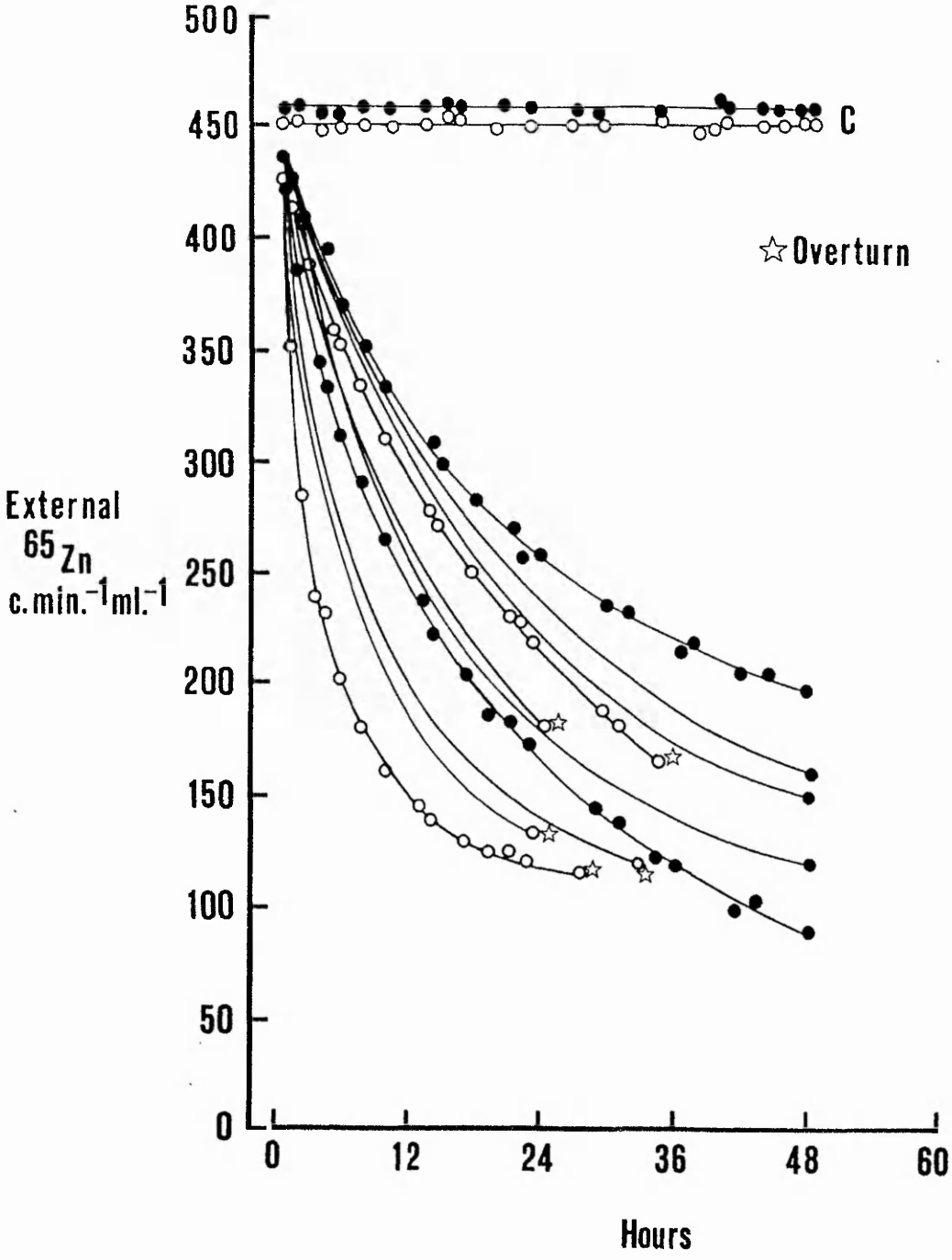

500
450
400
350
300
250
200
150
100
50
0
External
65Zn
c.min.-1ml.-1
(0·512)
(0·508)
pH 6
(0·511)
(0·520)
(0·160)
pH 8
(0·111)
0
12
24
36
48
60
Hours

Figure 21 Influx curves of ^{65}Zn for hard (●) and soft water fish (○) exposed to 0.50 mg Zn l^{-1}. Influx curves are sometimes presented with only two points purely for the purpose of clarity. Overturn (☆) indicates death of fish and typical plots selected from five controls (no fish) are also shown.


500
450
400
350
300
250
200
150
100
50
0
C
☆Overturn
External
65Zn
c.min.-1ml.-1
0
12
24
36
48
60
Hours

was evident at this exposure. However, there was a significant ($P < 0.05$) difference between the mean rates of zinc influx in the two fish groups as shown in Fig. 22. This zinc exposure proved to be lethal to all the soft water fish within 30-35 h but no hard water fish died during the 48 h test period.

Tissue ^{65}Zn accumulations are shown in Fig. 23 for hard water fish exposed to zinc for 48 hours and soft water fish exposed for 30-35 hours and sampled at overturn. Soft waters fish showed significantly greater ^{65}Zn burdens for all tissues compared with hard water fish irrespective of their shorter exposure times. In both groups of zinc exposed fish the prime target tissues for ^{65}Zn were the gills.

4.3.2 Sublethal exposure to zinc

4.3.2.1 Properties of zinc in control apparatus and media.

For sublethal ^{65}Zn-labelled zinc concentrations of 0.05 mg l^{-1} the degree of sequestration of zinc in fish-free control media at pH 6 are shown in Fig. 24. Measured concentrations of total zinc present in these media at the start and the end of an experiment are shown in parentheses. Provided the pH was maintained close to pH 6 neither the concentrations of radiozinc nor the stable isotope were altered in the controls.

Figure 22 Ratio of zinc influx ($\bar{x} \pm$ s.e., n = 5) in hard (▨) and soft water fish (□) exposed to 0.50 mg Zn l^{-1}. Students t-test *P < 0.05.

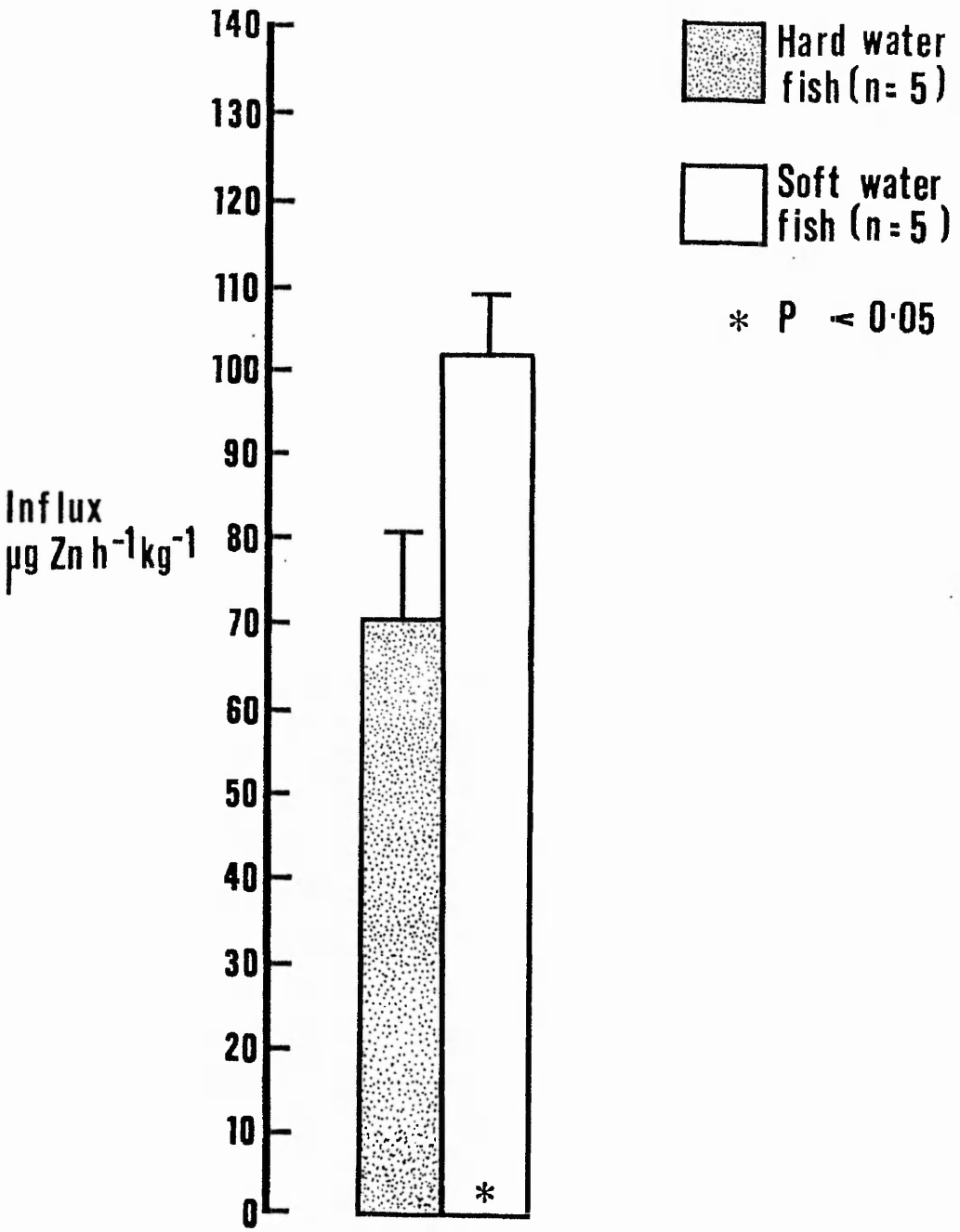

140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Influx
µg Zn h⁻¹kg⁻¹
*
Hard water fish (n = 5)
Soft water fish (n = 5)
* P ≤ 0·05

Figure 23 Tissue accumulation of ^{65}Zn ($\chi \pm$ s.e., n = 10) after exposure to 0.50mg Zn l^{-1} in hard (▩) and soft waters (□). Students t-test *P < 0.05 or **P < 0.01.

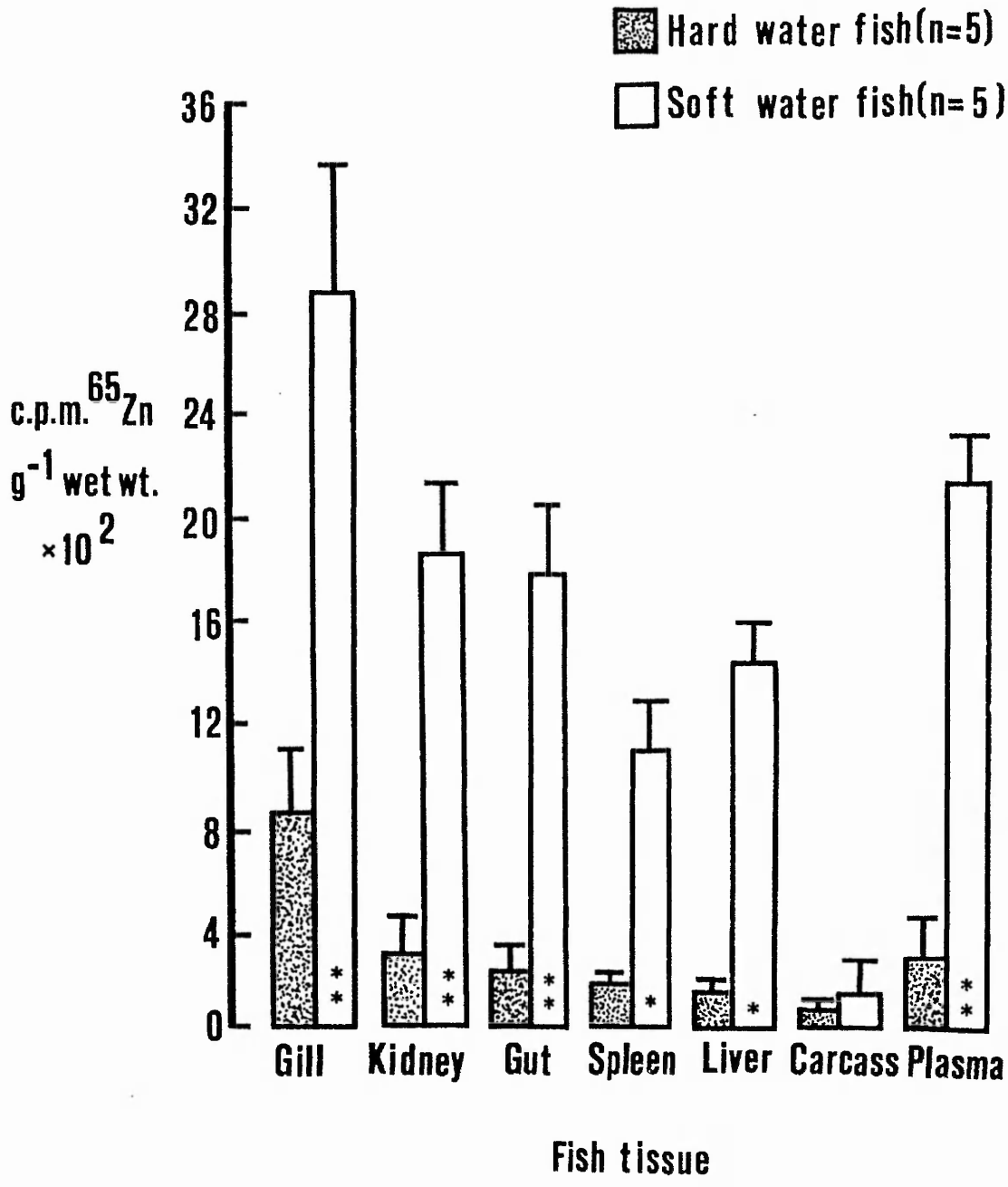
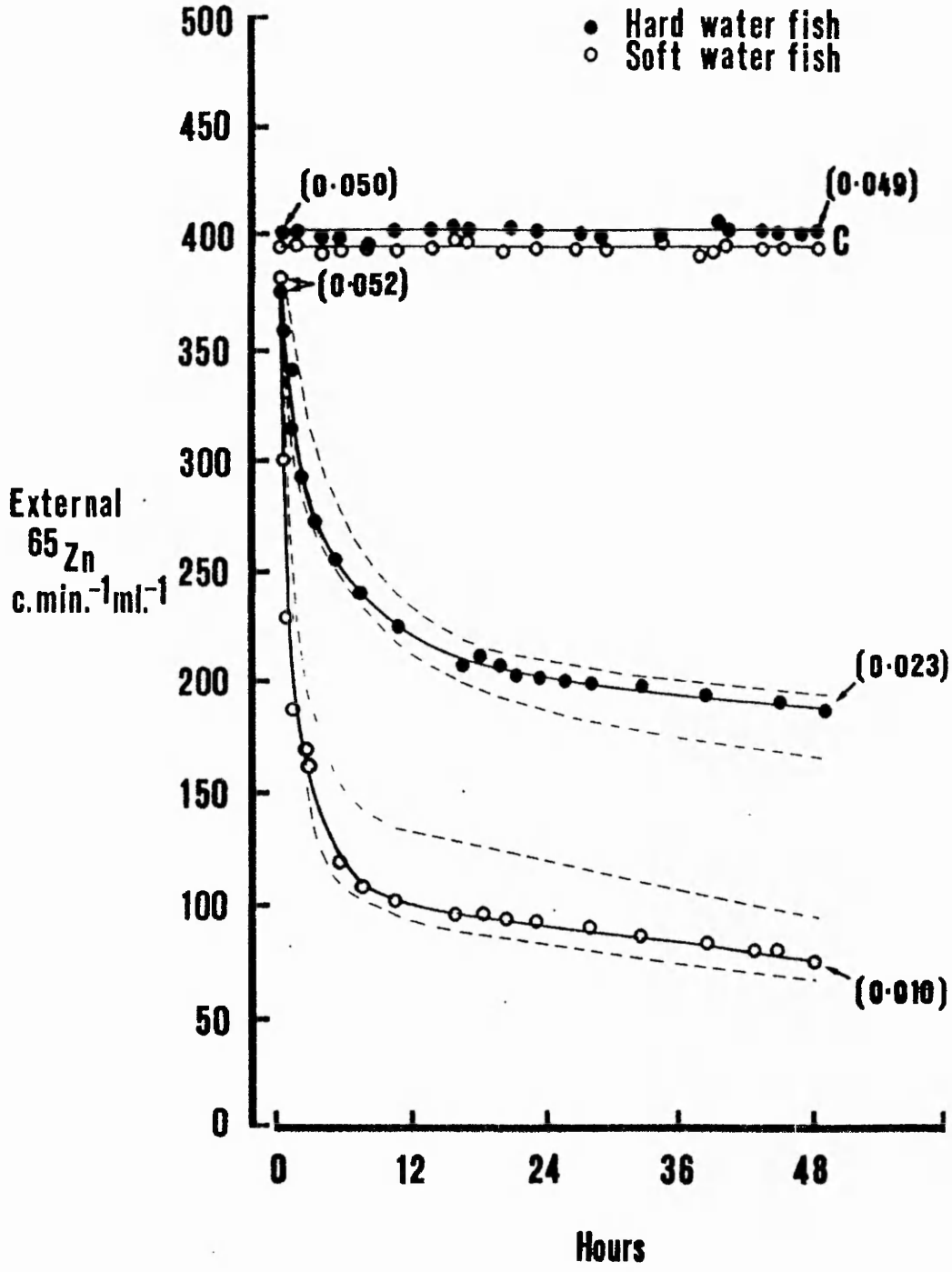

Hard water fish(n=5)
Soft water fish(n=5)
c.p.m. ^{65}Zn
g^{-1} wet wt.
×10^{2}
36
32
28
24
20
16
12
8
4
0
Gill
Kidney
Gut
Spleen
Liver
Carcass
Plasma
Fish tissue

Figure 24 Influx curves of ^{65}Zn for hard (●) and soft water fish (○) exposed to 0.05mg Zn l^{-1}. Control groups represented by C, dotted lines indicate the range of curves from 10 fish in each group and total measured zinc in mg l^{-1} is shown in parentheses.


Hard water fish
Soft water fish
(0·050)
(0·049)
C
(0·052)
(0·023)
(0·010)
External
65Zn
c.min.-1ml.-1
500
450
400
350
300
250
200
150
100
50
0
0
12
24
36
48
Hours

4.3.2.2. Zinc uptake in the fish.

In the first series of experiments under static conditions the total ammonia (NH_3 + NH_4+) concentrations in test media at the end of the 48 hours had been determined. Total ammonia levels from both control (n = 5) and zinc-exposed fish media (n = 5) did not exceed 0.042 mg l^{-1} (pH 6, 14°C) and were thus considerably below levels likely to result in toxic effects (Alabaster and Lloyd, 1980).

Typical examples of zinc influx curves and the range of values from groups of ten fish exposed to 0.05 mg Zn l^{-1} in hard and soft waters are shown in Fig. 24. Measured concentrations of total zinc at time zero and at 48 hours are shown in parentheses reflecting the net exchange between the fish and the medium. During the first 48 hours of sublethal zinc exposure under static conditions there was a net influx of zinc onto/into the fish from the medium.

