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THE TOXICITY OF ZINC TO THE BROWN TROUT SALMO TRUTTA L.: MODIFICATION BY EXTERNAL CALCIUM AND MAGNESIUM

by

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Thesis submitted to the Council for National Academic Awards in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

March, 1989

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TO MY PARENTS

ABSTRACT

The toxicity of zinc to the brown trout Salmo trutta L.: modification by external calcium and magnesium

J. F. Simmons

The toxicity of zinc, an important pollutant of aquatic ecosystems, was investigated to provide information concerning the mechanisms of toxicity and the modification of toxicity by external calcium and magnesium.

The acute toxicity of zinc was considerably lower in hard than in soft water. Increases in external calcium concentration resulted in progressive reductions of toxicity. The composition of the acclimation medium before zinc exposure had little influence on toxicity. Toxicity was governed mainly by the composition of the exposure medium.

Radioisotope studies at a sublethal zinc concentration demonstrated that both external calcium and magnesium reduced zinc influx. External calcium was of major importance and exerted greatest effect at a concentration of 0.15 mM. Acclimation medium composition had less influence than exposure medium composition in modifying zinc influx. Most zinc was accumulated by the gills, plasma, kidney and liver. The influence of external divalent ion concentration on zinc influx was reflected in radiozinc appearance in these tissues.

Hypocalcaemia in fish exposed to zinc at the lowest external calcium concentration suggested a disruption of calcium balance. This disruption was investigated using radiocalcium. Acclimation to low external calcium stimulated calcium influx. Apparent saturation of the uptake mechanism was taken as evidence for active calcium transport. External zinc caused a strong dose-dependent inhibition of calcium influx. This persisted immediately after the removal of zinc but had ceased after a further 48 h. Calcium efflux was unaffected by zinc.

External lanthanum inhibited both calcium and zinc influx suggesting a common apical membrane uptake route for these two metals.

The results are reviewed and a model proposed whereby external zinc is considered to inhibit calcium influx by blocking calcium transport across the basolateral membrane. The possibility of other heavy metals, in particular cadmium, exerting their sublethal toxic action through similar means is also discussed.

DECLARATION

Whilst registered as a CNAA candidate for the degree of PhD, the author has not been a registered candidate for another award from CNAA or a university.

No work from this thesis has been submitted, nor will be submitted in candidature for any other degree.

Due acknowledgement has been made to those who assisted during the study.

Signed .....
(Candidate)

Signed .....
(Director of Studies)

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ABBREVIATIONS

SI units are used throughout this thesis. Other abbreviations which appear in the main text are explained below.

[X]	Concentration of X
AAS	Atomic absorbtion spectrophotometry
LC_{50}	Median lethal concentration, the concentration of a toxicant lethal to 50% of a test population in a given time.
LT_{50}	Median lethal time, the resistance time of 50% of a test population in a given concentration of toxicant.
TEP	Transepithelial electrical potential, the electrical potential difference across a given membrane.
$\bar{x}$	Sample mean
se	Standard error of the mean.
P	Statistical probability.
^{65}Zn	Radioactive isotope of zinc.
^{45}Ca	Radioactive isotope of calcium.
c min^{-1}	Radioactive counts per minute.
d min^{-1}	Radioactive disintegrations per minute.
V_{max}	Maximum velocity of a reaction.
K_m	Michaelis constant, the substrate concentration at which the velocity of a reaction is equal to half of the maximal velocity (V_{max}).

1 INTRODUCTION

Zinc, the 30th element of the periodic table and a member of the first period of transition elements occurs fairly abundantly in nature and is estimated to represent 0.004% of the Earth's crust (Rice, 1961). As a result of its ubiquitous distribution it is present in measurable quantities in natural waters not subject to any inputs from the activities of man. The background level of zinc within an individual body of water is largely dependent on the geology of the surrounding area, the weathering of bedrock and overlying soils providing the major natural input but with some subsidiary deposition from airborne terrigenous dust. As a result the background level of zinc in unpolluted waters covers a wide range of values, a review of data from pristine soft-water lakes in North America and Scandinavia by Henriksen and Wright (1978) producing extremes of 8 and 460 nM (0.5 and 30 $\mu g\ l^{-1}$) with the majority of data tending towards the lower end of this range. The authors point out however, that even these background concentrations from supposedly unpolluted areas are likely elevated by an unknown amount due to increased atmospheric deposition from industrial activity over the last two centuries. In support of this they cite data from Greenland ice samples indicating a 2.5 fold increase in zinc levels since the turn of the century (Weiss *et al.*, 1975).

Such activities as the mining and processing of metal bearing ores, the use of zinc in a variety of industrial processes, leaching from mine waste dumps and human excretion all have the potential to release large quantities of zinc to the environment (reviews: Forstner and Wittman, 1979; Weatherley et al., 1980). The routes of entry of zinc into the environment from these sources are diverse (reviews: Leland et al., 1978; Vymazal, 1985). Exceptionally high concentrations have been reported in areas subject to mine drainage. On a larger geographical scale however, the atmosphere probably provides the major route of entry of zinc into the environment, background levels as discussed earlier being considered artificially elevated through this mechanism. In addition, serious local pollution can result through atmospheric fallout from point sources of pollution. The metal smelters at Sudbury, Ontario and Flin Flon, Manitoba have been particularly thoroughly studied in this respect, deposition being both dry in the form of dust, and wet in rain and snowfall (Van Loon and Beamish, 1977; Hutchinson and Whitby, 1977). The latter category is of particular interest as precipitation containing elevated levels of heavy metals is also frequently characterised by being of low pH. This phenomenon, now popularly known as "acid rain", has primarily arisen through the release of large quantities of sulphur dioxide and nitrogen oxides to the atmosphere. The

resulting low pH rainfall is able to titrate the low buffering capacity of the soft-water lakes characteristic of much of Scandinavia, the north-eastern United States and eastern Canada. In addition to the already elevated atmospheric input of heavy metals the acidification process may itself further increase the metal content of the water through the mobilisation of metals previously bound within sediments (Beamish and Van Loon, 1977), the chemical form (speciation) of zinc and other metals being strongly pH dependent (Hem, 1972; Farmer et al., 1979; Campbell and Stokes, 1985).

The literature documenting both the acidification of natural waters and the possible reasons for the resulting decline in the populations of fish and other species is extensive (reviews: Fromm, 1980; Harvey et al., 1981; Spry et al., 1981; McDonald, 1982; Wood and McDonald, 1982). Fish population decline is generally characterised by long-term chronic mortality and short-term abrupt episodes of mortality, the latter response typically being caused by a rapid reduction of pH accompanying rainstorms or snowmelt, accumulated acids being released in a short and concentrated burst (Jeffries et al., 1979). Many laboratory studies have been completed investigating the effects on fish of such short-term excursions to pH levels of 4.3 and below. The major mechanism of toxic action at such acutely toxic pH is generally considered to be a large disturbance of

ionoregulation, particularly sodium and chloride balance (McWilliams and Potts, 1978; McDonald et al., 1980, 1983; McDonald and Wood, 1981; McDonald, 1983; Hobe et al., 1984a,b). Similar results have been obtained from field studies, the most notable being the much cited work of Leivestad and Muniz (1976) on a meltwater-induced excursion to pH levels as low as 4.0 in the Tovdal River and its tributaries in southern Norway.