Both groups of fish showed a similar pattern of zinc uptake with an initial fast component over 0 - 5 hours followed by a second slower component over some 5 - 48 hours. Stable zinc remaining in the external media at the end of 48 hours reflected proportionally the gross uptake of ^{65}Zn, indicating there was no selective uptake between radiozinc or stable zinc.

Semi-logarithmic plots of the influx curves shown in Fig. 25 suggested the presence of two distinct phases of

zinc influx in both groups of fish. Mean rates of zinc influx for the fast and slow components in the two media are given in Fig. 26. In hard water fast and slow components were 52 ± 0.6 and 1.2 ± 0.1 $\mu g\ h^{-1}\ kg^{-1}$ respectively, with corresponding influxes in soft water of 121 ± 12.3 and 2.3 ± 0.3 $\mu g\ h^{-1}\ kg^{-1}$. Both fast and slow influxes were significantly ($P < 0.01$) different between trout exposed to the two media.

Approximately 80% of radiozinc and stable zinc was removed from the external medium in soft waters compared with 46% from hard waters. This was reflected in the significantly ($P < 0.05$) greater accumulation of ^{65}Zn in the gill, kidney, gut, liver, spleen, carcass and plasma of soft water fish compared with hard water fish as shown in Fig. 27. Table 13 shows total body burdens of ^{65}Zn as total radioactivity in the fish compared with total radioactivity lost from the external medium. In both groups of fish > 95% of the ^{65}Zn that disappeared from the external media appeared in the fish. 2.5% of the total radioactivity was unaccounted for and this was possibly lost by adsorption onto apparatus and into mucus removed from the fish during sampling.

Since both radioactive and stable zinc disappeared in similar proportions from the external media it can be assumed that > 95% of stable zinc was in the fish. Other workers have also assumed that the two forms of zinc are similarly distributed amongst the fish tissue.

Figure 25 Semi-logarithmic plots of the fast (0-5 h) and slow (12-48 h) components of the zinc influx for hard (●) and soft water fish (o).

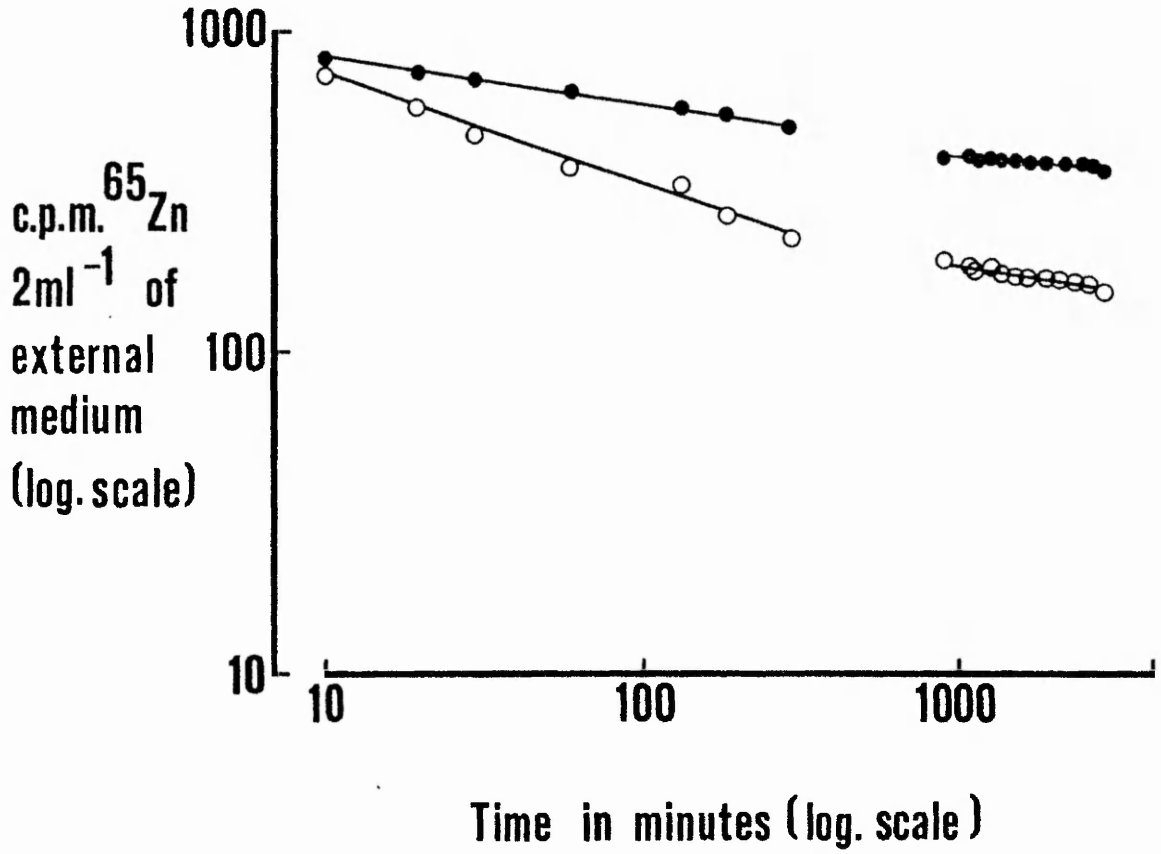

1000
100
10
c.p.m. ^{65}Zn $2ml^{-1}$ of external medium (log. scale)
10
100
1000
Time in minutes (log. scale)

Figure 26 Rates of zinc influx ($\chi \pm$ s.e., n = 10) in hard (▩) and soft water fish (□) exposed to 0.05 mg Zn l^{-1} during the fast and slow influx periods. Students t-test **P < 0.01.

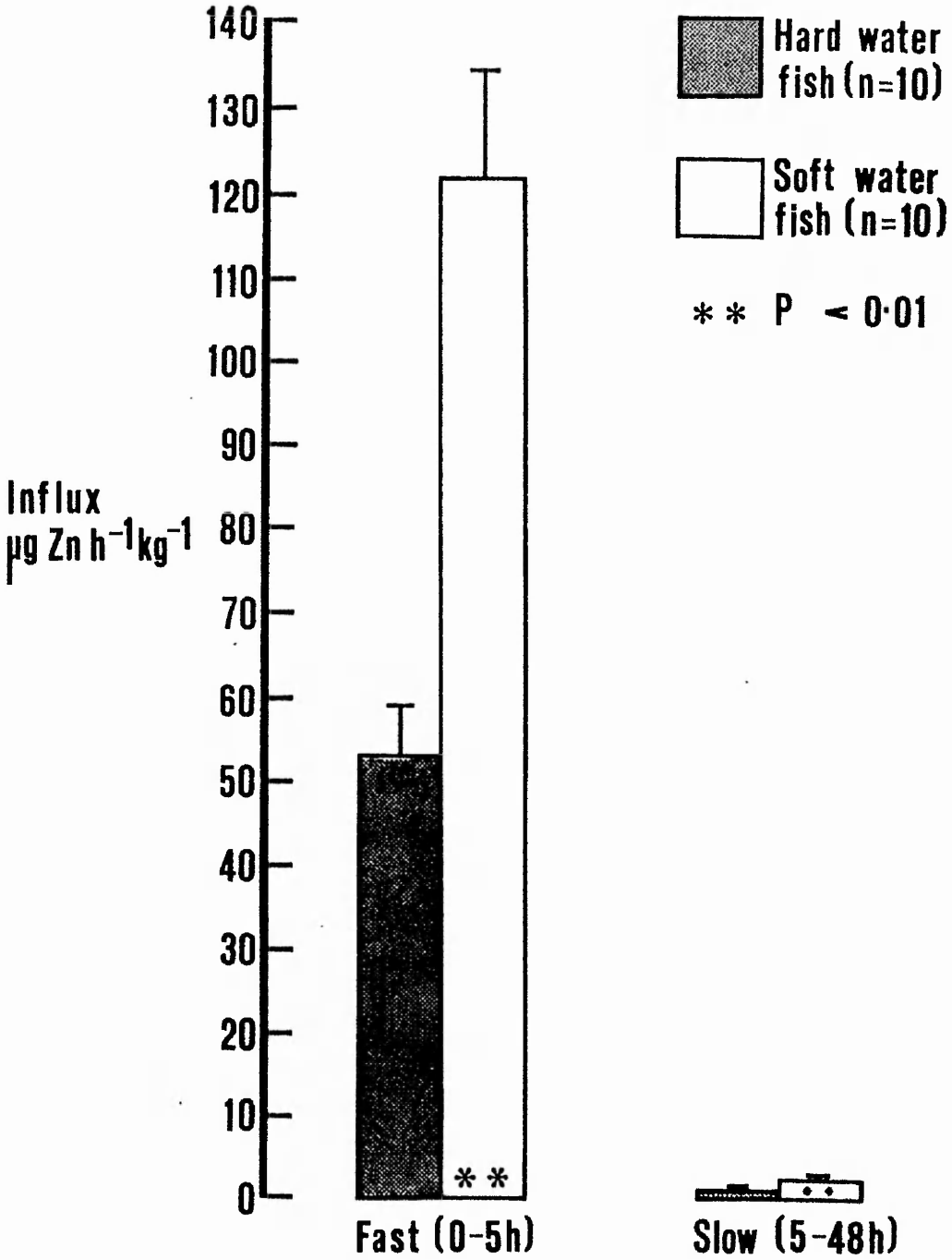

140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Influx
µg Zn h⁻¹kg⁻¹
Hard water fish (n=10)
Soft water fish (n=10)
** P < 0·01
**
Fast (0-5h)
Slow (5-48h)

Figure 27 Tissue accumulation of ^{65}Zn ($\chi \pm$ s.e., n = 10) after exposure to 0.05 mg Zn l^{-1} in hard (▩) and soft waters (□). Students t-test *P < 0.05, **P < 0.01 and ***P < 0.001.

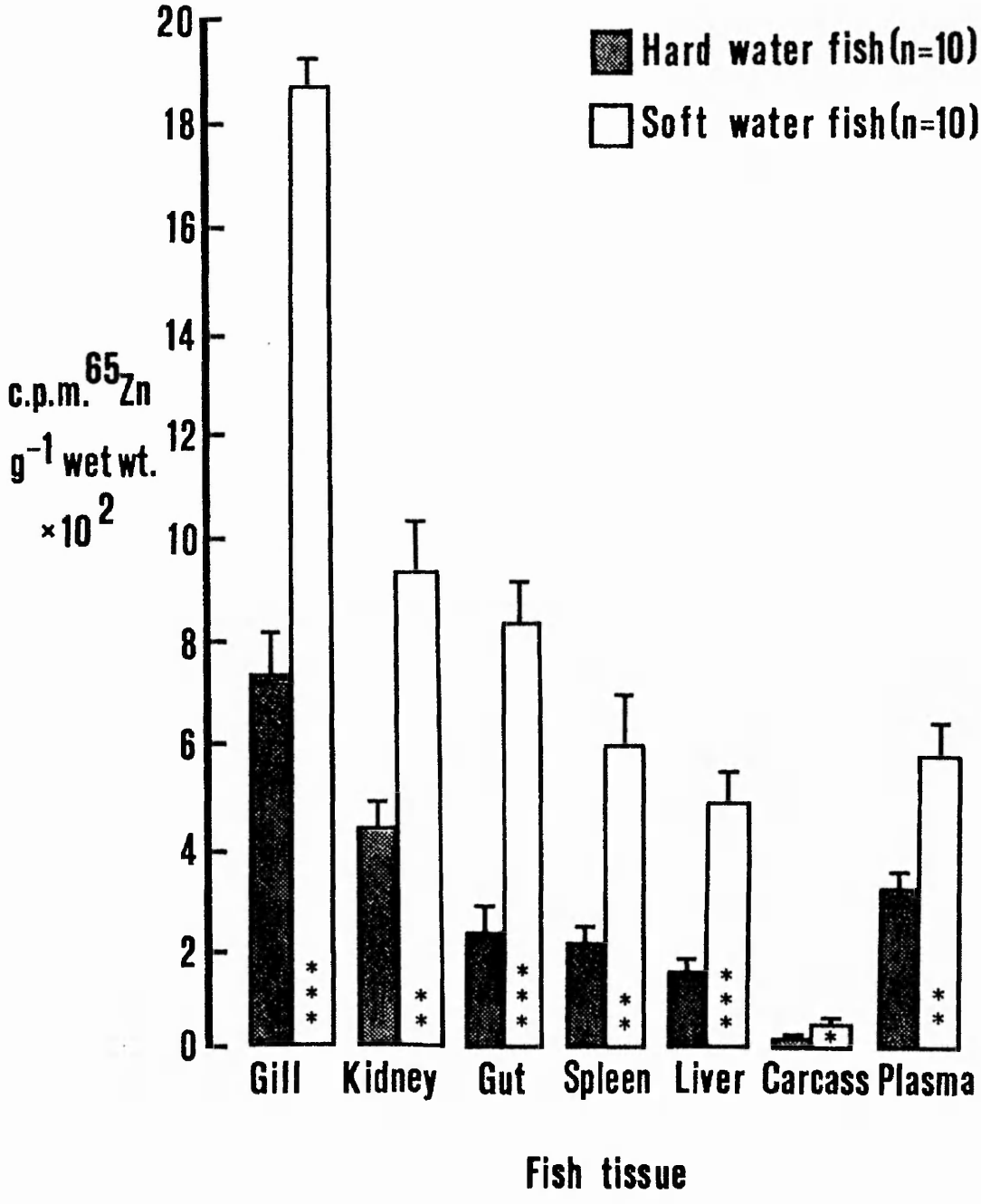

Hard water fish (n=10)
Soft water fish (n=10)
c.p.m. ^{65}Zn g^{-1} wet wt. $\times 10^2$
20
18
16
14
12
10
8
6
4
2
0
Gill
Kidney
Gut
Spleen
Liver
Carcass
Plasma
Fish tissue

Table 13 Comparison of ^{65}Zn loss from the test waters and the ^{65}Zn burden in the fish.

^{65}Zn distribution	α Radioactivity ^{65}Zn c min^{-1} (μCi)	
	Hard water (n = 10)	Soft water (n = 10)
Gross ^{65}Zn added at times o to ext. medium.	279636 700 ml^{-1} (10 μCi)	281013 700 ml^{-1} (10 μCi)
^{65}Zn lost from ext. medium	-128633 700 ml^{-1} (4,6 μCi)	-224810 700 ml^{-1} (8,0 μCi)
Gross ^{65}Zn per fish.	+121973 (46 g) $fish^{-1}$	+221470 (39 g) $fish^{-1}$
Net balance	-6660	-3340
% ^{65}Zn unaccounted for.	5,2%	1,5%
% of ^{65}Zn (zinc) uptake appearing in the fish.	94,8%	98,5%

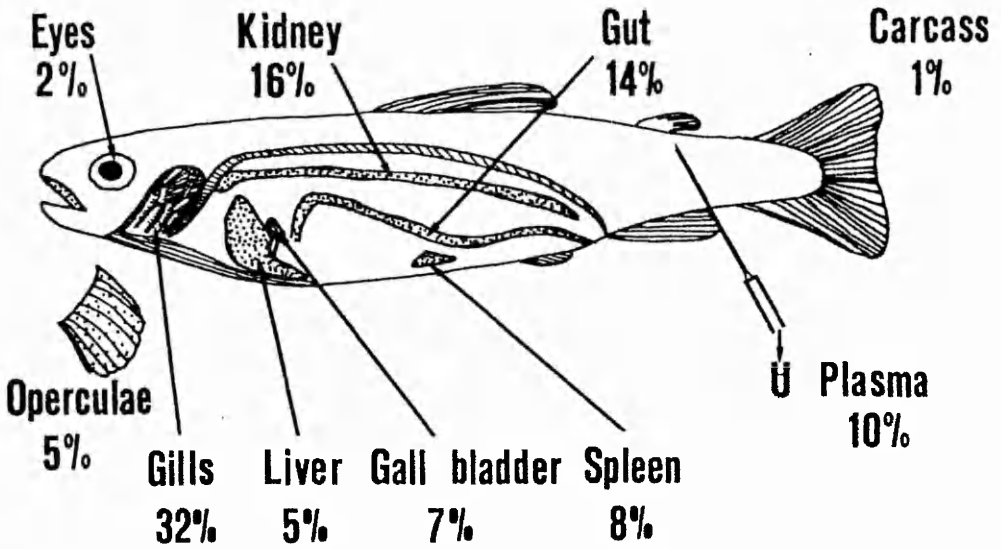
(Matthiessen and Brafield, 1977; Bradley and Sprague, 1985a). Fig. 28 summarises the body distribution of ^{65}Zn and stable zinc in fish following the 48h sublethal exposure in hard and soft water. In both sets of fish the gills were the major site of zinc accumulation with the kidney and gut as sites of lesser importance. Liver, gall bladder, spleen and plasma were also important sites. The residue of the fish included skin, skeleton, brain, muscle, fins and did not contain significant concentrations of zinc.

4.3.2.3 The 0-5 h rapid influx of zinc

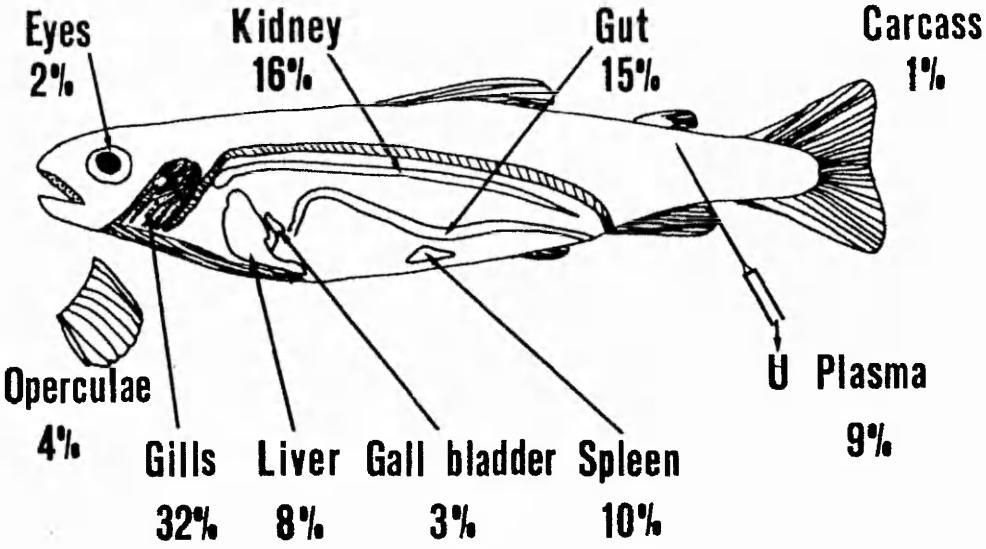
Further studies carried out on the fast influx component revealed an essentially consistent pattern of zinc uptake for both groups of fish as shown in Fig. 29. At the end of the 5 h exposure, ^{65}Zn analysis of fish tissues in Fig. 30 showed significant ($P < 0.05$) differences between ^{65}Zn contents of gills, kidney, gut and plasma of hard water fish compared with soft water fish. The quantities of zinc in the gill and plasma were not significantly different from values in fish sampled at 48 hours. However, kidney and gut zinc levels were significantly ($P < 0.05$) less than corresponding tissue concentrations after 48 hours.