With regard to the chronic mortality of fish in acidified waters however, a number of studies have demonstrated the mortality of fish in laboratory experiments and extinction of fish populations in field surveys at pH levels not normally considered to be particularly toxic. Craig and Baksi (1977) for example, noted reduced reproductive success of American flagfish (Jordanella floridae) at pH 6.0 and stated that pH 6.5 should be considered a critical level for unimpaired fish reproduction. Similarly, the experimental acidification of a small lake in Ontario resulted in recruitment failure of the fathead minnow (Pimephales promelas) population at pH 5.6 (Mills, 1984). Heavy metals have often been demonstrated to enhance stresses already imposed by exposure to low pH (Dalziel et al., 1986; Reader et al., 1988) and in a survey of the density and biomass of fish populations in relation to water quality in sixty upland streams in North Wales and the southern Pennines it was concluded that the importance of low pH

per se may not be paramount. The absence or scarcity of salmonid species in the streams examined was correlated with high levels of labile monomeric aluminium or copper, lead and zinc (Turnpenny et al., 1987). A similar conclusion was reached by Hutchinson and Sprague (1986) working on J. floridae. They observed no detrimental effect of pH 5.8 alone on this species. Following the addition of a mixture of heavy metals at concentrations known to be sublethal on individual addition however, a considerable decline in reproductive success was noted. Of the metals tested aluminium, copper and zinc were found to be the most toxic and it was suggested that the toxicities of these three, plus that due to the hydrogen ion itself, were additive or close to additive in their effects.

It is unlikely therefore, that the problem of fish population loss following acidification can be wholly ascribed to the reduction in pH itself. Heavy metals are probably of major importance in this process, their toxicity to aquatic life being well documented. In this respect zinc is a metal of particular interest as it is frequently present at elevated concentrations in acidified waters, and is of significant toxicity to aquatic life (reviews: Skidmore, 1964; Alabaster and Lloyd, 1980).

Although zinc is a known toxicant when present at elevated levels it is also an essential trace element

necessary for the survival of living organisms. Among many of its functions it is a constituent of a number of enzymes and is involved in the synthesis of nucleic acids. Fish fed a zinc-deficient diet have been shown to exhibit reduced rates of growth, decreased plasma protein concentration, cataracts, fin erosion and, in extreme cases, have died (Ogino and Yang, 1978; Satoh et al., 1987a,b; Spry et al., 1988).

Though an essential trace element the toxicity of zinc when present at elevated concentrations is well established. Many of the early data from work concerning the toxicity of zinc were obtained at concentrations unrealistically high in terms of the concentrations found in the majority of polluted waters. The mortalities which invariably occured in experiments of this type were initially attributed to the coagulation of large quantities of mucus at the gill surface thereby causing acute hypoxia (Jones, 1938; Westfall, 1945). Later experiments of this type revealed no such effect at lower, though still acutely toxic concentrations however. Mortality in these experiments was still largely attributed to hypoxia. Histological examination of zinc-exposed gill tissue suggested that increased blood-water diffusion distance due to partial sloughing of the epithelium of the secondary lamellae and stagnation of the blood flow within the secondary lamellae were the causes of the hypoxia (Skidmore, 1970;

Skidmore and Tovell, 1972; Matthiesen and Brafield, 1973; Tuurala and Soivio, 1982; Frain, 1983). Physiological evidence for tissue hypoxia was provided by Burton et al. (1972) who measured an increased concentration of lactic acid and a decreased concentration of pyruvic acid in rainbow trout (Salmo gairdneri) exposed to 40 mg l^{-1} zinc and by Tort et al. (1985) who reported an increased lactate concentration following short-term exposure of dogfish (Scyliorhinus canicula) to 10 mg l^{-1} zinc. Similarly, reduced arterial oxygen concentration in rainbow trout exposed to a lower, though still acutely toxic [Zn^{2+}] have been reported (Sellers et al., 1975; Tuurala, 1983).

Osmoregulatory disturbances have also been reported in these investigations of acute zinc lethality. Skidmore (1970) noted small changes in plasma osmolarity and ion concentrations on exposure of rainbow trout to 40 mg l^{-1} zinc but considered these unimportant in contributing to death when compared to the large disturbances in all respiratory parameters measured. In contrast, death in the channel catfish (Ictalurus punctatus) was attributed largely to zinc-induced changes in serum osmolarity (Lewis and Lewis, 1971). More recent work at lower concentrations of zinc has revealed little or no disturbance of normal respiratory activity, disruption of acid-base balance and ionoregulation being more evident (Spry and Wood, 1984, 1985). Even these most recent

experiments in this field were still undertaken at a zinc concentration unrealistically high in terms of all but a few natural waters however. One of the major aims of the present study therefore, was to examine the toxicity of a sublethal concentration of zinc to brown trout with a view to elucidating the mechanism through which it exerts its toxic action.

Concentrations of zinc reported to be toxic to fish cover a very wide range. Part of this variation is a result of interspecific differences in zinc tolerance, salmonid species generally being considered to be less tolerant than other freshwater fish (Ball, 1967). Within salmonid species themselves, the group from which the majority of data concerning the toxicity of zinc has been obtained, further interspecific differences are apparent (Nehring and Goettl, 1974; Chapman, 1978). Intraspecific variation introduces another complicating factor. In particular, susceptibility to zinc has frequently been shown to vary with the stage of the life cycle, eggs, juveniles and adults all exhibiting differing tolerances to zinc (Sinley et al., 1974; Zitko and Carson, 1977; Chapman, 1978; Holcombe et al., 1979).

By far the greatest cause of the variation in levels of zinc reported to be toxic to fish is due to variations of water quality between different studies. A number of water quality parameters have been demonstrated to influence strongly the toxicity of zinc. These have been

the subject of a number of extensive reviews (Skidmore, 1964; Lloyd, 1965; Alabaster and Lloyd, 1980; Weatherley et al., 1980; Sprague, 1985). Briefly, zinc toxicity is affected by such parameters as photoperiod (McLeay and Munro, 1979), temperature (Lloyd, 1960; Hodson, 1975; Hodson and Sprague, 1975), dissolved oxygen (Lloyd, 1960; Hughes and Flos, 1978), organic matter (Sprague, 1968; Muramoto, 1980; Hutchinson and Sprague, 1987) and previous exposure to zinc (Bradley et al., 1985). The major parameters influencing the toxicity of zinc in natural waters however, are pH and water hardness.