Figure 28 Tissue distribution of ^{65}Zn and net accumulation of zinc after exposure to 0.05 mg Zn l^{-1} in hard and soft waters. Zinc distribution expressed as mean (n=10) percentage of total body accumulation in hard (A) and soft water fish (B). Also expressed as mean (n = 10) percentage of the total zinc uptake from the medium in hard (C) and soft water fish (D).

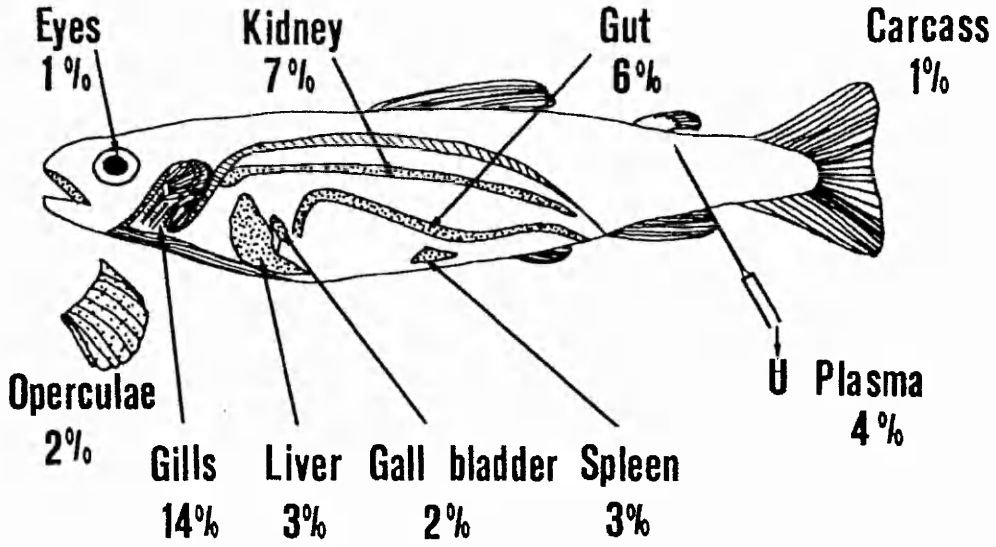
A


Eyes
2%
Kidney
16%
Gut
14%
Carcass
1%
Operculae
5%
Gills
32%
Liver
5%
Gall bladder
7%
Spleen
8%
U Plasma
10%

B


Eyes
2%
Kidney
16%
Gut
15%
Carcass
1%
Operculae
4%
Gills
32%
Liver
8%
Gall bladder
3%
Spleen
10%
U Plasma
9%

C


Eyes
1%
Kidney
7%
Gut
6%
Carcass
1%
Operculae
2%
Gills
14%
Liver
3%
Gall bladder
2%
Spleen
3%
U Plasma
4%

D

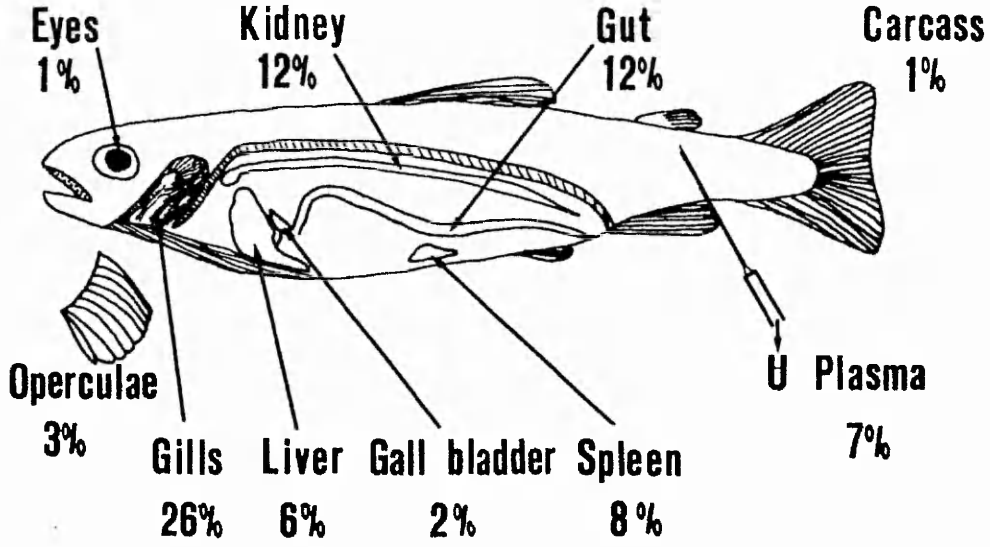

Eyes
1%
Kidney
12%
Gut
12%
Carcass
1%
Operculae
3%
Gills
26%
Liver
6%
Gall bladder
2%
Spleen
8%
U Plasma
7%

Figure 29 Influx curves of ^{65}Zn for hard (●) and soft water fish (○) exposed to 0.05 mg Zn l^{-1} during the fast uptake period. Control groups represented by C and dotted line indicates the range of curves from 10 fish in each group.

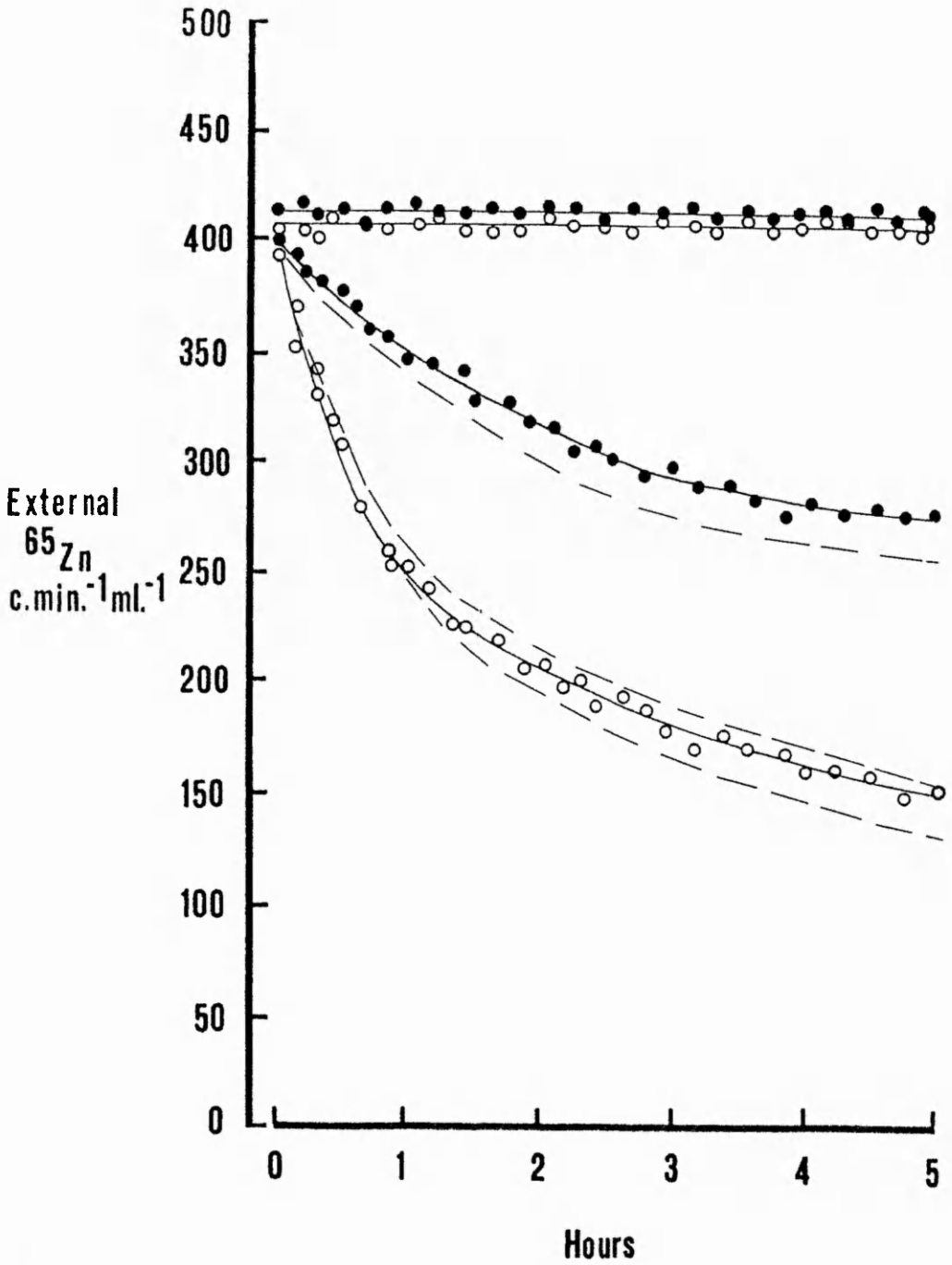
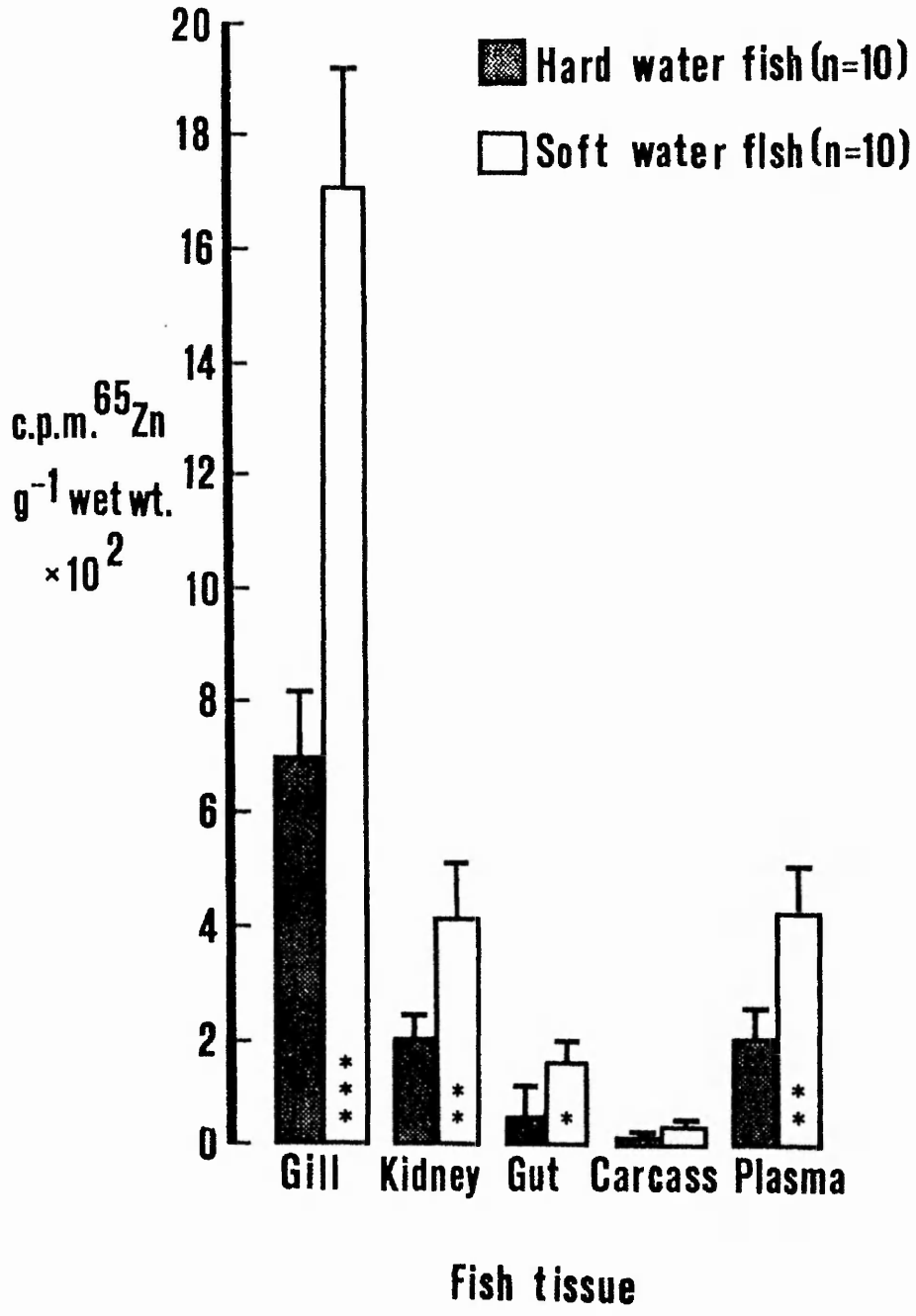

500
450
400
350
300
250
200
150
100
50
0
External
65Zn
c.min.-1ml.-1
0
1
2
3
4
5
Hours

Figure 30 Tissue accumulation of ^{65}Zn ($\chi \pm$ s.e., n = 10) after exposure to 0.05 mg Zn l^{-1} during the fast uptake period in hard (▩) and soft waters (□). Students t-test *P < 0.05, **P < 0.01 and ***P < 0.001.


Hard water fish (n=10)
Soft water fish (n=10)
c.p.m. ^{65}Zn
g^{-1} wet wt.
×10^{2}
20
18
16
14
12
10
8
6
4
2
0
***
**
*
**
**
Gill
Kidney
Gut
Carcass
Plasma
Fish tissue

4.3.2.4 The 5-48 h slow influx of zinc.

The effects of increasing the external zinc concentration on uptake of the metal during the slow influx period of hard and soft water fish is shown in Fig. 31. Returning both the radioactive and stable zinc concentrations to original levels after 48 hours did not alter the pattern of zinc influx; nor were the zinc uptake rates significantly different before and after zinc renewal in the external medium.

4.3.2.5 The role of calcium acclimation on zinc uptake

The influence of calcium as a component of water hardness on zinc influx may have been mediated through either long-term physiological effects during acclimation or the short-term effects of its concentration in the test waters. In Fig 32 typical examples of zinc influx plots from hard water acclimated fish which were exposed to zinc in soft water and soft water acclimated fish with zinc exposure in hard water are shown. The fish in both cases exhibited zinc uptake patterns which were primarily associated with long term effects of acclimation and were not affected by the abrupt changes in calcium levels of test waters. This long term response was reflected in the measured rates of zinc uptake which are shown in Fig. 33. There was no significant difference in the mean rate of zinc uptake between hard water acclimated fish which were exposed to

Figure 31 Influx curves of ^{65}Zn for hard (●) and soft water fish (o) exposed to 0.05 mg Zn l^{-1} at $time_o$ and renewal at 48 hours. Typical plots selected from 8 fish in each group are shown and ↑ indicates the point where ^{65}Zn and stable zinc concentrations were renewed.

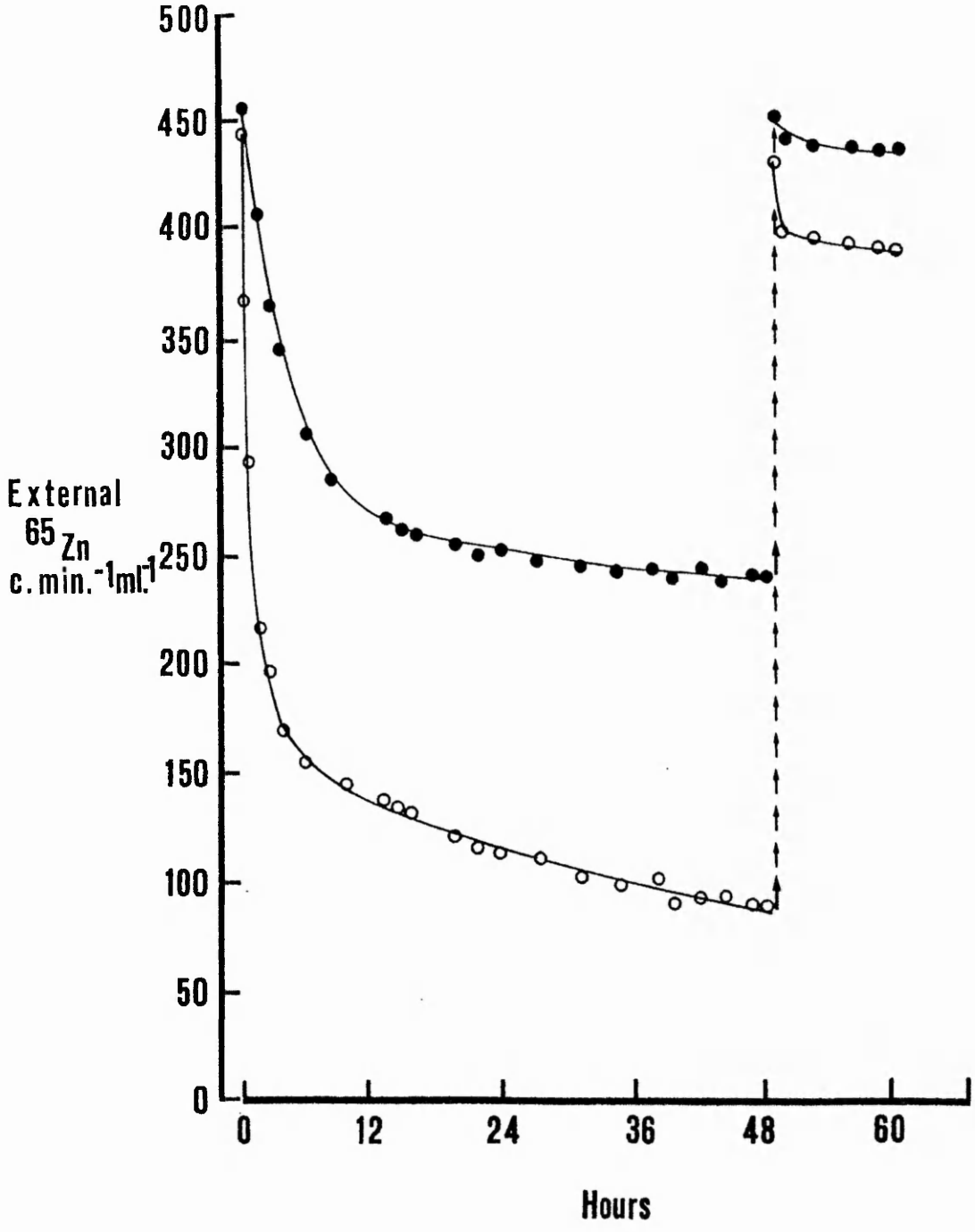

500
450
400
350
300
250
200
150
100
50
0
External
65 Zn
c. min.-1ml.-1
0
12
24
36
48
60
Hours

Figure 32 Influx curves of ^{65}Zn for hard (●) and soft water fish (○) exposed to 0.05 mg Zn l^{-1} in soft and hard waters respectively. Typical plots selected from 8 fish in each group are shown and a comparison with hard (▲) and soft water (△) acclimated and zinc exposed fish.

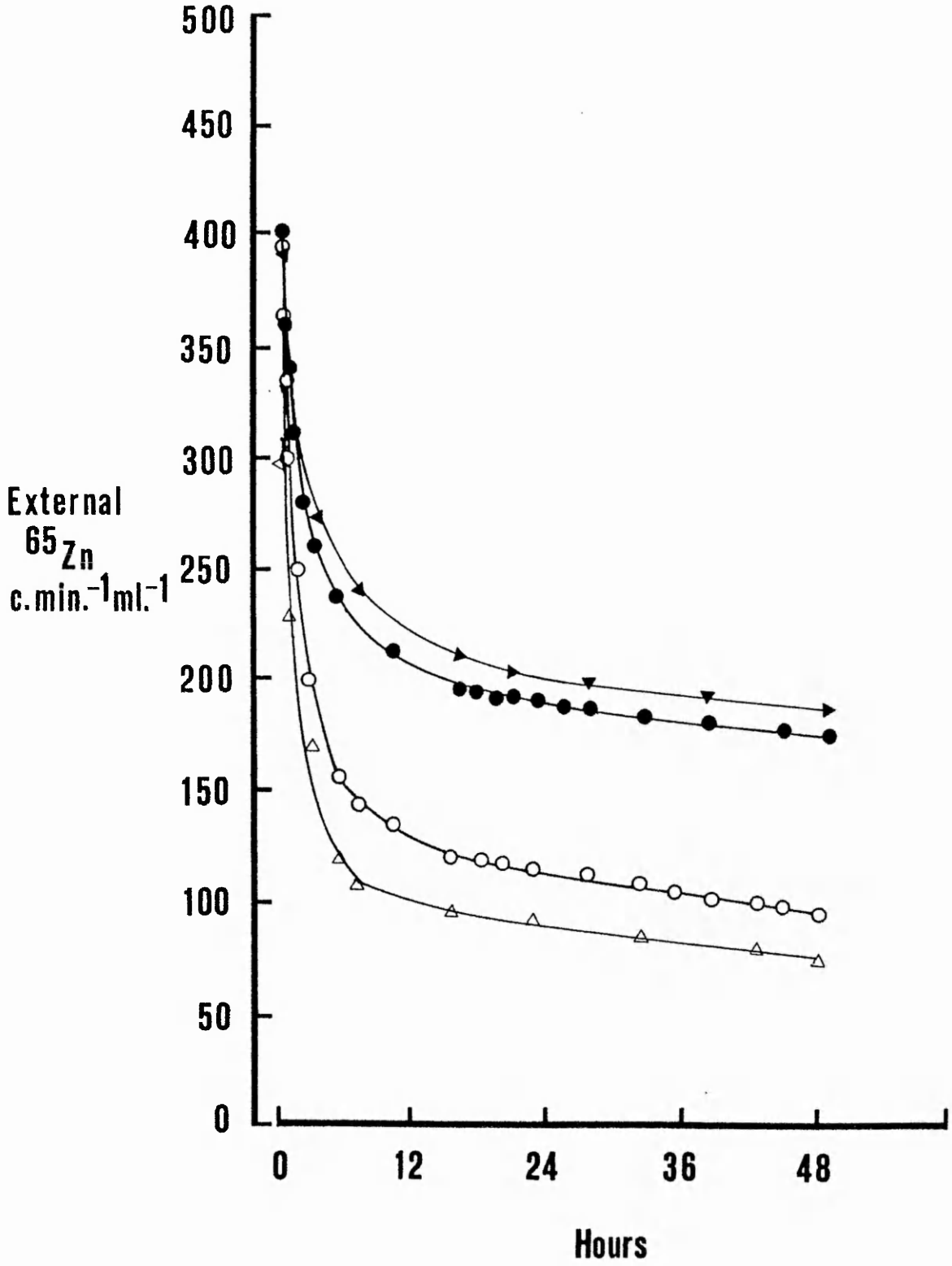
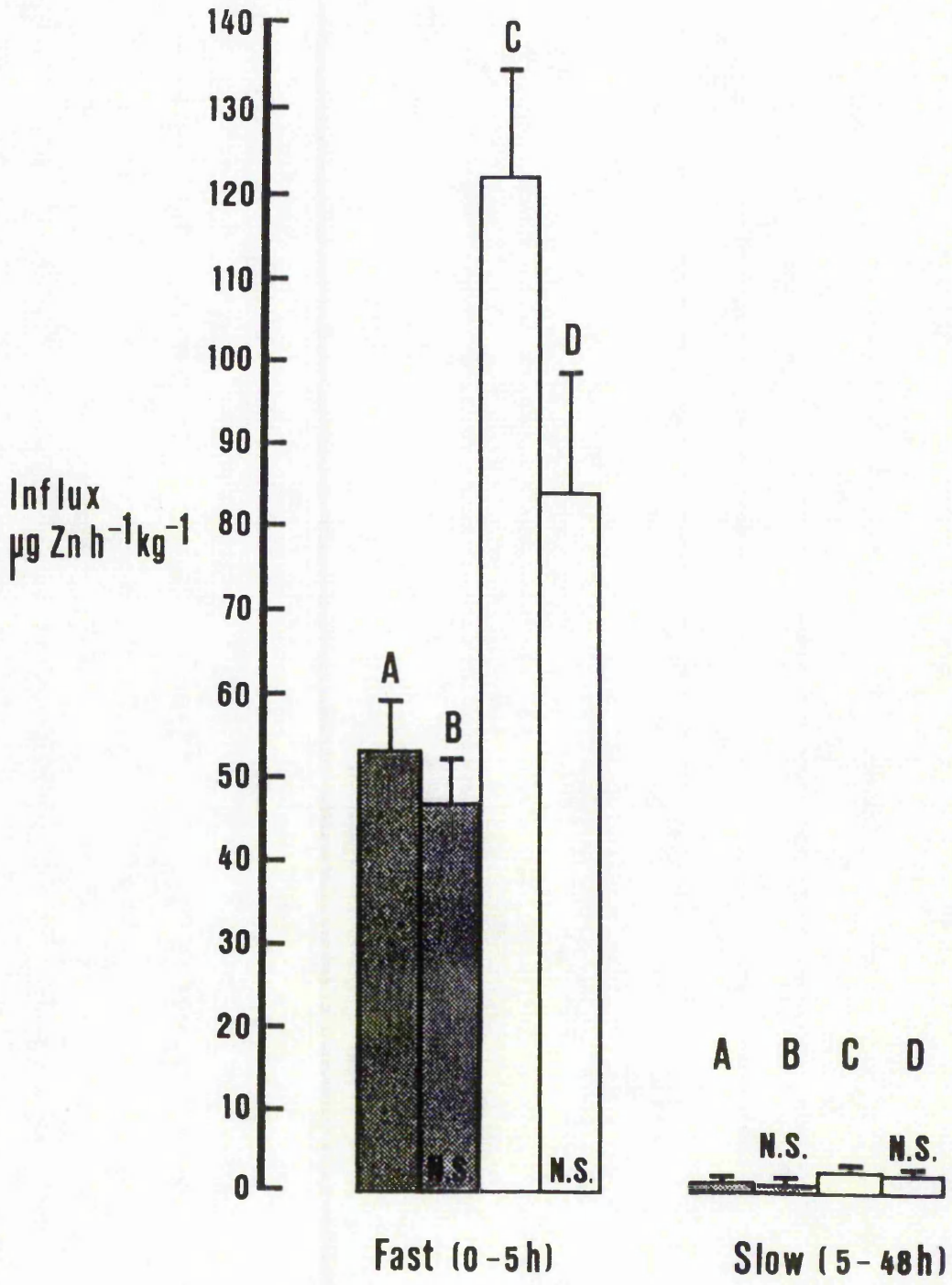

500
450
400
350
300
250
200
150
100
50
0
External
65Zn
c.min.-1ml.-1
0
12
24
36
48
Hours

Figure 33 Rates of zinc influx ($\chi \pm$ s.e., n = 10) in hard (A) and soft water fish (C) exposed to 0.05 mg Zn l^{-1} during the fast and slow influx period. In comparison, the rates of zinc influx ($\chi \pm$ s.e. n = 10) in hard (B) and soft water fish (D) exposed to 0.05 mg Zn l^{-1} in soft and hard waters respectively during the flux periods are also illustrated. Students t-test, N.S. is no significance between groups at any level of $P < 0.05$.


Influx
µg Zn h⁻¹kg⁻¹
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D
N.S.
N.S.
A B C D
N.S.
N.S.
Fast (0-5h)
Slow (5-48h)

zinc in soft or hard waters and soft water acclimated fish exposed to zinc in hard or soft water.

4.3.2.6 Zinc excretion

Shown in Fig. 34 are typical plots from hard and soft water fish during uptake and excretion phases with ^{65}Zn. During the first 48 hours both groups of fish showed characteristic patterns of zinc influx. Fish were 'loaded' with twice the radioactivity used in the influx studies to facilitate sufficient external media counts of ^{65}Zn during the excretion phase. After 48 hours when both sets of fish were switched to zinc-free media they exhibited a short fast zinc efflux lasting 1-4 hours. It was not possible to determine if this was simply due to surface washing or internal excretion processes. Over the remaining 44 hours there was a rapid release of zinc from hard water fish (5.6 ± 0.4 $\mu g\ h^{-1}\ kg^{-1}$) whereas there was a negligible loss from soft water fish (0.1 ± 0.07 $\mu g\ h^{-1}\ kg^{-1}$). Hard water fish rapidly lost zinc from tissues compared with soft water fish as illustrated by Fig. 35.

The body distribution of zinc was determined for the tissue data and is compared with net uptake and accumulation results in Fig. 36. In both groups of excreting fish there was a pronounced redistribution of zinc from a variety of tissue sites to the kidney and gut. This was evident in hard water fish with higher

Figure 34 Influx and efflux curves of ^{65}Zn for hard (●) and soft water fish (○) exposed to 0.05 mg Zn l^{-1}. Fish rinse indicate when the fish were bathed in zinc-free test waters and maintained in these media for a further 48 hours. Typical plots selected from 8 fish in each group are shown.

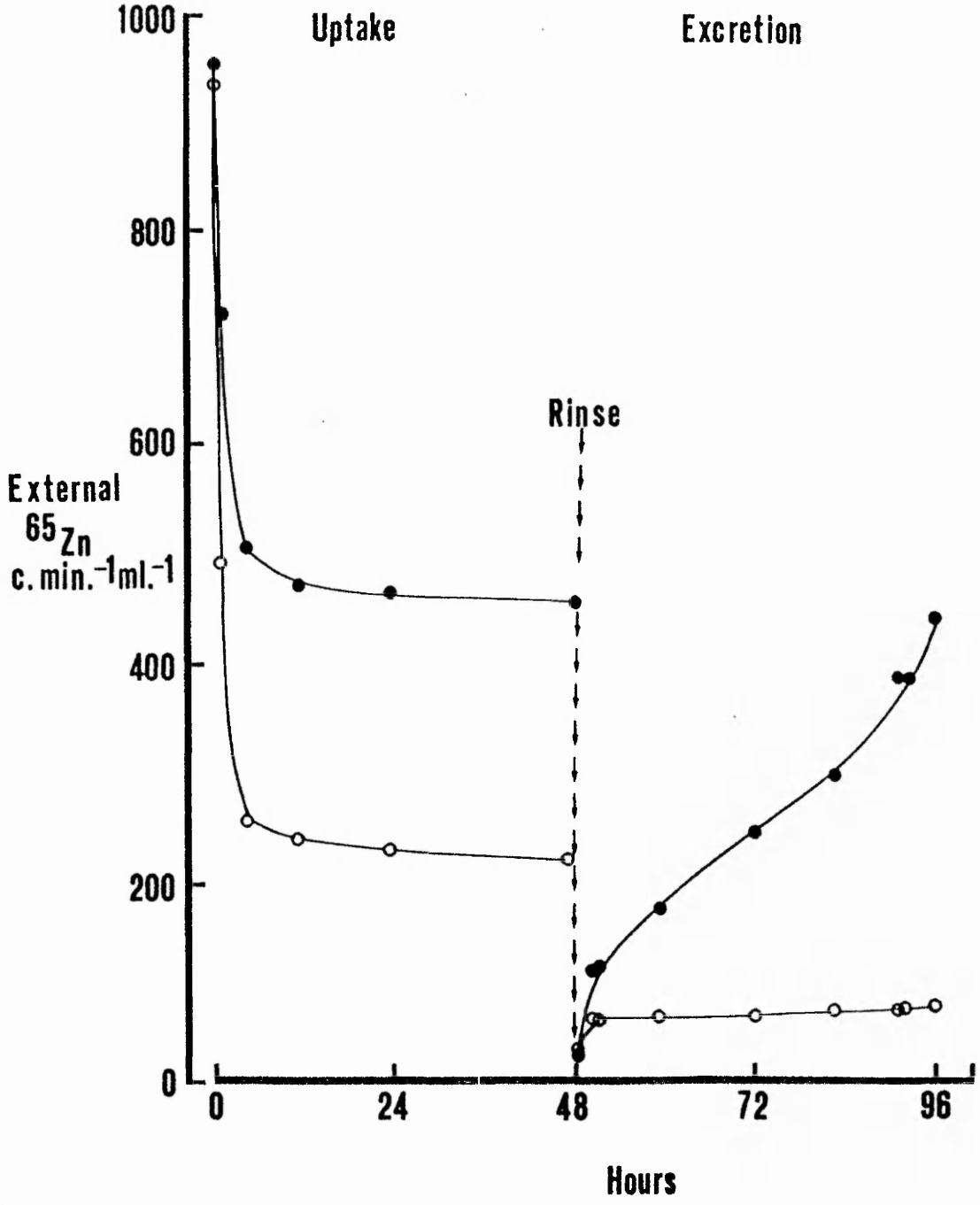

1000
800
600
400
200
0
Uptake
Excretion
Rinse
External
65Zn
c. min.-1ml.-1
0
24
48
72
96
Hours

Figure 35 Tissue loss of ^{65}Zn ($\chi \pm$ s.e., n = 8) following exposure to 0.05 mg Zn l^{-1} in hard (▩) and soft waters (□) Students t-test **P < 0.01 ***P < 0.001.

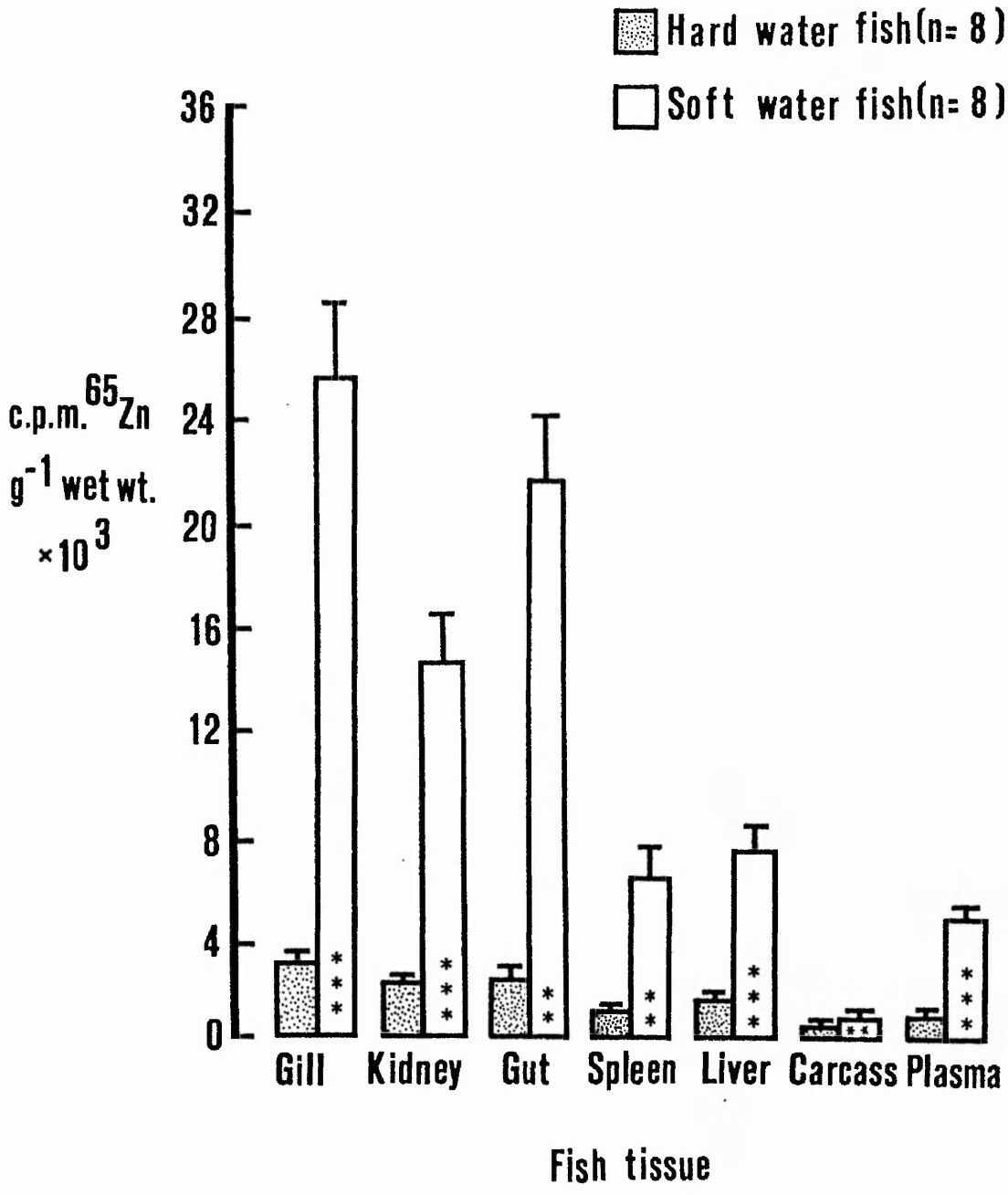
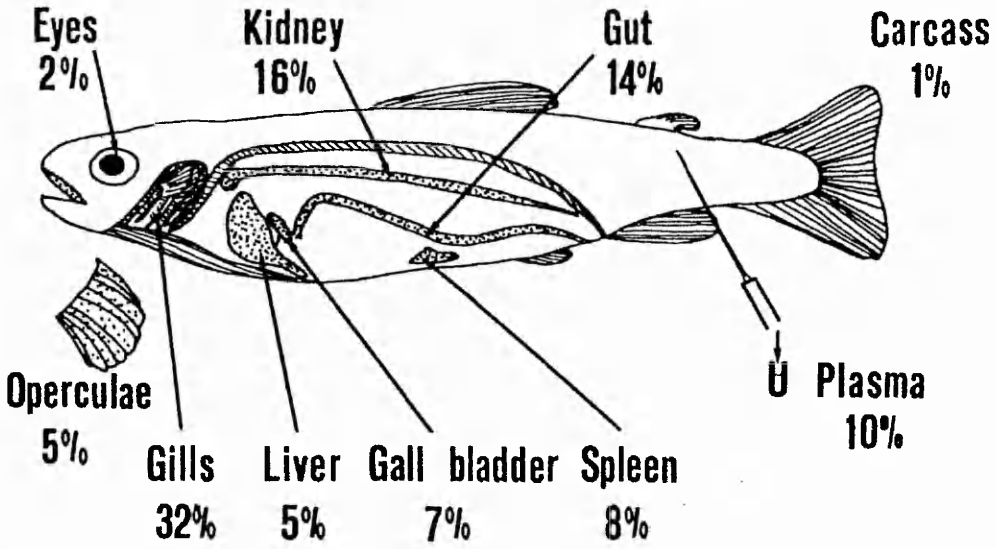
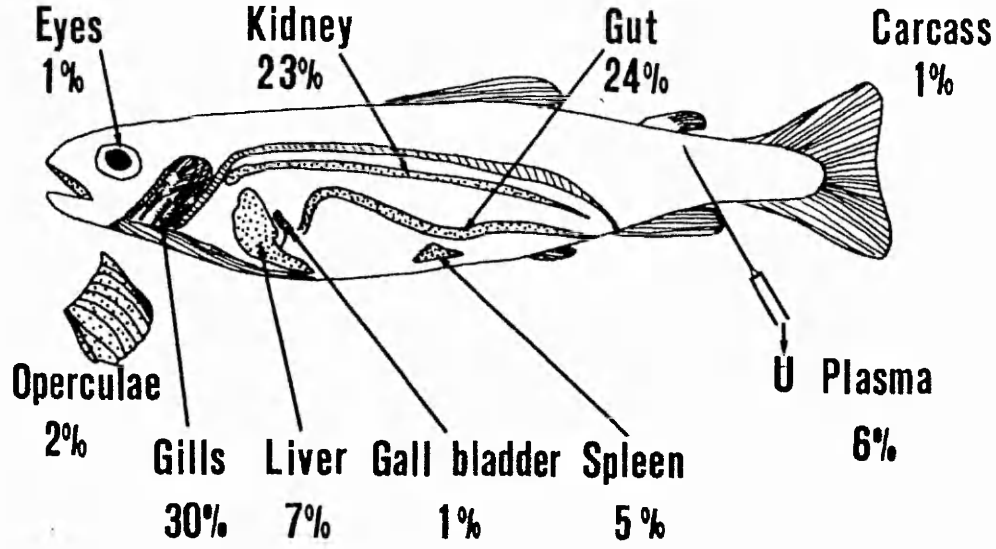