Until recent years the toxicity of zinc in relation to external pH was little studied. It was briefly mentioned earlier that pH is known to influence strongly the speciation of zinc in water. This is manifested as an increase in the proportion of zinc present as the aquo-ion $[Zn(H_2O)_6]^{2+}$ relative to that in the form of insoluble hydroxide and carbonate precipitates as external pH is lowered. This effect is such that below pH 7 almost all zinc is present as the aquo-ion (Campbell and Stokes, 1985). The relative toxicity of zinc in the precipitated or aquated form has been the subject of considerable speculation, the overall view being complicated by the fact that much of the early work in this field was completed in static water conditions. Under such conditions precipitates rapidly settle out of suspension so leading to uncertainties surrounding the

true concentration of zinc in the water. Lloyd (1960) proposed that precipitated zinc was of greater toxicity than soluble zinc. Mount (1966) suggested that the mechanical accumulation of precipitate between the gill filaments might be accompanied by a downward shift in pH due to the excretion of carbon dioxide. Such a pH shift might then dissolve the precipitated zinc resulting in a high concentration of soluble zinc in the region of the "gill micro-environment". Such a downward pH shift has recently been reported for rainbow trout, expired water having a significantly lower pH than inspired. Acidification was thought to be a result of the catalysis of excreted CO_2 to HCO_3^- and H^+ by carbonic anhydrase isolated from mucus covering the body surface and thought to be of similar composition to that covering the gills (Wright et al., 1986). Bradley and Sprague (1985a) however, have proposed that precipitated zinc is of low toxicity. They noted that below pH 7 dissolved zinc became increasingly toxic with increased pH. At pH 9 however, the competing mechanism of zinc precipitation had reduced the toxicity of zinc. Cusimano et al. (1986) also reported the exacerbation of zinc toxicity with increased pH over the range 4.7-7.0. In both of these studies toxicity was observed to increase as pH rose over a range at which the speciation of zinc was considered to be unchanged, all of the metal being present in the soluble aquated form. This effect was considered to

reflect an interference with zinc uptake by hydrogen ions as the concentration of the latter increased at more acid pH. In an earlier study at this institution (Everall, 1987) results were in broad agreement with these studies in those experiments conducted at circumneutral pH and below. Above this pH however, precipitated zinc was found to be of considerable toxicity, this difference in results being assigned to differences in precipitate composition between these studies. Everall (1987) considered zinc carbonate to be the main precipitate present in his work, this being of greater toxicity than the zinc hydroxide reported to predominate by Bradley and Sprague (1985a).

At the present time therefore, pH is clearly known to exert a considerable influence over the toxicity of zinc but further work is required to elucidate the importance of zinc precipitate formation in this process. Work of this type is frequently hampered by the lack of thermodynamic data necessary for the accurate prediction of metal speciation and by the potentially inaccurate and time consuming analytical methods currently available for the direct measurement of different metal species (Florence and Batley, 1977; Florence 1980).

In contrast to the uncertainties surrounding the modifying influences of pH on the toxicity of zinc, that of water hardness is well known, many workers having clearly demonstrated a reduction of metal toxicity in

hard water relative to that in soft water (eg. Lloyd, 1960; Mount, 1966; Sinley et al., 1974; Brafield and Wilshaw, 1981; Bradley and Sprague, 1985a). The major constituents of water hardness are the alkaline earth metals calcium and magnesium. The relative importance of these two ions in the reduction of zinc toxicity has been the subject of a number of studies. On the basis of chemical binding constants direct competition between magnesium and zinc for binding sites on fish tissues with little effect due to calcium has been proposed as the major factor governing the toxicity of zinc to Atlantic salmon (Salmo salar) (Zitko and Carson, 1976). Other studies, both on zinc and a number of other heavy metals however, have proposed calcium as being of more importance than magnesium. The toxicity of cadmium to brook trout (Salvelinus fontinalis) for example was reduced most effectively by the addition of calcium, magnesium and a number of other ions having little effect (Carroll et al., 1979). Cadmium transfer through the perfused gills of rainbow trout was reduced by both calcium and magnesium but with the equivalent effect of magnesium exerted at concentrations four to five times as high as that of calcium (Part et al., 1985). Addition of calcium to soft water reduced the toxicity of zinc to fathead minnows (Judy and Davies, 1979) and of aluminium to brown trout (Brown, 1983).

These uncertainties concerning the relative

importance of calcium and magnesium in the reduction of zinc toxicity are also apparent in work investigating the mechanism by which the protective effect is exerted (Spear, 1981). A number of conclusions concerning the manner of protection have been drawn from the results of a few "switch" type experiments. Rainbow trout transferred from hard to soft water and exposed to an acutely toxic zinc concentration responded in a similar manner to those acclimated and exposed in hard water. A response typical of soft water-acclimated fish was only elicited following a five day acclimation period in soft water (DSIR, 1958). Lloyd (1965) proposed what has now become known as the "biological mechanism" for the protective action of water hardness, protection being conferred through some internal mechanism, this mechanism requiring time to be both acquired and lost on transfer of fish between waters of differing hardness. In support of this Calamari *et al*. (1980) working on the toxicity of cadmium to rainbow trout, acclimated fish to hard water before exposure to the metal in soft water and obtained a 48 h LC_{50} intermediate in value between that from fish acclimated and exposed in hard water and those acclimated and exposed in soft water.

Pagenkopf (1983) however, proposed a "chemical mechanism" for the action of water hardness. His "Gill Surface Interaction Model" attempted to explain the variation of trace metal toxicity to fish with varying

water hardness, pH and complexing capacity on the basis of chemical competition between trace metals and the divalent hardness ions for sites of uptake at the gill surface.

Bradley and Sprague (1985b) proposed that both calcium and magnesium were involved in the reduction of zinc toxicity, magnesium mainly or wholly exerting its effect through chemical means and calcium through biological effects. The work presented in this thesis was initiated with the primary aim of clarifying the mechanisms through which water hardness modifies the toxicity of zinc. More general effects of hardness were to be investigated initially but with the specific importance of calcium and magnesium in these processes to be examined subsequently. An initial series of experiments of similar design to standard toxicity test procedures was intended to clarify the influence of acclimation to waters of differing hardness on the subsequent toxicity of zinc. The specific effect of calcium in this process was then investigated.

Subsequent work used ^{65}Zn, a radioactive isotope of zinc, as a biological tracer in experiments investigating the uptake of zinc by the fish in relation to external $[Ca^{2+}]$ and $[Mg^{2+}]$. The use of ^{65}Zn also allowed examination of the distribution of zinc throughout the various body tissues. It was thought that these data, together with those provided by measurements of a number

of physiological parameters (essentially plasma ion concentrations), would provide an insight into the mechanism by which zinc exerts its toxic action.