Hard water fish(n= 8)
Soft water fish(n= 8)
c.p.m. ^{65}Zn g^{-1} wet wt. $\times 10^3$
36
32
28
24
20
16
12
8
4
0
***
***
**
**
***
***
Gill
Kidney
Gut
Spleen
Liver
Carcass
Plasma
Fish tissue

Figure 36 Tissue distribution, accumulation and loss of ^{65}Zn and stable zinc after exposure in hard and soft waters. Zinc concentrations expressed as mean (n = 10) percentage of total body accumulation in hard (A) and soft waters (C) or as mean (n = 8) percentage of total body loss in hard (B) and soft waters (D).

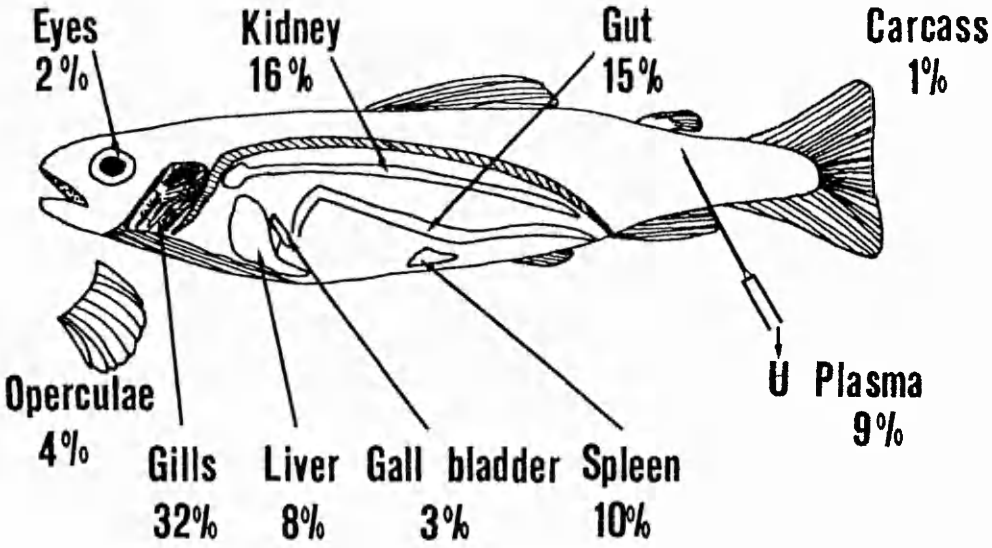
A


Eyes
2%
Kidney
16%
Gut
14%
Carcass
1%
Operculae
5%
Gills
32%
Liver
5%
Gall bladder
7%
Spleen
8%
U
Plasma
10%

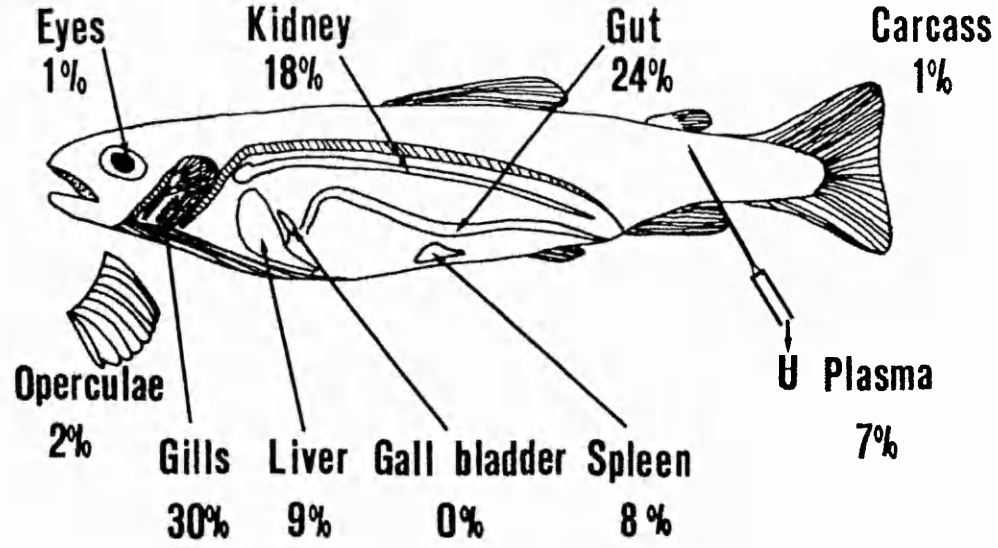
B


Eyes
1%
Kidney
23%
Gut
24%
Carcass
1%
Operculae
2%
Gills
30%
Liver
7%
Gall bladder
1%
Spleen
5%
U
Plasma
6%

C


Eyes
2%
Kidney
16%
Gut
15%
Carcass
1%
U Plasma
9%
Operculae
4%
Gills
32%
Liver
8%
Gall bladder
3%
Spleen
10%

D


Eyes
1%
Kidney
18%
Gut
24%
Carcass
1%
U Plasma
7%
Operculae
2%
Gills
30%
Liver
9%
Gall bladder
0%
Spleen
8%

excretion rates and reflected in the kidney zinc levels. However, liver zinc concentrations rose and spleen levels fell in a similar pattern for both groups of excreting fish. Plasma and gall bladder zinc levels were reduced in both hard and soft water fish during excretion. Gill zinc levels were not significantly altered in excreting fish when compared with fish during uptake and suggested a possible excretory function. Surface tissue zinc levels were also reduced in excreting fish but zinc levels in the carcass remained unaltered from uptake levels.

4.4. DISCUSSION

Present studies of zinc uptake at pH 6 were undertaken to determine the mechanisms by which the toxicity of the metal to the fish may be influenced by external water hardness. Data regarding the sensitivity of metal interactions at biological surfaces were not readily available in the relatively few reports to be found in the literature (Campbell and Stokes, 1985). Often the pH of the external medium has been varied experimentally with no consideration of the possible effects of this change on the speciation of the adsorbate in solution. pH was closely controlled in the present study in order to study the biological response of fish through zinc uptake and accumulation in hard of soft waters and to avoid responses due to or involving chemical speciation of the metal. As previously reported the biological-protective effect of increased calcium levels is evident from toxicological studies (Lloyd, 1965 and Calamari et al., 1980) and zinc could be toxic to fish because of either its surface activity/biological sensitivity at cell surfaces as well as the internal effects it might have. To elicit a biological response from a fish and to accumulate within this animal, a given metal must obviously interact with or traverse a cell membrane.

Tissue sites for the possible surface interactions and absorption of zinc include the mucosa, buccal and opercular cavities, the gut and the gill. No evidence from this study or those of other workers has been produced to implicate the skin as an important route for zinc adsorption or absorption. Dietory absorption has been suggested as a major route for heavy metal contamination in aquatic animals (Bryan, 1976). In crabs (Carcinus maenas) and shrimp (Lysmata seticaudata), absorption of ^{65}Zn from food appeared to be unimportant compared with direct uptake from the water (Renfro et al., 1975). Since the brown trout were starved before and during tests drinking was the only source of zinc for uptake via the gut. Rainbow trout in fresh water have a low drinking rate of 1.43 ml kg^{-1} h^{-1} (Bath and Eddy, 1979) which is reduced (0.26 ml kg^{-1} h^{-1}) in zinc-exposed trout (Lovegrove and Eddy, 1982). Joyner (1961) concluded that swallowed water did not significantly contribute to gastro-intestinal zinc uptake and accumulation for bullheads (Ictalurus nebulosus). Thus in the brown trout the gut was probably not a significant route for zinc uptake.

Other workers have shown the gills to be the major site of zinc uptake in fish (Joyner, 1961; Slater, 1961; Matthiessen and Brafield, 1977; Bradley and Sprague, 1985a). Both acute and sublethal studies of zinc uptake from this work showed the gills were the major site of

metal influx and accumulation in hard and soft waters. The mechanism leading to a biological effect may involve the reaction of the metal ion at a metabolically active site on the gill surface, resulting in a direct biological response (Pagenkopf, 1983). Alternatively. binding at the gill surface sites may simply precede transport into the cells, the reaction with the metabolically active centre occurring intracellularly (Harrison and Morel, 1983).

Assuming the majority of zinc uptake occurred via the gills, then branchial metal influx appeared to be composed of two rate components. A fast phase of zinc influx over 0-5 hours was followed by a slower rate over 5-48 hours. The first component of zinc uptake was thought to involve adsorption of the metal ion to metabolically active sites at the gill surface and influx i.e. absorption into the cells. Initial adsorption effects may simply be an effect due to the volume of the external medium. However, secondary uptake was unaffected by changes in external zinc concentrations and probably also occurs during the fast phase but is masked by the effects of surface adsorption. It appeared that both surface adsorption and influx of zinc were reduced in the presence of high external calcium concentrations.

Joyner (1961) suggested the mechanisms of zinc uptake may involve adsorption of zinc to external gill mucus

followed by inward diffusion and eventual adsorption of the metal to unspecified tissue sites. The present work suggested that diffusion was probably occurring during both the slow and fast phases of zinc uptake as determined by the blood and tissue accumulation of the metal during both phases. However, differences between low and high zinc influxes in high and low calcium concentrations suggested a rate-limiting mechanism was operating. The mechanism may be rapidly established during the surface adsorption/binding phase at the gill, where in the presence of high external (medium) or internal (tissues) calcium levels the transport of zinc through branchial channels or across cell membranes may be inhibited. The protective effects of calcium appeared to be internal, hard water acclimated fish exposed to zinc in soft waters exhibited influx rates associated with hard water fish. Hence, the internal effects of acclimation to high calcium levels (70 mg l^{-1}) were not overcome by surface effects of low calcium concentrations (1 mg l^{-1}) when bathing the gills for 48 hours.

The mechanism of zinc absorption may be mediated by internal calcium levels at the gills by transcellular or paracellular pathways. Diffusion of ions other than trace metals is thought to occur largely via a transcellular route (Sardet et al., 1979) but paracellular movement may be important (Steen and Stray-

Pederson, 1975). Active transcellular routes of membrane transport for zinc could involve mobile protein carriers at apical and/or basolateral membranes or protein-lined channels spanning apical membranes of the branchial epithelium. Paracellular routes involve extracellular fluid filled intercellular channels in the outer (mucosal) epithelial layer which appear to be regulated by apical tight junctions and desmosomes (Morgan and Tovell, 1973; Sardet et al., 1979). The present studies suggested that reduced zinc influx at high calcium levels was caused by depressed branchial permeability. A possible calcium-mediated mechanism may operate through paracellular routes involving the extracellular channels. These channels are controlled by tight-junctions whose permeability is restricted, at least in part, by calcium bound to fixed negative charges in the intercellular cement (Oschman, 1978). Calcium is well documented for its effects on biological systems in the bound state because of its ability to provide reversible cross links between proteins and other cell wall structures (review: Williams, 1976). The mechanism leading to a biological effect might then involve the reaction of zinc ions at these metabolically-active internal branchial sites resulting in the response of different influxes of the metal. So, calcium might be liberated from paracellular pathways by zinc and so open these channels to zinc influx and ionic

effluxes (as observed in brown trout for sodium and chloride). So, the effects of water hardness on zinc uptake may depend upon the degree of calcium saturation of these channels during acclimation. Initial metal adsorption during the fast uptake phase might reflect zinc exchange for calcium in branchial channels and may be integral to the protective mechanism of calcium in hard waters.

In hard waters, gill permeability and consequently zinc influx were significantly reduced and appeared to be uninfluenced by changes in external metal concentration. However, if the uptake mechanism for the metal was dependent upon surface permeability changes altering the diffusion rate of the metal then increasing the external zinc concentration might be expected to have increased the influx rate. Since this did not occur in either hard or soft waters it is again suggested that the influx of the metal was dependent upon preconditioning of the uptake mechanism by calcium apparently during hardness acclimation.

With acute zinc exposures the pattern of zinc influx was difficult to discern and it was not clear which period of uptake may have involved surface adsorption. These observations were in accord with other workers who described the pattern of zinc uptake in other species as constant (Joyner, 1961; Slater, 1961). Unless stated otherwise further discussion of the zinc uptake

mechanism will be confined to observations from sublethal studies.

Tissue zinc levels at the end of the initial fast uptake phase indicated that the metal had penetrated the tissues and established tissue pools. The extent of adsorption vs influx during this initial phase could not be determined but it is suggested that influx rate of the metal during the second phase would involve exchange between tissue pools and excretion. Joyner (1961) suggested such exchanges would initially depend upon diffusion gradients established between the gill and the blood. In brown trout, zinc levels in the gill were much higher than those of the blood after 5 and 48 hours. Potential diffusion gradients between tissue compartments were thus established during the initial influx phase and remained so, during the secondary influx period.

Once within the blood, evidence suggests the majority of zinc is unbound (80%) and thus available for exchange with body tissues (Pentreath, 1973). During the slow secondary phase, zinc uptake would also be dependent upon tissue influx from the blood and eventually excretion processes. Influx rates of the metal were reflected by tissue accumulations and were consequently higher in soft water fish. It was suggested by Joyner (1961) that absorption may be at a rate just sufficient to replace zinc removed from the blood by adsorption,

exchange with tissue components and possibly excretion. Internal protective effects by calcium may therefore also be important in terms of zinc exchange between tissue compartments and the excretion of the metal.

Muscle, skin, bone and eyes accounted for only a very small proportion of the total zinc uptake in hard and soft water fish. Liver and spleen appeared to be tissue pools of some secondary importance to gut and kidney. These bioaccumulation and tissue studies may indicate that liver, spleen and kidney are important sites of detoxification possibly due to their phagocytic and associated storage properties. Spleen had a high melano-macrophage concentration in both groups of zinc exposed fish and may be important in terms of tissue metal burdens and possible detoxification. It was suggested earlier in this work that an immunological response may be important in the process of zinc detoxification and it has been noted that black coloured fish absorbed a large amount of zinc in their pigment layer (Slater, 1961). Possible combinations of zinc with melanin or associated intermediates are worthy of further investigation particularly in relation to metal uptake and tissue accumulation in fish. This may be a separate protective mechanism against metal toxicity in addition to the role of water hardness. The liver had a high zinc content in both groups of exposed fish and so did the gall bladder fluid which suggests an excretory

function via bile salts through the intestine. This may partly account for the high zinc levels in the intestine of starved fish following a 48 h exposure to the metal.

Studies of zinc uptake and accumulation suggested that reduced metal uptake at high hardness resulted from an internal calcium-induced reduction in branchial permeability. In conjunction, accumulation and possibly detoxification were respectively reduced and enhanced in fish acclimated to high hardness. Both factors of zinc influx and tissue accumulation had an effect on intrinsic toxicity with acute exposures and suggest an important long-term toxic action with sublethal exposure. To complete the basic understanding of the mechanisms involved in the protective action of high calcium levels upon zinc toxicity, the process of excretion was studied.

Zinc efflux was greater in metal exposed fish from hard waters, after being placed in zinc-free hard water. Efflux of zinc in soft water fish appeared to be negligible . It is suggested that hardness acclimation may have an important bearing upon the ability of fish to excrete zinc following exposure to the metal. Calcium might facilitate zinc excretion, a process which may, however, be dependent on either internal protective effects of this ion or concentrations in the external media or on both. The influence of hardness on the sites of tissue excretion during zinc efflux remain to

be determined. Fish probably excrete zinc by way of the gut and gills (Matthiessen and Brafield, 1977) with some evidence in favour of the gut of brown trout recorded during the period of net influx. Hard water trout excreted some 40 - 50% of their accumulated zinc after only 48 hours. In these fish the gut and kidney zinc concentrations increased by a factor of 2 compared with fish during a period of net uptake but gill levels in both uptake and excreting groups changed little. This indicated that the gills may also be important as an excretory route along with the gut and the kidney. Such observations support Nakatini's (1966) conclusion that rainbow trout removed ingested ^{65}Zn via the gills rather than the urine. Matthiessen and Brafield (1977) further proposed that the chloride cells may have a general excretory role in the removal of zinc from sticklebacks, but Pentreath (1973) found no evidence of ^{65}Zn concentrations in such cells of excreting plaice. Chloride cells are probably important in calcium uptake in rainbow trout (Payan et al., 1981) but the rates of zinc influx and efflux obtained in brown trout from sublethal studies are several orders of magnitude lower than the values for calcium fluxes. Whilst the gills appear to play some role in zinc excretion it seems unlikely that chloride cells are involved in this process.