On the basis of the results obtained in the work using ^{65}Zn it was considered that an investigation of how calcium balance is modified by zinc would be of value in the consideration of how zinc exerts its toxic effects. The final experiments presented in this thesis therefore, used ^{45}Ca, a radioactive isotope of calcium, in an investigation of calcium turnover in relation to external $[Zn^{2+}]$. Lanthanum, a specific calcium-channel blocker, was also employed in this group of experiments to investigate similarities in the uptake of calcium and zinc by the fish.

Zinc was chosen as the metal to be studied due to its previously discussed importance in waters subject to acidification, its known toxicity to aquatic life and the uncertainties surrounding its mode of toxic action. It was considered particularly important to examine the effects of zinc at environmentally relevant sublethal concentrations, as toxicity under these conditions is likely to be exerted through a different mechanism to that at a more acute concentration. Through comparisons with similar work undertaken on other metals, particularly cadmium, it was considered that similarities in the mode of toxic action should become apparent. In this way it was aimed to propose a model explaining the

mechanism of sublethal toxicity of a number of environmentally relevant heavy metals and the modifying effects of water hardness in general, and specifically its constituent ions calcium and magnesium.

The brown trout was chosen as the species to be studied as it is a salmonid native to the British Isles and is of significant ecological and economic importance. It is also a species for which toxicological data are somewhat scarce in comparison to its North American counterpart the rainbow trout, although most of the evidence available points to it being the more resistant of the two species (Alabaster and Lloyd, 1980).

CHAPTER 2

The modifying influence of water hardness and calcium on the acute toxicity of zinc.

2.1 INTRODUCTION

In Chapter 1 the conclusions drawn from previous investigations of zinc toxicity in relation to water hardness in general, and calcium and magnesium in particular, have been summarised. From these studies it was apparent that although the toxicity of zinc is reduced by increased water hardness or by increased concentrations of its major constituent ions calcium and magnesium, there are diverse opinions as to the mechanism(s) by which this protective action is exerted. The work which is reported in this chapter examines the effect of varying the time of acclimation of fish to either hard or soft water on the subsequent toxicity of zinc and was undertaken with the aim of clarifying the role of acclimation history. It was also necessary to investigate the time course of the acclimatory process so that in subsequent experiments, both at acute and sublethal zinc concentrations, the minimum effective acclimation period could be employed. In the later experiments described in this chapter the specific influence of calcium on the toxicity of zinc was examined.

All of the experiments reported here, and indeed elsewhere in this thesis, were completed at an external pH of 6. This particular pH was chosen as one at which zinc is considered to exist almost entirely as aquo zinc

ions (Chapter 1) thereby avoiding practical problems and theoretical uncertainties associated with the insoluble hydroxide and carbonate precipitates. This slightly acid pH is also representative of many natural waters subject to elevated levels of zinc. As the primary aim of this work was the investigation of zinc toxicity it was also desirable that the media pH *per se* should be of no significant toxicity. This has previously been demonstrated for brown trout held at pH 6 (McWilliams, 1980).

2.2 MATERIALS AND METHODS

2.2.1 Stock collection and maintenance

Brown trout of weight 5-30 g, fork length 6-15 cm were obtained from Leadmill Trout Farm, Hathersage, Derbyshire. The geology of this area is predominantly millstone grit, producing a fairly soft water. Fish were maintained at the polytechnic in c.1100 l circular GRP tanks of sloping bottom, centre drain design. They were vigorously aerated and received a continuous supply of dechlorinated mains water. Dechlorination was achieved by passing all incoming mains water through an activated carbon filter (Filtromat AC1, Elga Ltd). Water quality characteristics for this and the fish farm water are given in Table 1. Holding tanks were situated outside the building so received the prevailing natural photoperiod. Fish were fed ad libitum once a day on Fingerling 1 or Standard Expanded 4 (BP Nutrition) trout pellets.

2.2.2 Water chemistry analysis

A variety of chemical analyses were performed on the water samples collected. The techniques used are described in this section and unless otherwise stated are applicable to all work presented in this thesis.

Immediately after collection the temperature,

Table 1

Water quality characteristics of the fish supplier, Leadmill Trout Farm, and the stock holding water at Trent Polytechnic

		Leadmill	Polytechnic
pH		7.59 ± 0.09	7.77 ± 0.02
Total Hardness	mg l^{-1} $CaCO_3$	72 ± 3	208 ± 3
Alkalinity	mg l^{-1} $CaCO_3$	31 ± 5	122 ± 2
Conductivity	µS cm^{-1}	206 ± 7	562 ± 13
Calcium	mM	0.39 ± 0.02	1.65 ± 0.02
Magnesium	mM	0.31 ± 0.01	0.40 ± 0.01
Sodium	mM	0.41 ± 0.02	1.42 ± 0.05
Potassium	mM	0.06 ± 0.01	0.09 ± 0.01
Chloride	mM	0.31 ± 0.03	1.40 ± 0.04
Sulphate	mM	0.31 ± 0.03	0.78 ± 0.02
Zinc	µM	<0.06	<0.15
Aluminium	µM	<2.40	<2.40
Cadmium	µM	<0.002	<0.006
Chromium	µM	<0.04	<0.08
Copper	µM	<0.016	<0.08
Iron	µM	<0.72	<0.90
Lead	µM	<0.005	<0.005
Manganese	µM	<0.18	<0.18
Nickel	µM	<0.034	<0.034

Data expressed as $\bar{x}$ ± standard error

Heavy metal levels are maxima recorded in samples analysed by STWA.

dissolved oxygen, total hardness and pH of samples were measured. Dissolved oxygen was measured using a portable oxygen meter, pH with a portable pH meter fitted with a combination electrode (Russell pH, no. CE7L) and total hardness by EDTA titration. The alkalinity and conductivity of some samples were also measured, alkalinity by titration to pH 4.5 with 0.02 M HCl (HMSO, 1981) and conductivity with a portable conductivity meter. A 150 ml sample was stored in a polythene bottle at 4^{o}C after acidification with 3.5 M nitric acid in a 1:100 ratio. This procedure was considered adequate to avoid problems of heavy metal adsorption onto the container surfaces (Henriksen and Wright 1977). Calcium, magnesium and zinc concentrations were measured by atomic absorbtion spectrophotometry (Perkin Elmer model 1100) after appropriate dilution with double-distilled water. To prevent interference with elements forming stable oxysalts lanthanum oxide was added to give a final concentration of 0.25% lanthanum for all AAS calcium analyses. All measurements were performed against standards made with AAS grade 'Spectrosol' (BDH Chemicals) reagents diluted with double-distilled water and with lanthanum or nitric acid added as appropriate. Sodium was measured on a Corning model 400 flame photometer and chloride on a Corning model 920 chloride titrator.

In addition to these analyses a number of samples

were sent to the Severn-Trent Water Authority analytical laboratory, primarily for the determination of all major heavy metal concentrations, but also to allow independent verification of analyses.