Gut and kidney zinc concentrations rose in both groups of excreting fish compared to fish during the phase of net influx. Zinc appeared to be translocated from other tissue pools (e.g. spleen and blood) perhaps in a similar manner to the kidney of brook trout (Holcombe et al., 1979). Increased gut and kidney levels of the metal suggested excretory roles for these tissues but in mammals, excretion of endogenous zinc takes place predominantly through the faeces (Miller, 1971). Pentreath (1973) has proposed that such a route may supplement renal excretion in teleosts. Since fish in the present excretion studies were starved and zinc accumulated in the gut, the case for active excretion seems unlikely. Greater zinc losses may have occurred if brown trout had been fed (Bryan, 1967) but then greater uptake would also probably have taken place (Hoss, 1964; Pentreath, 1976). However, the kidney may have had an active role in zinc excretion along with the gills. Efflux of ^{65}Zn through the kidney of plaice was found to be very high, second only in importance to the gill (Pentreath, 1973).

Low and negligible efflux rates of zinc in hard and soft water fish respectively seemed indicative of metal-transfer mechanisms with slower turnovers than indicated by the influx mechanisms operating during metal uptake. It has not been determined if the processes of zinc excretion are active or passive but they appeared to be

limited by either external or internal protective effects of calcium at high hardness.

Renfro et al., (1974a) concluded that in adult fish refractory pools of zinc exist which exchange slowly if at all, with zinc absorbed from water or food. The nature of these refractory pools remains largely undetermined but at tissue level zinc has general binding properties for compounds such as proteins, polysaccharides and amino acids (Bryan 1976). Coombs (1974) found that in normal oysters (Ostrea edulis), about 60% of the zinc was firmly bound to cell debris but was readily exchangeable with zinc ions, 18% was bound less firmly and 40% was weakly bound to small molecular weight compounds such as taurine, lysine and A.T.P.ases. Zinc is also strongly bound to intracellular storage proteins like metallothioneins (Bradley et al., 1985) and in extracellular melano-macrophage aggregates of brown trout, which may both serve as detoxification agents. Calcium ions may interfere with zinc binding to proteins by competing for binding sites and thereby preventing zinc from making irreversible bonds with enzymes. In this way calcium may directly protect enzymatic biochemical processes at cell level.

Fish acclimated to high hardness have increased cellular content of calcium (Houston, 1959) which may help to explain the greater zinc efflux in hard water

fish. However, the formation of stable or insoluble storage products of zinc in the tissues of exposed fish would suggest a tendency for zinc loss in the absence of metal exposure to be small, which was found to be the case in the present studies.

The long-term toxicity of zinc to fish at environmentally relevant concentration of 0.05 mg l^{-1}, particularly in soft waters (Burns et al., 1984; Wright and Henriksen, 1978; Spry et al., 1981; Campbell and Stokes, 1985), was dependent upon the mechanisms of uptake, accumulation and excretion of the metal. The mechanisms governing zinc toxicity appear to involve reduced branchial permeability at high hardness resulting in reduced metal uptake. This process may depend upon internal branchial protection of calcium resulting from acclimation to high hardness levels in the external media. A further internal protective mechanism appears to exist involving the storage and possibly the detoxification of the metal by melano-macrophage centres. This process was independent of calcium protective effects at tissue level. Zinc excretion also appears to be calcium-mediated but it was not determined if this process was due to internal calcium levels or externally via surface effects of calcium in the external medium.

The uptake of other trace metals (cadmium and manganese) has also been shown to be reduced at high

hardness and has been attributed to calcium induced effects (Reader, 1986). Manganese concentrations of 2.2 μmol l^{-1} were the only trace metal levels reasonably comparable to zinc in the present study of 0.76 μmol l^{-1} (0.05 mg l^{-1}). Calcium concentrations used in the studies on cadmium and manganese were 0.8 and 20.4 mg Ca l^{-1} for artificial soft and hard waters respectively. These conditions are reasonably comparable with the 1.0 and 60.0 mg Ca l^{-1} used for artificial soft and mains hard water in the studies on zinc. Both studies used brown trout from the same source and test conditions varied by ± 5°C and ± 0.8 of a pH unit between studies. However, uptake of cadmium, manganese and zinc were all markedly greater at the lowest calcium concentrations.

It was proposed that cadmium and manganese enter the fish primarily by competition with calcium for sites on the mechanism for the active uptake of calcium but that passive uptake components may be involved (Reader, 1986). In terms of zinc uptake and toxic effects it is difficult to determine how closely related the processes of trace metal and calcium uptake might be. However, zinc and other trace metals may cause intoxication due to disturbances in calcium uptake and homeostasis (Spry and Wood, 1985; Reader 1986). Reduction in calcium uptake by exposure to 0.8 mg Zn l^{-1} and an associated fall in plasma calcium levels have been recorded for rainbow trout (Spry and Wood, 1985). It is possible

that zinc may displace calcium as a substrate for the Ca-ATPase found in fish gill (Fenwick, 1976). The route of calcium transport is not certain but is thought to occur by way of the chloride cells (Payan et al., 1981). The activity of Ca-ATPase has been shown to be dependent upon calmodulin, a Ca^{2+}-transport regulator protein (reviewed by Cheung, 1980; Moore and Dedman, 1982). Lock et al., (1985) reported that cadmium (Cd^{2+}) severely depressed calcium uptake in rainbrow trout which was demonstrated to be due to Cd^{2+} displacing Ca^{2+} from Ca^{2+}-binding sites on calmodulin. This resulted in a decreased affinity of the phosphodiesterase enzyme for the Cd^{2+}-calmodulin complex and a decreased stimulation of calcium uptake. However, the inhibition process appeared to be specific to cadmium and zinc does not appear to behave chemically in the same way (Lock, et al., 1985). Possibly the cause of the disruption in calcium transport mechanisms by zinc (Spry and Wood, 1985) may be determined by biochemical studies on isolated enzyme systems from fish gills (Watson and Beamish, 1980; Nieminen et al., 1982).

Present studies with zinc indicated that increased water hardness protects fish from both acute and long-term toxic effects of the metal by reducing metal uptake and excretion. The ameliorating influence of hardness was attributed to the effects of calcium during acclimation and exposure. Unlike observations on calcium

(Lock, et al., 1985; Reader, 1986), zinc uptake and calcium showed little evidence of a direct relationship. However, physiological effects of zinc exposure such as disturbances to calcium metabolism should be considered in assessing the long-term toxicity of the metal, particularly in waters of low calcium content.

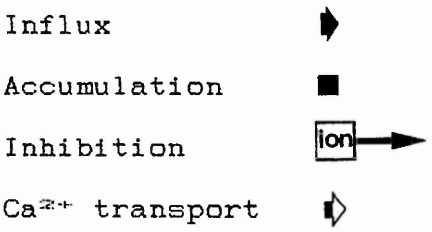
The effects of pH on zinc uptake were not determined from this study but guidelines for work of this nature are given by Campbell and Stokes (1985). McDonald et al., (1986) state that copper and cadmium uptake by rainbow trout during short exposures (< 24h) is reduced at pH 5 compared to pH 7 and may largely reflect a reduction in surface adsorption of the metal but not true tissue uptake. However, the toxicity of these metals to rainbow trout is sharply reduced at acid pH (pH 4.7) compared with more alkaline pH values (pH 5.7 and 7.0; Cusimano et al., 1986). During what appeared to be a partial adsorption phase in the pattern of zinc uptake in brown trout at pH 6 actual tissue uptake also occurred. Therefore, for zinc uptake in the pH range 5 - 7 the pattern of metal influx could not be explained simply by surface adsorption alone. Further studies of branchial surface-binding and uptake of trace metals over a range of pH and calcium levels are required to determine the mechanisms of pH, calcium and metal influx interactions.

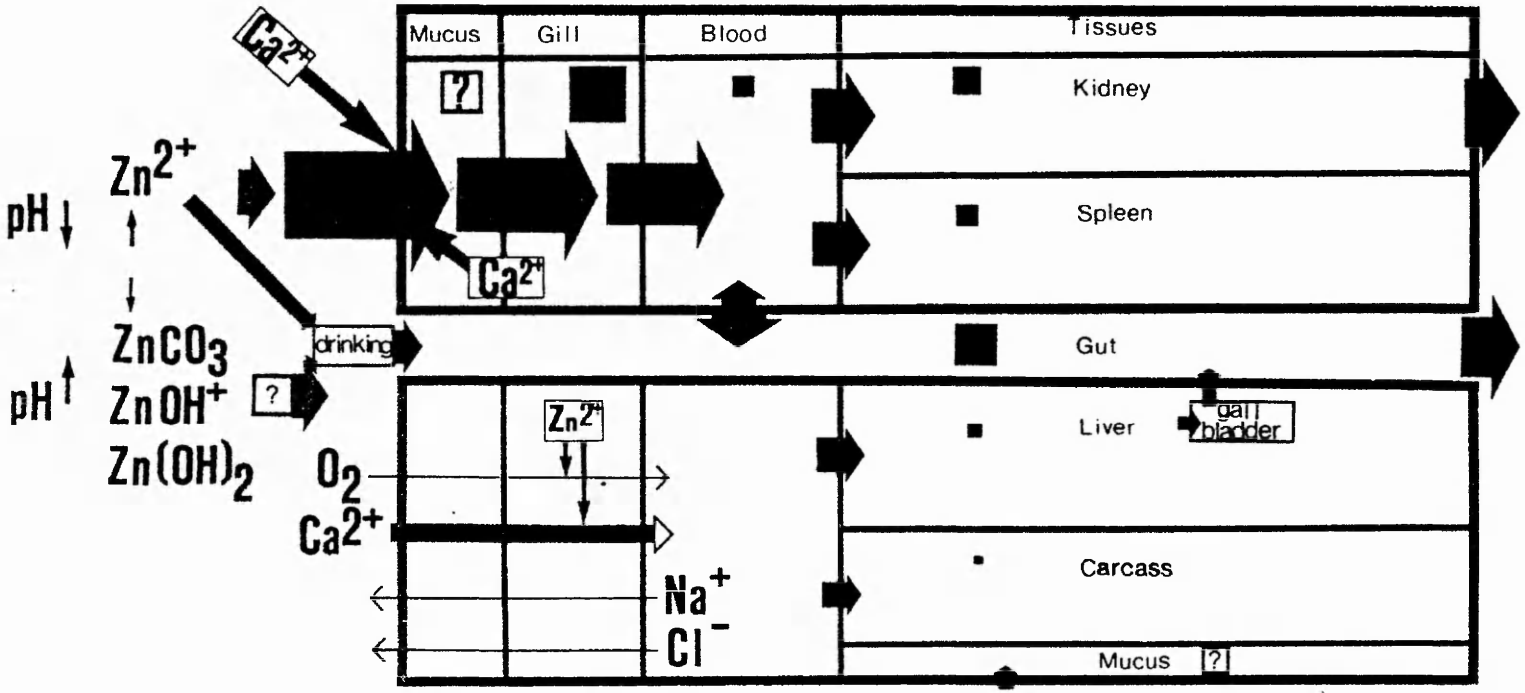
5. SUMMARY

The main structural target for the toxic effects of zinc on fish appeared to be the gills and the main physiological targets were respiratory gas exchange and branchial ion regulation (see Fig. 37). The surface activity of the metal at the gill was dependent upon the water chemistry, significantly the pH, external calcium concentration (hardness) and the complexing capacity of zinc.

Uptake, excretion and hence acute and chronic toxicity of zinc were ameliorated in the presence of high calcium concentration in the external medium. Reduced metal uptake was thought to be the main protective effect of calcium and was attributed to a lowering of branchial permeability caused by high calcium levels in gill channels inhibiting the influx of zinc and thereby determining both acute and chronic toxicity of the metal. Protective effects of calcium at the gills appeared to result from high hardness acclimation as opposed to the surface effects of calcium and zinc interactions in the media at the time of metal exposure. However, surface binding or adsorption of the metal to the fish appeared to occur but true absorption also took place and suggested that the chronic toxicity

Figure 37 Physiological and environmental interactions of water hardness (calcium concentration) and pH upon structural targets and metabolic pathways for zinc in brown trout.




Mucus
Gill
Blood
Tissues
Kidney
Spleen
Gut
Liver
gall bladder
Carcass
Mucus
?
Ca^{2+}
Zn^{2+}
pH ↓
pH ↑
$ZnCO_3$
$ZnOH^+$
$Zn(OH)_2$
drinking
?
Zn^{2+}
O_2
Ca^{2+}
Na^+
Cl^-

of zinc was likely to rise as the duration of exposure increases.

For surface waters with low but environmentally relevant concentrations of calcium (1 mg l^{-1}) and zinc (0.05 mg l^{-1}) the uptake and chronic toxicity of the metal in the fish were enhanced compared with waters of greater ambient calcium concentration (60 mg l^{-1}). At higher metal concentrations, more typical of surface waters in mine drainage areas (Vymazal, 1985), the deleterious effects of zinc on ionoregulatory and respiratory processes of the fish became apparent but were also ameliorated in the presence of high calcium levels.

The absence of marked deleterious effects of pH at or above pH 4 in hard and soft waters of low trace metal content, in this and similar studies with a high degree of control over the composition of the medium, contrasted with reports of results in natural waters or in media contaminated with toxic levels of trace metals (discussed in Chapter 1). In attempting to determine the cause of fish population loss in acid waters, it was clearly necessary to distinguish the effects of pH *per se* from those of associated changes in the environment, particularly elevation of trace metal concentrations.

The major effects of water pH were upon metal toxicity such that dissolved zinc became increasingly toxic over the pH range 5 - 7, but at high pH 7 - 9 Zn^{2+}

was replaced by metal complexes some of which appeared to be more toxic than dissolved zinc within this pH range. At the slightly acidic pH 6 differences between the acute toxicities of the metal in hard and soft waters could be accounted for by a biological response in zinc uptake which may be calcium-dependent and involved absorption of zinc. Further studies with sublethal metal concentrations revealed that both adsorption and absorption may be important in the process of calcium-mediated influx of zinc at the gills in slightly acidic media. Long-term or chronic toxicity was therefore thought to be dependent upon the absorption rate of the metal which was reduced in hard waters of high calcium content. Both acute and chronic toxicity of the metal also appeared to be dependent upon the absorption rate of the metal, which was concentration-dependent and therefore higher with acute exposures.

pH is also thought to affect the solubility of the metal and lowering of pH below pH 6 caused reduced zinc uptake and accumulation in fish (Campbell and Stokes, 1985). However, the present study has shown uptake of the metal to be both concentration and calcium-dependent. Therefore in soft acid waters in the absence of calcium protective effects where acidification tends to mobilise metals, any ameliorating influence of H^+ on

zinc toxicity may be counteracted by increasing concentrations of the metal in the water.

Mechanisms of toxic action for zinc upon fish appeared to be complex and significantly dependent upon the hardness (calcium concentration), pH and complexing capacity of test waters. Indeed, a combined biological and chemical hypothesis is required to explain the influence of water hardness and pH upon metal-fish interactions. Both calcium-induced changes in branchial permeability (biological) and pH-induced speciation of the metal (chemical) appeared to be of major influence upon the acute and chronic toxicity of zinc to brown trout. In conclusion, there is a clear need for experimental investigation to explore the mechanisms of the toxic action of metals to fish at sublethal or chronic concentrations in media that are environmentally relevant. This type of study should be extended to those metals which have not received much attention but are known to be elevated in polluted surface waters e.g. acidified waters, namely Zn, Pb, Ni, Cu and Fe. Particular emphasis should be placed upon determining the protective role of calcium and how pH exerts its toxic and ameliorative effects upon metal solubility and speciation with respect to biological responses of the fish. Studies of this type may help to resolve the many conflicting views in the literature of metal toxicity to

fish at alkaline pH, the toxic mechanisms of which are not understood (Mount, 1966).

Studies of fish population loss in the field due to toxic acid and trace metal levels should be extended to all stages in a species life cycle, with particular attention to reports of recruitment failure (Beamish et al., 1975). Evidence from such investigations will enable more effective management and protection of inland waters and their fishery resources. Indeed, the human race is not immune to the deleterious effects of elevated trace metal levels in water supplies (Wills and Savory, 1985) and so fish may serve as sensitive indicators of the quality of surface waters which is so essential to all life.

APPENDIX

Water quality conditions of brown trout holding waters at Leadmill Trout Farm and The Trent Fish Culture, Derbyshire.

Water Quality Factor		Leadmill Trout Farm [1]	Trent Fish Culture [2]
pH		7,00	7,60
Total water hardness as	mg l^{-1} $CaCO_3$	62	260
Alkanity as	mg l^{-1} $CaCO_3$	18	138
Calcium	mg l^{-1} Ca^{2+}	16	76
Magnesium	mg l^{-1} Mg^{2+}	10	74
Chloride	mg l^{-1} Cl^-	52	62
Sulphate	mg l^{-1} SO_4^{2-}	13	110
Zinc	µg l^{-1} Zn^{2+}	4	8
Aluminium	µg l^{-1} Al^{3+}	0,01	<0,01
Cadmium	µg l^{-1} Cd^{2+}	<0,1	0,2
Chromium	µg l^{-1} Cr^{2+}	1	1
Copper	µg l^{-1} Cu^{2+}	0,02	0,02
Iron	µg l^{-1} Fe^{2+}	0,01	0,05
Lead	µg l^{-1} Pb^{2+}	4	2
Manganese	µg l^{-1} Mn^{2+}	8	1

1 Water source was from underground gritstone spring.

2 Water source was surface limestone run-off.

Both 1 and 2 seasonal fluctuations in water quality factors were unknown.

REFERENCES

ABRAM, F.S.H. (1960). An automatic dosage apparatus. Lab. Practice 9, 796-797.

ALABASTER, J.S. & LLOYD, R. (1980). Water Quality Criteria for Freshwater. Fish, London : Butterworths.

ALDERDICE, D.F. & McLEAN, W.E. (1982). A Review of the Potential Influence of Heavy Metals on Salmonid Fishes in the Campbell River, Vancouver Island, B.C. Can. Tech. Rep. Fish. aquat. Sci 1104.

BATH, R.N. & EDDY, F.B. (1979). Salt and water balance in rainbow trout (Salmo gairdneri) rapidly transferred from freshwater to sea water. J. exp. Biol. 83, 193-202.

BEAMISH, R.J. (1976). Acidification of lakes in Canada by acid precipitation and the resulting effects on fishes. Water Air Soil Polut. 6, 501-514.

BEAMISH, R.J., LOCKHART, W.L., VAN LOON, J.C. & HARVEY, H.H. (1975). Long-term acidification of a lake and resulting effects on fishes. Ambio. 4, 98-103.

BRADLEY, R.W. & SPRAGUE, J.B. (1985a). Accumulation of zinc by rainbow trout as influenced by pH, water hardness, and fish size. Environ. Toxicol. Chem. 4, 685-694.

BRADLEY, R.W. & SPRAGUE, J.B. (1985b). The influence of pH, water hardness and alkalinity on the acute lethality of zinc to rainbow trout (Salmo gairdneri). Can. J. Fish. Aquat. Sci. 42, 731-736.

BRADLEY, R.W., DuQUESNAY, C. & SPRAGUE, J.B. (1985). Acclimation of rainbow trout, Salmo gairdneri Richardson, to zinc : kinetics and mechanism of enhanced tolerance induction. J. Fish Biol. 27, 367-379.

BROWN, D.J.A. (1981). The effects of various cations on the survival of brown trout, Salmo trutta at low pHs. J. Fish Biol. 18, 31-40.

BROWN, D.J.A. (1982a). The effect of pH and calcium on fish and fisheries. Water Air Soil Pollut. 18, 343-351.

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. & LYNAM, S. (1983). The effect of calcium and aluminimum concentrations on the survival of brown trout (Salmo trutta) at low pH. Bull. environ. Contam. Toxicol. 30, 583-587.

BROWN, D.J.A. & SADLER, K. (1981). The chemistry and fishery status of acid lakes in Norway and their relationship to European sulphur emissions. J. appl. Ecol. 18, 433-441.

BRYAN, G.W. (1967). Zinc regulation in the fresh-water crayfish (including some comparative copper analyses) J. exp. Biol. 46, 281-296.

BRYAN, G.W. (1976). Some aspects of heavy metal tolerance in aquatic organisms. In Effects of Pollutants on Aquatic Organisms, ed. A.P.M. Lockwood, pp7-34. Cambridge University Press.

BURNS, J.C., COY, J.S., TERVET, D.J., HARRIMAN, R., MORRISON, B.R.S. & QUINE, C.P. (1984). The Loch Dee Project : A study of the ecological effects of acid precipitation and forest management on an upland catchment in South-West Scotland. Fish. Mgmt, 15, 145-167.

CALAMARI, D. MARCHETTI, R. & VAILATI, G. (1980). Influence of water hardness on cadmium toxicity to Salmo gairdneri Rich. Water Res. 14, 1421-1426.

CAMPBELL, P.G.C. & STOKES, P.M. (1985). Acidification and toxicity of metals to aquatic biota. Can. J. Fish. Aquat. Sci. 42, 2034-2049.

CARROLL, J.J. ELLIS, S.J. & OLIVER, W.S. (1979). Influences of hardness constituents on the acute toxicity of cadmium to brook trout (Salvelinus fontinalis) Bull. environ. Contam. Toxicol. 22, 575-581.

CHAPMAN, G.A. (1978). Effects of continuous zinc exposure on sockeye salmon during adult-to-smolt freshwater residency Trans. Am. Fish. Soc. 107, 828-836.

CHEUNG, Y.A. (1980). Calmodulin (a Review). Science 207, 19-27.

CHIPMAN, W.A., RICE, T.R. & PRICE, T.J. (1958). Uptake and accumulation of radioactive zinc by marine plankton, fish and shellfish. US Fish and Wild. Serv. Fish. Bull. 135, 279-292.

COOMBS, T.L. (1974). The nature of zinc and copper complexes in the oyster Ostrea edulis. Mar. Biol. 28, 1-10.

CROSS, F.A., HARDY, L.H., JONES, N.Y. & BARBER, R. (1973). Relation between total body weight and concentrations of manganese iron, copper, zinc and mercury in white muscle of bluefish (Pomatomus saltatrix) and a bathy-demersal fish Aritimora rostrata. J. Fish. Res. Board Can. 30, 1287-1291.

CUSIMANO, R.F., BRAKKE, D.F. & CHAPMAN, G.A. (1986). Effects of pH on the toxicities of cadmium, copper and zinc to steelhead trout (Salmo gairdneri). Can. J. Fish. Aquat. Sci. 43, 1497-1503.

CUTHBERT, A.W. & MAETZ, J. (1972). The effects of calcium and magnesium on sodium fluxes through the gills of Carassius auratus L. J. Physiol. 22, 633-643.

DALE, T., HENRIKSEN, A., JORANGER, E., & KROA, S. (1974). Vannog nedborkjemiske studier i Birkenesfelter for perioden 20 juli 1972 til 30. April 1973. Res. Rep. TN 1/74. Oslo As. Norway : S.N.S.F. Project.

DALZIEL, T.R.K. (1986). The effects of aluminium in low conductivity acid waters on the ionic regulation and early development of Salmo trutta L. Ph.D., Thesis : University of Nottingham.

DALZIEL, T.R.K., MORRIS, R. & BROWN, D.J.A. (1985a). The effects of low pH, low calcium concentrations and elevated aluminium concentrations on sodium fluxes in brown trout, Salmo trutta Res. Note TPRD/2861/N85. Leatherhead, Surrey : Central Electricity Research Laboratories.

DALZIEL, T.R.K., MORRIS, R. & BROWN, D.J.A. (1985b). Further investigations into sodium uptake inhibition in brown trout (Salmo trutta L.) exposed to elevated aluminium concentrations at low pH. Res. Note TPRD/L/2908/N85. Leatherhead, Surrey : Central Electricity Research Laboratories.

DAYE, P.G. & GARSIDE, E.T. (1976). Histopathological changes in surficial tissues of brook trout Salvelinus fontinalis (Mitchell) exposed to acute and chronic levels of pH. Can. J. Zool. 54, 2140-2155.

EDDY, F.B. (1975). The effect of calcium on gill potentials and on sodium and chloride fluxes in the goldfish, Carassius auratus. J. Comp. Physiol. 96, 131-142.

EDDY, F.B. & FRASER, J.E. (1982). Sialic acid and mucus production in rainbow trout (Salmo gairdneri Richardson) in response to zinc and seawater. Comp. Biochem. Physiol. 73C, 357-359.

EUROPEAN INLAND FISHERIES ADVISORY COMMISSION (E.I.F.A.C.) (1970). Water quality criteria for European freshwater fish. Report on ammonia and inland fisheries. EIFAC tech. Pap. 11.

EIFAC (1973). Water quality criteria for European freshwater fish. Report on zinc and freshwater fish EIFAC tech. Pap. 21.

ELLIS, A.E. & DeSOUSA, M.A.B. (1974). Phylogeny of the lymphoid system. I. A study of the fate of circulating lymphocytes in plaice. Eur. J. Immun. 4, 338-343.

ELLIS, A.E., MUNROE, A.L.S. & ROBERTS, P.J. (1976). Defence mechanisms in fish. 1. A study of the phagocytic system and the fate of intraperitoneally injected particulate material in the plaice (Pleuronectes platessa L.) J. Fish Biol. 8, 67-78.

EVERALL, N.C. & MACFARLANE, N.A.A. (1985). Zinc toxicity to brown trout in waters of differing hardness and pH. Abstract from the 4th Symposium on Fish Physiology. Aug. 1985. Göteborg p45.

FARMER, G.J., ASHFIELD, D. & SAMANT, H.S. (1979). Effects of zinc on juvenile atlantic salmon Salmo salar: acute toxicity, food intake, growth and bioaccumulation. Environ. Pollut. 19, 103-117.

FENWICK, J.C. (1976). Effect of stanniectomy on calcium activated adenosine triphosphatase activity in the gills of fresh water adapted North American eels (Anguilla rostrata) Le Seur. Gen. comp. Endocr. 29, 383-387.

FINNEY, D.J. (1971). Probit analysis. 3rd ed. Cambridge University press, Cambridge, England. 333p.

FORSTNER, U. & WITTMAN, G.T.W. (1981). Metal Pollution in the aquatic environment, 2nd. edn. New York : Springer-Verlag.

FRAIN, W.J. (1983). Ionic regulation and the effects upon it of Heavy Metals inthe Minnow Phoxinus phoxinus (L). (Teleostei : Cyprinidae). Ph.D. Thesis : University of Newcastle Upon Tyne.

FRANZIN, W.G. & McFARLANE, G.A. (1981). Elevated Zn, Cd and Pb concentrations in waters of ice-covered lakes near a base metal smelter during the snow melt, April 1977. Environ. Poll. (Series B)., 2, 11-19.

FRANZIN, W.G. McFARLANE, G.A. & LUTZ, A. (1979). Atmosopheric fallout in the vicinity of a base metal smelter at Flin Flon Manitoba, Canada. Environ . Sci. Technol. 13, 1513-1522.

FROMM, P.O. (1980). A review of some phsyiological and toxicological responses of freshwater fish to acid stress. Environ. Biol. of Fishes 5, 79-93.

GARRELLS, R.M. & CHRIST, C.L. (1965). Solutions, Minerals, and Equilibria. Harper and Row, New York, 450 pp.

GARRELLS, R.M. & THOMPSON, M.E. (1962). A Chemical Model for Sea Water at 25°C and One Atmosphere Total Pressure. Am. J. Sci. 260, 57-66.

GIRARD, J.P. & PAYAN, P. (1980). Ion exchanges through respiratory and chloride cells in freshwater and seawater - adapted teleosteans. Am. J. Physiol. 238, R260-268.

GOODYEAR, C.P. & BOYD, C.E. (1972). Elemental composition of largemouth bass (Micropterus salmoides) Trans. Am. Fish. Soc. 101, 545-547.

GRAHAM, M.S. & WOOD, C.M. (1981). Toxicity of environmental acid to the rainbow trout : interactions of water hardness, acid type and exercise. Gen. J. Zool. 59, 1518-1526.

GUBELI, A.O. & STE-MARIE, J. (1967). Stability of hydroxide complexes and metal hydroxide solubility products. I : Silver and zinc. Can. J. Chem. 45, 827-832.

HAHNE, H.C.H. & KROONTJE, W. (1973). Significance of pH and chloride concentration on behaviour of heavy metal pollutants. Mercury (II), cadmium (II), zinc (II) and lead (II). J. environ. Qual. 2, 444-450.

HAINES, T.A. (1981). Acidic precipitation and its consequences for aquatic ecosystems : a review. Trans. Am. Fish. Soc. 110, 669-707.

HARRISON, G.I. & MOREL, F.M.M. (1983). Antagonism between cadmium and iron in the marine diatom Thalassiosira weissflogii. J. Phycol. 19, 495-507.

HARRISON, R.M. & WILLIAMS, C.R. (1982). Airborne cadmium, lead and zinc at rural and urban sites in North-West England. Atmospheric Environment 16, 2669-2681.

HASWELL, M.S., RANDALL, D.J. & PERRY, S.F. (1980). Fish gill carbonic anhydrase : acid-base regulation or salt transport Am. J. Physiol. 238, R240-245.

HEATH, A.G. & HUGHES, G.M. (1973). Cardiovascular and respiratory changes during heat stress in rainbow trout (Salmo gairdneri). J. exp. Biol. 59, 323-338.

HEM, J.D. (1972). Chemistry and occurrence of cadmium and zinc in surface water and groundwater. Wat. Resour. Res. 8, 661-679.

HENRIKSEN, A. SKOGHEIM, O.K. & ROSSELAND, B.O. (1984). Episodic changes in pH and aluminium-speciation kill fish in a Norwegian salmon river. Vatten 40, 255-260.

HENRIKSEN, A. & WRIGHT, R.F. (1978). Concentrations of heavy metals in small Norwegian lakes. Wat. Res. 12, 101-112.

HIBIYA, T. (1981). An Atlas of Fish Histology. Normal and pathological features. Kodansha Ltd. p.140.

HODSON, P.V. (1974). The effect of temperature on the toxicity of zinc to fish of the genus Salmo. PhD. Thesis. Univ. Guelph, Guelph. Ont. 189p.

HODSON, P.V. & SPRAGUE, J.B. (1975). Temperature-induced changes in acute toxicity of zinc to Atlantic salmon (Salmo salar). J. Fish. Res. Board Can. 32 (1), 1-10.

HOLCOMBE, G.W. & ANDREW, R.W. (1978). The acute toxicity of zinc to rainbow and brook trout : comparisons in hard and soft water. U.S. Environ. Prot. Agency Ecoligical Res. Series. EPA-60013-78-094.

HOLCOMBE, G.W.., BENOIT, D.A. & LEONARD, E.N. (1979). Long-term effects of zinc exposures on brook trout (Salvelinus fontinalis). Trans. Am. Fish. Soc 108, 76-87.

HOSKINS, E.R. & HOSKINS, M.M. (1918). The reaction of selachii to injections of various non-toxic solutions and suspensions (including vital dyes) and to excretory toxins. J. exp. Zool. 27, 101-141.

HOSS, D.E. (1964). Accumulation of Zn-65 by flounder of the genus Paralichthys. Trans. Am. Fish. Soc. 93, 364-368.

HOUSTON, A.H. (1959). Osmoregulatory adaptation of steelhead trout (Salmo gairdneri Richardson) to sea water. Can. J. Zool. 37, 729-748.

HUGHES, G.M. (1979). Scanning electron microscopy of the respiratory surface of trout gills. J. Zool. 188, 443-453.

HUGHES, G.M. & ADENEY, R.J. (1977). The effects of zinc on the cardiac and ventilatory rhythms of rainbow trout (Salmo gairdneri, Richardson) and their responses to environmental hypoxia. Wat. Res. 11(12), 1069-1077.

HUGHES, G.M. & FLOS, R. (1978). Zinc content of the gills of rainbow trout (S. gairdneri) after treatment with zinc solutions under normoxic and hypoxic conditions. J. Fish Biol. 13, 717-728.

HUGHES, G.M. & GRAY, I.E. (1972). Dimensions and ultra-structure of toadfish gills. Biologic. Bull. Mar. Biol. Lab. Woods Hole, 143, 150-161.

HUGHES, G.M. & WRIGHT, D.E. (1970). A comparative study of the ultrastructure of the water/blood pathway in the secondary lamellae of teleost and elasmobranch fishes - benthic forms. Zeitschrift fur Zellforshung and Mikroskopische Anatomie. 104, 478-493.

INTERNATIONAL ELECTRIC RESEARCH EXCHANGE, (I.E.R.E.) (1981). Effects of SO_2 and its Derivatives on Health and Ecology. 2 : Natural Ecosystems, Agriculture, Forestry and Fisheries. Leatherhead, Surrey : Central Electricity Research Laboratories.

JEFFRIES, D.S. & SNYDER, W.R. (1981). Atmospheric deposition of heavy metals in central Ontario. Water Air Soil Pollut. 15, 127-152.

JENNE, E.A. (1968). Controls on Mn, Fe, Co, Ni, Cu and Zn concentrations in soils and water : The significant role of hydrous Mn and Fe oxides in trace inorganics in water, Advan. Chem. Ser. 73, 337-387.

JONES, J.R.E. (1938). The relative toxicity of salts of lead, zinc and copper to the stickleback (Gasterosteus aculeatus L.) and the effect of calcium on the toxicity of lead and zinc salts. J. exp. Biol. **15**, 394-407.

JONES, J.R.E. (1940). A study of the zinc-polluted river Ystwyth in North Cardiganshire, Wales. Ann. appl. Biol. **27**, 368-378.

JONES, J.R.E. (1958). A further study of the zinc-polluted river Ystwyth J. Anim. Ecol. **27**, 1-14.

JOYNER, R. (1961). Exchange of zinc with environment solutions by the brown bullhead. Trans. Am. Fish. Soc. **90**, 444-448.

JUDY, R.O. & DAVIES, P.H. (1979). Effects of calcium addition as Ca $(NO_3)_2$ on zinc toxicity to fathead minnows, Pimephales promelas Rafinesque. Bull. Environ. Contam. Toxicol. **22**, 88-94.

KAGI, J.H.R. & NORDBERG, M. (eds). (1979). Metallothionein. Proceedings of the 1st International Meeting on Metallothionein and Other Low Molecular Weight-Binding Proteins. Boston : Birkhauser Verlag.

KERSTETTER, T.H., KIRSCHNER, L.B. & RAFUSE, D.D. (1970). On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (Salmo gairdneri) J. Gen. Physiol. **56**, 342-359.

KERSTETTER, T.H. & KIRSCHNER, L.B. (1974). HCO_3^--dependent ATPase activity in the gills of rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. **48B**, 581-589.

KLAVERKAMP, J.F., McDONALD, W.A., DUNCAN, D.A. & WAGEMANN, R. (1984). Metallothionein and acclimation to heavy metals in fish : A review In Contaminent effects on Fisheries (ed. Nriagu, J.). **16**, 100-113.

KODAMA, M., OGATA, T. & YAMAMORI, K. (1982). Haemolysis of erythrocytes of rainbow trout Salmo gairdneri exposed to zinc polluted water. Bull. Japan. Soc. Sci. Fish. **48(4)**, 593.

LAZARUS, A.L.., LORANGE, E. & LODGE Jr., J.P. (1970). Lead and Other Metal Ions in US Precipitation. Environ. Sci. Technol. **4**, 55-58.

LANTZY, R.J. & MACKENZIE, F.T. (1979). Atmospheric trace meals : Global cycles and assessment of man's impact. Geochim. Cosmochim. Acta. **43**, 511-525.

LEBEDEVA, G.D. & KUZNETSOVA, G.A. (1967). Absorption of radioactive zinc by freshwater fish (an experimental investigation). Gig. Sanit. **34**, 151-153.

LEE, G.F. (1975). Role of hydrous metal oxides in the transport of heavy metal in the environment. In Heavy Metals in the Aquatic Environment, Oxford, Permagon Press, P.A. Krenkel (Ed.)., 137-147.

LEIVESTAD, H. & MUNIZ, I.P. (1976). Fish kill at low pH in a Norweigian River. Nature **259**, 391-392.

LELAND, H.V., LUOMA, S.N., ELDER, J.F. & WILKES, D.J. (1978). Heavy metals and related trace elements. J. Water Pollut. Contr. Fed. **50**, 1469-1516.

LITCHFIELD, J.T. & WILCOXON, F. (1949). A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Therapeut. **97**, 99-113.

LIPKE, E.J. (1971). Effects of environmental parameters on the uptake of radioisotopes in freshwater fish. PhD. Thesis Univesity of Michigan. Ann. Arbor MI.

LLOYD, R. (1960). The toxicity of zinc sulphate to rainbow trout. Ann. Appl. Biol. **48**, 84-94.

LLOYD, R. (1961). The toxicity of mixtures of zinc and copper sulphates to trout (Salmo gairdneri Richardson). Ann. appl. Biol. **49**, 535-538.

LLOYD, R. (1965). Factors that effect the tolerance of fish to heavy metal poisoning. Biological Problems in Water Pollution. Publ. Hlth. Serv., Wash. 99-WP-25, 181-187.

LLOYD, R. & SWIFT, D.J. (1976). Some physiological responses by freshwater fish to low dissolved oxygen, high carbon dioxide, ammonia and phenol with particular reference to water balance. In Effects of Pollutants on Aquatic Organisms ed. A.P.M. Lockwood, pp.47-69. Cambridge University Press.

LOCK, R.A.C., PART, P, WINKEL VAN DE, J.G.J. & FLICK, G. (1985). Cadmium interaction with calmodulin-stimulated phosphodiesterase activity. Abstract from the 4th Symposium on Fish Physiology Aug. 1985. Göteborg p71.

LOVEGROVE, S.M. & EDDY F.B. (1982). Uptake and accumulation of zinc in juvenile rainbow trout, Salmo gairdneri.Env. Biol. Fish. **7(3)**, 285-289.

MAETZ, J. & BORNANCIN, M. (1975). Biochemical and biophysical aspects of salt secretion by chloride cells in teleosts. International Symposium on Excretion. Fortschritte der Zoologie, 28, Heft 2/3, 322-362.