2.2.3 Fish acclimation

A group of fish was placed in a c.400 l tank supplied with 0.6 l min^{-1} dechlorinated water maintained at 15 ± 1°C by two chemically inert vitreous silica (Vitreosil) immersion heaters contained in a header tank above the experimental room. Water in the acclimation tanks was maintained at pH 6.0 ± 0.2 by the dosing of 2 M sulphuric acid through a single channel peristaltic pump controlled by a pH recorder-controller (Analytical Measurements 'Digitrol' model) fitted with separate glass and reference electrodes (type SWL and SRR, Russell pH). To avoid pockets of low pH the tanks were vigorously aerated. This also maintained dissolved oxygen levels above 90% saturation. For reasons discussed earlier all work presented in this thesis was conducted at a pH of 6.

Water supplied during this acclimation period was either unmodified (except for dechlorination) mains water or an artificially softened water produced by passing dechlorinated mains water through a cation exchange water softener (ELA3, Elga Ltd.). The softening process involved the exchange of the divalent water hardness ions

calcium and magnesium for sodium. The sodium ions of the ion exchange resin were replenished every 24 h by backflushing with concentrated brine followed by a thorough rinsing with 200 l of softened water to remove any excess NaCl. Typical compositions of the hard and soft waters are listed in Table 2. Apart from the obvious changes of decreased $[Ca^{2+}]$ and $[Mg^{2+}]$ and increased $[Na^{+}]$ the softening process was also characterised by generally decreased background heavy metal levels, Alkalinity was unaffected by softening but the vigorous aeration and constant titration to pH 6 in the 400 l acclimation tanks, combined with the relatively slow turnover of water in these tanks resulted in a lowering of alkalinity levels to below 8 mg l^{-1} $CaCO_3$ compared to the levels of approximately 40 mg l^{-1} in the non-aerated toxicity testing tanks. The resulting medium was soft only in respect of the very low calcium and magnesium levels. In comparison with naturally derived soft waters it could not be categorised as soft on the basis of its overall ion status.

During both acclimation and exposure all fish were subject to a 12:12 h photoperiod. They were fed once a day during acclimation but were starved for 48 h before, and during subsequent testing. In any one test the longest fish used did not exceed the length of the smallest by more than 50% (Sprague 1973).

Table 2

Typical composition of the hard and artificially softened waters used in the zinc toxicity studies

		Hard Water	Soft Water
pH		6.00 ± 0.20	6.00 ± 0.20
Total Hardness	mg l^{-1} $CaCO_3$	208 ± 3	<10 (usually <4)
Alkalinity	mg l^{-1} $CaCO_3$		
Toxicity testing tanks		40 ± 8	38 ± 5
Acclimation tanks		<8	<8
Conductivity	µS cm^{-1}	562 ± 13	683 ± 9
Dissolved Oxygen	% saturation	>90%	>90%
Temperature	^{o}C	15 ± 1.0	15 ± 1.0
Calcium	mM	1.65 ± 0.02	<0.03
Magnesium	mM	0.40 ± 0.01	<0.013
Sodium	mM	1.42 ± 0.05	6.0 ± 0.14
Potassium	mM	0.09 ± 0.01	0.09 ± 0.01
Chloride	mM	1.40 ± 0.10	1.30 ± 0.10
Sulphate	mM	1.94 ± 0.03	1.94 ± 0.03
Zinc	µM	<0.15	<0.10
Aluminium	µM	<2.4	<2.4
Cadmium	µM	<0.006	<0.004
Chromium	µM	<0.08	<0.04
Copper	µM	<0.08	<0.11
Iron	µM	<0.90	<0.36
Lead	µM	<0.005	<0.001
Manganese	µM	<0.18	<0.18
Nickel	µM	<0.035	<0.017

Data expressed as $\bar{x}$ ± standard error

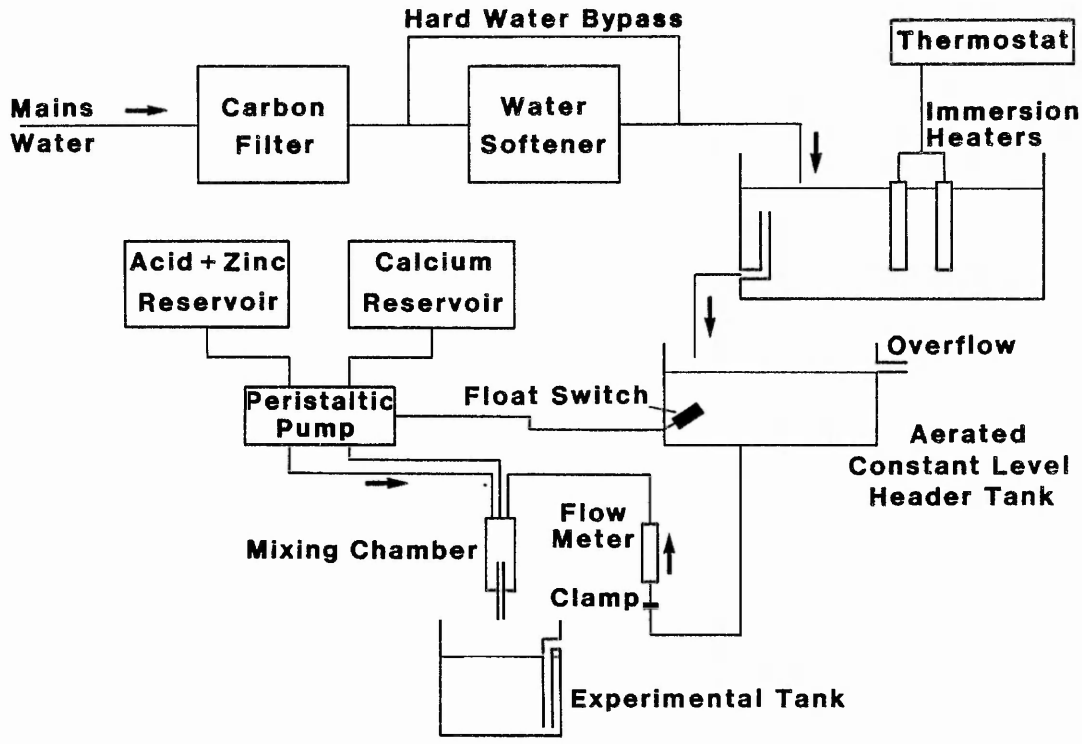
Heavy metal levels are maxima recorded in samples analysed by STWA

2.2.4 The toxicity testing apparatus

The apparatus used for the toxicity tests is illustrated in Figure 1. The dechlorinated, heated, hard or softened water flowed into a vigorously aerated header tank in the controlled environment fish room. Through supplying an excess flow it was possible to maintain a constant level in this tank. From this point the system was split into six replicate dosing systems. In each system water flowed from the header tank via a flow meter to a mixing chamber. Flow was regulated to 0.5 l min^{-1} with a tubing clamp. Zinc was dosed as a series of concentrated reagent-grade $ZnSO_4 . 7H_2O$ solutions through a multi-channel peristaltic pump (502S with 501M pumphead, Watson Marlow Ltd.) into the mixing chamber. A measured concentration of reagent grade sulphuric acid was dosed through the same system to maintain the test tanks at pH 6 ± 0.2. This also ensured that the concentrated zinc stock solutions were highly acidic so no precipitation occurred. In later experiments a separate set of reservoirs were set up to dose measured quantities of reagent-grade $CaCl_2 . 6H_2O$ solutions via the same pump. A float switch in the constant level header tank ensured the switching off of the dosing pump on the few occasions when the mains water was unexpectedly cut off. Thus the experimental tanks never received a concentrated zinc/ acid/ calcium mixture.

Figure 1

Apparatus employed in the flow-through experiments investigating the acute toxicity of zinc to brown trout.


Hard Water Bypass
Thermostat
Mains Water
Carbon Filter
Water Softener
Immersion Heaters
Acid + Zinc Reservoir
Calcium Reservoir
Overflow
Peristaltic Pump
Float Switch
Aerated Constant Level Header Tank
Flow Meter
Mixing Chamber
Clamp
Experimental Tank

From the mixing chamber a siphon arrangement delivered c.1.0 l volumes of water-toxicant mixture to the 120 l experimental tanks. A second siphon arrangement in these tanks drew excess water from the bottom rather than the surface so simultaneously carried away any waste matter.

The flow rate of 0.5 l min^{-1} into the 120 l tanks ensured 90% molecular replacement of the water in 9 h, well within the limits recommended by Sprague (1969).

All apparatus in contact with the experimental water was made of, or coated with, silicone rubber or polythene. Both of these materials were chosen for their low zinc adsorption properties (Florence, 1980). For 24 h before experimentation the system was allowed to flush through under experimental conditions. Thus zinc, calcium and pH levels were allowed to stabilise before addition of the fish. The various calcium and/ or zinc concentrations were randomly allocated amongst the six tanks to avoid any possible bias due to tank position. Due to the increased quantity of water required during toxicity testing the water softener was regenerated every 12 h as opposed to every 24.

2.2.5 Experimental design

In section 2.1 it was mentioned that a series of experiments were to be undertaken investigating the time

course of the process of acclimation to water hardness on the subsequent toxicity of zinc so that in later experiments, at both acute and sublethal levels of zinc, the minimum effective acclimation period could be employed. A series of experiments were therefore proposed investigating this acclimation effect. A single [Zn^{2+}] was to be used in these experiments, the concentration decided on being the 96 h LC_{50} determined in hard water under the experimental conditions outlined. It was reasoned that this concentration would result in mortality of both hard and soft water acclimated fish whereas an LC_{50} determined in soft water might only cause mortality in soft water acclimated fish.

To determine the hard water LC_{50} a number of preliminary sighting tests were completed before the definitive experiment to establish the approximate range of concentrations to be employed. The definitive experiment involved the acclimation of 60 fish to pH 6, 15°C hard water for two weeks before exposure of randomly allocated groups of 10 fish to a logarithmic series of five zinc concentrations and a control. The nominal and actual zinc concentrations during the experiment are listed with the results of the experiment in section 2.3.1. The acclimation period of two weeks was chosen as it was considered that it would allow complete acclimation to changes in temperature, pH and water hardness (Lloyd, 1965; Peterson and Anderson, 1969;

McDonald et al., 1980).

Water samples were collected from the mid-water level of the tanks at 12 h intervals throughout the duration of the test for later analysis. The pH was also measured at these times and any adjustments necessary in the dosing system made immediately.

Observations on mortality were carried out over a logarithmic time sequence as outlined by Sprague (1973). Lack of opercular or body movement after 1-2 minutes gentle prodding was taken as the criterion for death, dead fish being removed immediately.

The 96 h LC_{50} and associated 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (1949). The resulting concentration was then used as the nominal $[Zn^{2+}]$ in the subsequent transfer experiments.

60 fish were acclimated as before to pH 6, 15°C hard water for two weeks. They were then moved to an adjacent acclimation tank containing soft water. 10 fish were removed immediately (day 0) for exposure to zinc in soft water. Further groups of 10 were removed 1, 2, 4 and 8 days after the transfer for identical treatment. Mortality was again noted over a logarithmic time sequence.

The opposite transfer was next completed involving two weeks acclimation to soft water before zinc exposure in hard water. On this occasion groups of 10 fish were zinc exposed at 0, 1, 2 and 4 days after the transfer. A

further group of 10 fish were held in hard water for two weeks following the transfer and then abruptly exposed to zinc in soft water, a repeat of the day 0 group in the first transfer experiment.

A similar experiment was devised to check the reproducibility of the results obtained in the initial transfer experiments. It involved a series of transfers of groups of 10 fish from hard to soft water and vice versa. The experimental protocol in total involved five groups of fish, a sixth serving as control group. Treatments received by these groups are summarised with the results of the experiment in Figure 2. In addition three groups were transferred from hard to soft water actually during zinc exposure. This transfer was completed within the experimental tank rather than by physical transfer between tanks. To facilitate the water change the tanks were partially drained by siphoning, complete replacement of hard with soft water being achieved in approximately 20 h by this method.

The emphasis of the work was now shifted away from water hardness in general to calcium in particular. The effect of a range of calcium concentrations on zinc toxicity was investigated in two experiments, the first over a wide range of $[Ca^{2+}]$ and the second over a narrower range centered around the point where calcium seemed to exert its greatest effect in the first experiment.

70 fish were acclimated to soft water for one week rather than the two week period used previously. This reduction was considered reasonable on the basis of the results obtained from the transfer experiments. It was considered sufficient for acclimation to pH and temperature. Six groups of 10 fish were then randomly allocated to 6 calcium concentrations each containing the 96 h hard water LC_{50} zinc concentration. The lowest $[Ca^{2+}]$ employed was the minimum achievable level from the water softening process. The remaining 10 fish continued to be held in the acclimation tank and served as a control group.

An identical protocol was followed in the second experiment. Mortality was noted at intervals no longer than 5 h during the first 48 h, and no longer than 12 h after this time.

2.3 RESULTS

2.3.1 The effect of acclimation to water hardness on the toxicity of zinc

The mortalities that occured during the test to determine the 96 h hard water LC_{50} are recorded in Table 3. From Litchfield and Wilcoxon (1949) the 96 h LC_{50} was calculated to be 48.8 µM (3.17 mg l^{-1}) $[Zn^{2+}]$ with 95% confidence limits of 34.2-69.7 µM. The slope function (S) of the plotted line was calculated as 1.51.

This zinc concentration was used as the nominal zinc concentration in the subsequent experiments investigating the effects of water hardness acclimation and external calcium concentration on zinc toxicity.