MAETZ, J. & GARCIA ROMEU, F. (1964). The mechanism of sodium and chloride uptake by the gills of a freshwater fish, Carassius auratus II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. J. Gen. Physiol. 47, 1209-1227.

MAGER, M., McNARY, W.F., Jr. & LIONETTI, F. (1953). The histochemical detection of zinc. J. Histochem. Cytochem. 1, 493-504.

MARRIER, J.R., NERI, L.C. & ANDERSON, T.W. (1979). Water hardness, human health, and the importance of magnesium. National Research Council of Canada, Assoc. Comm. on Sci. Criteria for Env. Quality. Report No. N.R.C.C. 17581, 119p.

MATTHIESSEN, P. & BRAFIELD, A.E. (1977). Uptake and loss of dissolved zinc by the stickleback (Gasterosteus aculeatus L.) J. Fish Biol. 5(5), 607-613.

McDONALD, D.G. (1983). The interaction of environmental calcium and low pH on the physiology of the rainbow trout, Salmo gairdneri. I. Branchial and renal net ion and H^+ fluxes. J. exp. Biol. 102, 123-140.

McDONALD, D.G., HOBE, H. & WOOD, C.M. (1980). The influence of calcium on the physiological responses of the rainbow trout, Salmo gaidneri, to low environmental pH. J. exp. Biol. 88, 109-131.

McDONALD, D.G., READER, J.P. & DALZIEL, T.K.R. (1986). The Combined Effects of pH and Trace Metals on Fish Ionoregulation. S.E.B. Seminar Series. In press.

McDONALD, D.G., WALKER, R.L. & WILKES, P.R.H. (1983). The interaction of environmental calcium and low pH on the physiology of the rainbow trout, Salmo gairdnei II. Branchial ionoregulatory mechanisms. J. exp. Biol. 102 , 141-155.

McWILLIAMS, P.G. (1980a). Effects of pH on sodium uptake in Norwegian brown trout (Salmo trutta) from an acid river. J. exp. Biol. 88, 259-267.

McWILLIAMS, P.G. (1980b). Acclimation to an acid medium in the brown trout Salmo trutta. J. exp. Biol. 88, 267-280.

McWILLIAMS, P.G. (1982a). A comparison of physiological characteristics in normal and acid-exposed populations of brown trout, Salmo trutta. Comp. Biochem. Physiol. **72A**, 515-522.

McWILLIAMS, P.G. (1982b). The effects of calcuim on sodium fluxes in the brown trout, Salmo trutta, in neutral and acid water. J. exp. Biol. **96**, 439-442.

McWILLIAMS, P.G. (1983). An investigation of the loss of bound calcium from the gills of the brown trout, Salmo trutta, in acid media. Comp. Biochem. Physiol. **74A**, 107-116.

McWILLIAMS, P.G. & POTTS, W.T.W. (1978). The effects of pH and calcium concentrations on gill potentials in the brown trout, Salmo trutta. J. comp. Physiol. **126**, 277-286.

MILLER, W.J. (1971). Zinc metabolism in farm animals. In Mineral studies with isotopoes in domestic animals (Rep. of FAO/IAEA Panel, Vienna, 1970), I.A.E.A, Vienna, 105-148.

MINISTRY OF TECHNOLOGY, U.K. (1971). In Water Pollution Research 1970, p63, London. H.M.S.O.

MOORE, P.B. & DEDMAN, J.R. (1982). Calcium binding proteins and cellular regulation. Life Sciences **31**, 2937-2946.

MOREAU, G., BARBEAU, C. FRENETTE, J.J., SAINT-ONGE, J. & SIMONEAU, M. (1983). Zinc, manganese and strontium in opercula and scales of brook trout (Salvelinus fontinalis) as indications of lake acidification. Can. J. Fish. Aquat. Sci. **40**, 1685-1691.

MORGAN, M. & TOVELL, P.W.A. (1973). The structure of the gill of the trout Salmo gairdneri (Richardson). Z. Zellforsch. Mikr. Anat. **142**, 147-162.

MOUNT, D.I. (1966). The effect of total hardness and pH on the acute toxicity of zinc to fish. Air Water Pollut. Int. J. **10**, 49-56.

NAKATINI, R.E. (1966). Biological response of rainbow trout (Salmo gairdneri) ingesting zinc-65. In Disposal of radioactive wastes in seas, oceans and surface waters, pp809-823. International Atomic Energy Agency, Vienna.

NIEMINEN, N., KORHONEN, I. & LAITINEN, M. (1982). Effects of pH on the gill ATPase activity and blood composition of whitefish (Coregonus peled) and trout (Salmo trutta Fario). Comp. Biochem. Physiol. 72A, 637-642.

N.R.C.C. (1981). Acidification in the Canadian Environment National Research Council of Canada, Assoc. Comm. on Sci. Criteria for Env. Quality. Report No. N.R.C.C. 18475, 369pp.

O'CONNOR, J.T. (1968). Fate of zinc in natural surface waters. Univ. Ill. Bull. Dep. Civ. Engng. Sanit. Engng Ser. 49.

O'GRADY, K.T. & ABDULLAH, M.I. (1985). Mobility and residence of Zn in brown trout Salmo trutta : Results of environmentally induced change through transfer. Environ. Pollut. (Series A) 38, 109-127.

O'NEILL, J.G. (1981). The humoral immune response of Salmo trutta L. and Cyprinus carpio L. exposed to heavy metals. J. Fish Biol. 19, 297-306.

OSCHMAN, J.L. (1978). Morphologial correlates of transport. In Membrane Transport in Biology. Vol III. Transport across Multi-membrane Systems Ed. Giesbisch, G., Tasteson, D.C. & Ussing, H.H. Springer Verlag N.Y. 55-84.

OWEN, B.B. & GURRY, R.W. (1938). The electrolytic conductivity of zinc sulphate and copper sulphate in water at 25°C. J. Am. Chem. Soc. 60, 3074-3078.

PACKER, R.P. (1979). Acid-base balance and gas exchange in brook trout (Salnelinus fontinalis) exposed to acidic environments. J. exp. Biol. 79, 127-34.

PAGENKOFF, G.K. (1980). Zinc speciation and toxicity to fish. In Zinc in the Environment, Park 2 : Health Effects (ed. Nriagu, J.O.), 353-361.

PAGENKOFF, G.K. (1983). Gill surface interaction for trace-metal toxicity to fishes : role of complexation, pH and water hardness. Environ. Sci. Toxicol. 17, 347-352.

PAGENKOFF, G.K., RUSSO, R.C. & THURSTON, R.V. (1974). Effects of complexation on toxicity of copper to fishes. J. Fish. Res. Board Can. 31, 462-465.

PART, P., SVANBERG, O. & KIESSLING, A. (1985). The availability of cadmium to perfused rainbow trout gills in different water qualities. Water res. 19, 427-434.

PATTERSON, J.W., SCALA, J.S. & ALLEN, H.E. (1975). Heavy metal treatment by carbonate precipitation, p132-150. In Proceedings of the 30th. Industrial Waste Conference, May 6-8, 1975 (ed. J.M. Bell). Purdue University, Lafayette, IN. Ann. Arbor Science Publishers Inc. Ann Arbor, MI. 1311p.

PAYAN, P. (1978). A study of the Na^+/NH_4^+ exchange across the gill of the perfused head of the trout (Salmo gairdneri). J. Comp. Physiol. 124, 181-188.

PAYAN, P., GIRARD, J.P. & MAYER-GOSTAN, N. (1984). Branchial ion movements in toleosts : the roles of respiratory and chloride cells. In Fish Physiology Vol. 10, Gills, Part B Ion and Water Transfer Ed. Hoar, W.S. & Randall, D.J. Academic Press, New York., 39-60.

PEARSE, A.G.E. (1953). Histochemistry Theoretical and Applied. London : J. & A. Churchill Ltd. pp 705-708. (Appendix 24, p946).

PENTREATH, R.J. (1973). The accumulation and retention of ^{65}Zn and ^{54}Mn by the plaice, Pleuronectes platesa L. J. exp. mar. Biol. Ecol. 12, 1-18.

PENTREATH, R.J. (1976). Some further studies on the accumulation and retention of ^{65}Zn and ^{54}Mn by the plaice, Pleuonectes platessa L. J. exp. mar. Biol. Ecol. 21, 179-189.

PERRIN, D.D. & SAYCE, I.G. (1967). Computer calculations of equilibrium concentrations in mixtures of metal ions and complexing species. Talanta 14, 833-842.

PERRY, S.F. & WOOD, C.M. (1985). Kinetics of branchial calcium uptake in the rainbow trout : effects of acclimation to various external calcium levels. J. exp. Biol. 116, 411-413.

POTTS, W.T.W. & FLEMING, W.R. (1971). The effect of environmental calcium and ovine prolactin on sodium balance in Fundulus kansae. J. exp. Biol. 54, 63-75.

PYTKOWICZ, R.M. & HAWLEY, J.E. (1974). Bicarbonate and carbonate ion-pairs and a model of seawater at 25°C. Limnol. Oceanogr. 19, 223-234.

READER, J.P. (1986). Effects of cadmium, manganese and aluminium in soft acid water on ion regulation in Salmo trutta L. Ph.D. Thesis. University of Nottingham.

RENFRO, W.C., FOWLER, S.W., HEYRAUD, M. & LA ROSA, J. (1974). Relative importance of food and water pathways in the bio-accumulation of zinc. Tech. Rep. IAEA-163, 11-20. International Atomic Energy Agency, Vienna.

RENFRO, W.C., FOWLER, S.W., HEYRAUD, M. & LA ROSA, J. (1974). Relative importance of food and water in long-term zinc-65 accumulation of marine biota. J. Fish Res. Board Can. 32, 1339-1346.

RICE, T.R. (1961). Review of Zinc in Ecology. In Proc. 1st. Natl. Symp. on Radioecology, 619-631.

ROBERTS, R.J. (1974). Melanin-containing cells of telost fish and their relation to disease. In Anatomic Pathology of Teleost Fish (Ed. Ribelin, W.R. & Mikagi, G). Madison, Wisconsin : University of Wisconsin Press.

SADLER, K. & LYNAM, S. (1984). Some effects of low pH and calcium on the growth and tissue mineral content of yearling brown trout (Salmo trutta). Res. Note TPRD/L/2789/N84. Leatherhead Surrey : Central Electricity Research Laboratories.

SADLER, K. & LYNAM, S. (1985b). An investigation into the importance of dietary calcium for the growth of brown trout at low pH and low ambient calcium concentrations. Res. Note TPRD/L/2818/N85 Leatherhead, Surrey : Central Electricity Research Laboratories.

SARDET, C., PISAM, M. & MAETZ, J. (1979). The surface of epithelium of teleostean fish gills. Cellular and junctional adaptations of the chloride cell in relation to salt adaptations. J. Cell Biol. 80, 96-117.

SEIJI, M., SHIMAO, K., BIRBECK, M.S.C. & FITZPATRICK, T.B. (1963). Subcellular localization of melanin biosynthesis. Ann. N.Y Acad. Sci 100, 497-533.

SILLEN, L.G. & MARTELL, A.E. (1964). Stability Constants. Chemical Society. Spec. Publ. 17, London

SIMKISS, K. (1976). Intracellular and extracellular routes in biomineralization. Symp. Soc. exp. Biol. 30, 423-444.

SINLEY, J.R., GOETTL, J.P., & DAVIES, P.H. (1974). The effects of zinc on rainbow trout (Salmo gairdneri) in hard and soft water. Bull. envir. contam. Toxicol 12 (2), 193-201.

SKIDMORE, J.F. (1970). Respiration and osmoregulation in rainbow trout with gills damaged by zinc sulphate. J. exp. Biol. 52, 481-494.

SKIDMORE, J.F. & TOVELL, P.W.A. (1972). Toxic effects of zinc sulphate on the gills of rainbow trout. Wat Res. 6, 217-230.

SKOGHEIM, O.K., ROSSELAND, B.O. & SEVALDRUD, I. (1984). Deaths of spawners of Atlantic salmon in River Ogna, S.W. Norway, caused by acidified aluminium-rich water. Inst. Freshw. Res. Drottingholm Rep. 61, 195-202.

SLATER, J.V. (1961). Comparative accumulation of radioactive zinc in young rainbow, cutthroat and brook trout. Copeia 1961 (2), 158-161.

SMITH, M.J. & HEATH, A.G. (1979). Acute toxicity of copper, chromate, zinc and cyanide to freshwater fish : Effect of different temperatures. Bull. Environ Contam. Toxicol 22, 113-119.

SMITH, R.M. & MARTELL, A.E. (1976). Critical Stability Constants, Vol. 4. Inorganic complexes. Plenum Press, New York, 257pp.

SNEKVIK, E., SELMER-OLSEN, A.R. NJOS, A. & BAERUG, R. (1973). Investigations on precipitation from various locations in Norway, 1965-1971. Meld Norg. Landbrhoisk 52, 1-19.

SOLBE, J.F. de L.G. (1973). The relation between water quality and the status of fish populations in Willow Brook. J. Soc. Wat. Treat. Exam, 22, 44-61.

SOLBE, J.F. de L.G. (1974). The toxicity of zinc sulphate to rainbow trout in very hard water. Wat Res. 8, 389-391.

SOLBE, J.F. de L.G. (1977). Water Quality, Fish and Invertebrates in Zinc-Polluted Stream. J.S. Alabaster Ed., In Biological Monitoring of Inland Fisheries: Proceedings of the European Inland Fisheries Advisory Commission. Helsinki, Finland, June 7-8, 1976. Food and Agriculture Organization, Rome; Applied Science Publisher, London.

SPRAGUE, J.,B. (1964). Lethal concentrations of copper and zinc for young Atlantic salmon. J. Fish. Res. Board Can. 21, 17-26.

SPRY, D.J. & WOOD, C.M. (1984). Acid-base, plasma ion and blood gas changes in rainbow trout during short term toxic zinc exposure. J. Comp. Physiol. **154B**, 149-158.

SPRY, D.J. & WOOD, C.M. (1985). Ion-flux rates, acid-base status, and blood gases in rainbow trout, Salmo gairdneri, exposed to toxic zinc in natural soft water. Can. J. Fish. aquat. Sci. **42**, 1332-1341.

SPRY, D.J., WOOD, C.M. & HODSON D.V. (1981). The effects of environmental acid on freshwater fish with particular reference to the soft water lakes in Ontario and the modifying effects of heavy metals. A literature review. Can. Tech. Rep. Fish. aquat. Sci. **999**, 149p.

STARK, G.T.C. (1967). An automatic dosing apparatus made with standard laboratory ware. Lab. Practice, **16 (5)**, 594-595.

STAURNES, M. SIGHOLT, T. & REITE, O.B. (1984). Reduced carbonic anhydrase and Na-K-ATPase activity in gills of salmonids exposed to aluminium-containing acid water. Experentia **40**, 226-227.

STEEN, J.B. & STRAY-PEDERSON, (1975). The permeability of fish gills with comments on the osmotic behaviour of cellular membranes. Acta Physiol. Scand. **95**, 6-20.

STUMM, W. & MORGAN, J.J. (1981). Aquatic chemistry - An introduction emphasizing chemical equilibria in natural waters. John Wiley and Sons, New York. 780p.

TABATA, K. (1969). Studies on the toxicity of heavy metals to aquatic animals and the factors to decrease the toxicity. 2. The antagonistic action of hardness components in water on the toxicity of heavy metal ions. Bull. Tokai Reg. Fish. Res. Lab, **58**, 215-232.

THE WATT COMMITTEE ON ENERGY (1984). Acid Rain. London : The Watt Committee on Energy Ltd. pp33-40.

TOMLINSON, G.H., BROUZES, R.J.P., McLEAN, R.A.N. & KADLECEK, J. (1980). The role of clouds in atmospheric transport of mercury and other pollutants. 1. The link between acid precipitation, poorly buffered waters, mercury and fish. In Proc. Int. Conf. Ecological Impact of Acid precipitation (Eds. D. Drables & Tollan, A.) pp.134-135. Oslo-As, Norway : S.N.S.F. Project.

TURNPENNY, A.W.H. (1985). The fish populations and water quality of some Welsh and Pennine streams. Res. Note TPRD/L/2859/N85. Leatherhead, Surrey : Central Electricity Research Laboratories.

ULTSCH, G.R. & GROS, G. (1979). Mucus as a diffusion barrier to oxygen : possible role in oxygen uptake at low pH in carp (Cyprinus carpio) gills. Comp. Biochem. and Physiol. **62A**, 685-689

VAN LOON, J.C. & BEAMISH, R.J. (1977). Heavy metal contamination by atmospheric fallout of several Flin Flon area lakes and the relation to fish populations. J. Fish. Res. Board Can. **34**, 899-906.

VARANASI, U, ROBISCH, P.A. & MALINS, D.C. (1975). Structural alterations in fish epidermal mucus produced by water-borne lead and mercury. Nature **258**, 431-432.

VYMAZAL, J. (1985). Occurrence and chemistry of zinc in freshwaters - Its toxicity and bioaccumulation with respect to algae : A review. Part 1: Occurrence and chemistry of zinc in freshwaters. Acta hydrochim. Hydrobiol. **13 (6)**, 627-654.

WAIWOOD, K.G. (1980). Changes in haematocrit of rainbow trout exposed to various combinations of water hardness, pH and copper. Trans. Am. Fish. Soc. **109**, 461-463.

WATSON, T.A. & BEAMISH, F.W.H. (1980). Effects of zinc on branchial ATPase activity in vivo in rainbow trout Salmo gairdneri. Comp. Biochem. Physiol. **66C**, 77-82.

WATSON, T.A. & BEAMISH, F.W.H. (1981). The effects of zinc on bronchial adenosine triphosphatase enzymes in vitro from rainbow trout, Salmo gairdneri. Comp. Biochem. Physiol **68C**, 167-173.

WEATHERLEY, A.H., LAKE, P.S. & ROGERS, S.C. (1980). Zinc pollution and the ecology of the freshwater enviornment. In Zinc in the Environment. Part 1: Ecological Cycling (Ed. Nriagu, J.O.), 338-403.

WEINER, J.G. & GEISY, J.P. (1979). Concentrations of Cd, Cu, Mn, Pb, and Zn in fishes in a highly organic softwater pond. J. Fish. Res. Board Can. 36, 270-279.

WILLIAMS, R.J.P. (1976). Calcium chemistry and its relation to biological function. In Calcium in Biological Systems SEB Symposium. Vol. XXX. pp1-17. Cambridge University Press.

WILLS, M.R. & SAVORY, J. (1985). Water content of aluminium, dialysis, dementia and osteomalacia. Environ. Health Perspectives 63, 141-147.

WRIGHT, R.F., DALE, T. GJESSING, E.T., HENDREY, G.R., Henriksen, A., Johannessen, M. & Muniz, I.P. (1975). Impact of acid precipitation on freshwater ecosystems in Norway : Res. Rep. FR3/75. Oslo-As, Norway : S.N.S.F. project.

WRIGHT, R.F. & HENRIKSEN, A. (1978). Chemistry of small Norwegian lakes, with special reference to acid precipitation. Limnol. Oceanogr. 23, 487-498.