The results of the two initial transfer experiments, from hard to soft water and vice versa are presented in Tables 4 and 5 respectively. The final column of Table 4 represents the group of ten fish in the second experiment that were transferred from hard water to soft water plus zinc in an attempt to repeat the results of the first transfer experiment. Measured zinc concentrations were within ±10% of the desired 48 µM. The maximum concentration difference between any two treatments was approximately 13% so in analysing the results it was reasonable to assume that treatments only differed in the type of acclimation water and duration of acclimation

Table 3

Zinc concentrations ($\bar{x}$ ± range) during, and percentage mortality at the end of, the experiment to determine the 96 h LC_{50} in hard water

Nominal $[Zn^{2+}]$ µM	Actual $[Zn^{2+}]$ µM	Mortality %
0	<0.15	0
12	14 ± 1	0
25	27 ± 2	0
50	54 ± 2	40
100	108 ± 8	100
200	222 ± 3	100

Table 4

Cumulative mortality of fish exposed to zinc in soft water following acclimation to soft water for varying times

		Duration of soft water acclimation period (days)					
		0	1	2	4	8	0
Duration of zinc exposure (h)	4	0	0	0	0	0	0
	6	0	2	0	0	0	0
	8	2	5	0	0	1	0
	12	5	7	1	1	8	4
	24	8	9	7	8	10	10
	30	10	10	10	10	10	10
$[Zn^{2+}]$ µM x ± range		48.7 ±1.0	48.4 ±0.1	52.3 ±0.1	47.7 ±0.1	50.4 ±1.2	47.4 ±0.9

Table 5

Cumulative mortality of fish exposed to zinc in hard water following acclimation to hard water for varying times

		Duration of hard water acclimation period (days)				
		0	1	2	4	
Duration of zinc exposure (h)	4	0	1	0	0	
	42	1	1	0	0	
	60	3	1	0	0	
	72	3	1	2	0	
	120	3	1	2	*	
	144	3	1	*		* - Test discontinued
	168	3	*			
$[Zn^{2+}]$ µM x ± range		47.4 ±0.9	45.4 ±2.0	50.2 ±1.5	48.0 ±0.5	

period before zinc exposure.

The results indicated that the composition of the acclimation water had little bearing on subsequent zinc toxicity which largely appeared to be governed by the water composition during zinc exposure. Evidence for this was reinforced by the results of the multiple transfer experiment (Figure 2). Again water composition during zinc exposure was more important than acclimation water in controlling zinc toxicity. In the three groups where a further hard to soft water transfer was undertaken during zinc exposure (groups i, ii and iv) death followed very rapidly. Group (i) fish had survived 266 h in hard water plus zinc with only one mortality. Following the c.20 h transfer to soft water plus zinc all had died after a further 74 h.

Fish exposed to zinc in hard water displayed a lower than expected mortality in these transfer experiments. The nominal [Zn^{2+}] chosen was such that approximately 50% of the fish were expected to die in 96 h when exposed to zinc in the most "optimal" conditions employed, that is hard water acclimated, hard water exposed fish. The results presented in Table 5 and Figure 2 show that this obviously did not occur. The significance of this will be discussed later in this chapter.

2.3.2 The effect of calcium on the toxicity of zinc

Figure 2

Cumulative percentage mortalities obtained during the multiple transfer experiments. Figures in parentheses represent the external $[Zn^{2+}]$ (μM, $\bar{x} \pm$ range) during the experiment. An arrow denotes the point at which groups (i), (ii) and (v) were transferred from hard to soft water during exposure to zinc. The treatments received by each group are outlined below:

60 fish acclimated to soft water (SW) for 14 days.

↓ Group (i): 10 exposed to zinc in hard water (HW) but transferred back to SW containing the same zinc concentration at 266 h.

50 held in HW for 7 days.

↓ Group (ii): 10 exposed to zinc in HW but transferred back to SW containing the same zinc concentration at 96 h.

40 kept in HW for a further 5 days (total 12 days in HW).

↓ Group (iii): 10 exposed to zinc in SW.

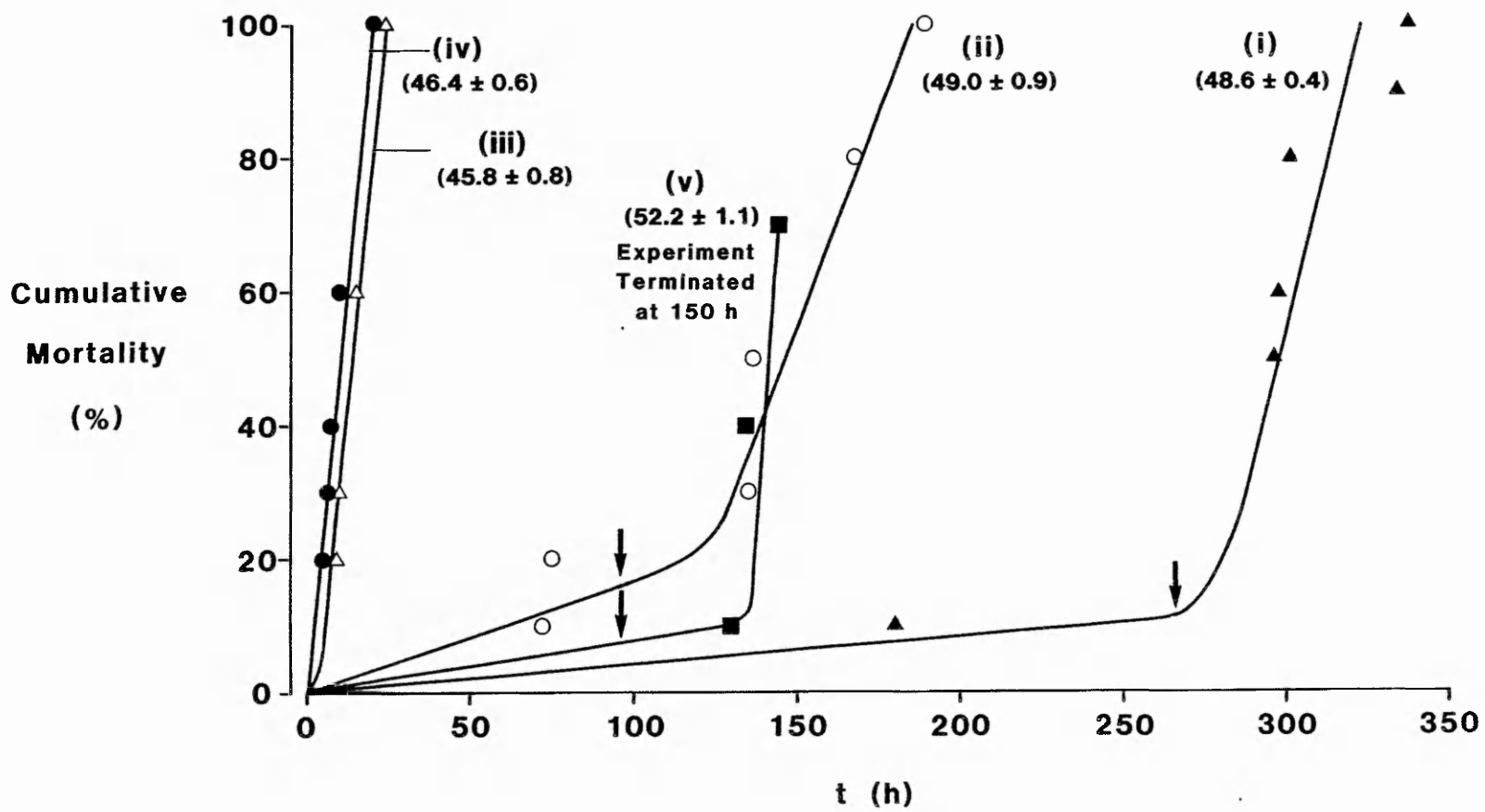
30 kept in SW for 7 days.

↓ Group (iv): 10 exposed to zinc in SW.

20 kept in SW for a further 3 days (total 10 days in SW).

↓ Group (v): 10 exposed to zinc in HW but transferred back to SW containing the same zinc concentration at 96 h.

The remaining 10 fish served as a control group examining whether the transfer process <u>per se</u> was deleterious to fish health.


Cumulative
Mortality
(%)
100
80
60
40
20
0
0
50
100
150
200
250
300
350
t (h)
(iv)
(46.4 ± 0.6)
(iii)
(45.8 ± 0.8)
(v)
(52.2 ± 1.1)
Experiment
Terminated
at 150 h
(ii)
(49.0 ± 0.9)
(i)
(48.6 ± 0.4)

The results of the two experiments conducted at a range of external calcium concentrations are presented in Figures 3 and 4. Also shown are the calcium and zinc concentrations experienced by all groups during the two experiments. Chloride concentrations are also listed due to the elevation in concentration of this ion as the counter-ion of calcium.

As in the tests investigating acclimation to water hardness all measured zinc concentrations are similar (<12% variation) so differences in zinc toxicity can be assigned to differences in the $[Ca^{2+}]$ of the media.

From Figures 3 and 4 it is evident that zinc toxicity is decreased by increasing external $[Ca^{2+}]$. The first experiment revealed a possible threshold in this effect between 1.25 and 2.50 mM but the second, employing the somewhat narrower range of calcium concentrations, suggested a dose-dependent response of zinc to increased external $[Ca^{2+}]$.

The data from Figures 3 and 4 are shown in Figure 5 in the form of median lethal times (LT_{50}), that is, the time required to kill 50% of the fish in a given concentration of toxicant. The dose-dependent response of zinc toxicity to external $[Ca^{2+}]$ is clearly shown in this figure. An apparent threshold $[Ca^{2+}]$ is evident between 1.0 and 1.5 mM. The extent to which this threshold is a real phenomenon is discussed in the next section.

Figure 3

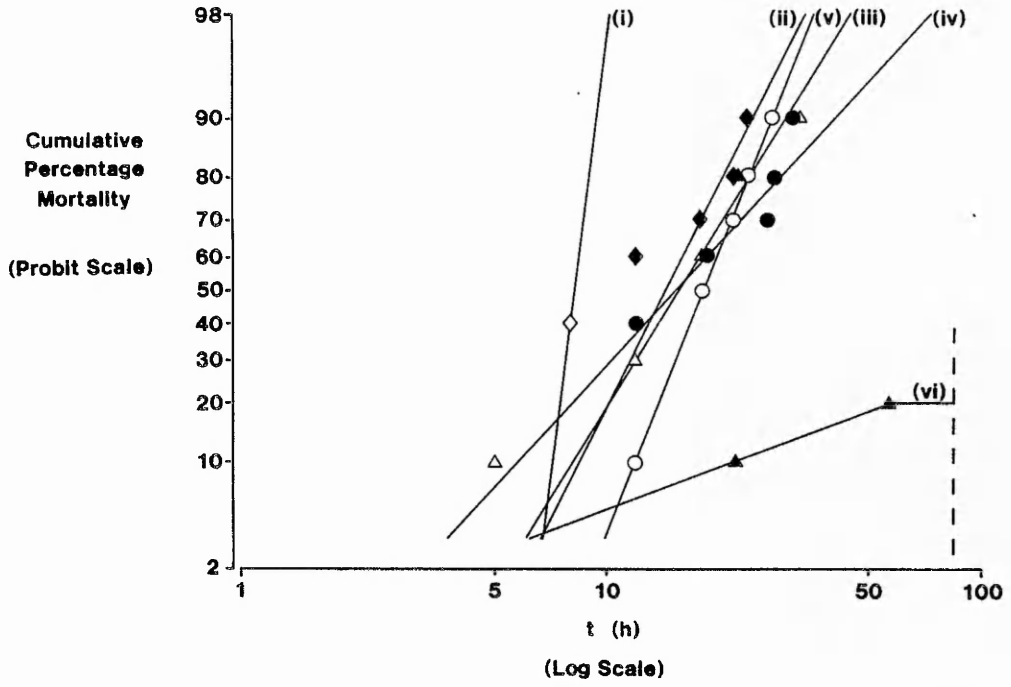
The effect of a range of external $[Ca^{2+}]$s on the cumulative percent mortality of brown trout exposed to a single external $[Zn^{2+}]$. Nominal and measured $[Ca^{2+}]$, its counter-ion $[Cl^{-}]$ and the $[Zn^{2+}]$ experienced by each group are listed below. (All values $\bar{x}$ ± range). This experiment was terminated at 84h.

Group	Nominal $[Ca^{2+}]$ mM	Actual $[Ca^{2+}]$ mM	$[Cl^{-}]$ mM	$[Zn^{2+}]$ µM
◇ (i)	0	0.004 ± 0.002	1.8 ± 0.1	42.0 ± 0.8
◆ (ii)	0.15	0.167 ± 0.003	2.0 ± 0.1	42.9 ± 5.7
△ (iii)	0.30	0.33 ± 0.01	2.4 ± 0.1	40.8 ± 3.9
● (iv)	0.62	0.62 ± 0.01	2.9 ± 0.0	41.6 ± 4.7
○ (v)	1.25	1.27 ± 0.03	4.0 ± 0.1	43.8 ± 1.1
▲ (vi)	2.50	2.57 ± 0.06	6.3 ± 0.1	46.1 ± 2.5

Figure 4

The effect of a narrower range of external $[Ca^{2+}]$s on the cumulative percent mortality of brown trout exposed to the same external $[Zn^{2+}]$. (All values $\bar{x}$ ± range). This experiment was terminated at 96 h.

Group	Nominal $[Ca^{2+}]$ mM	Actual $[Ca^{2+}]$ mM	$[Cl^{-}]$ mM	$[Zn^{2+}]$ µM
△ (i)	0.50	0.60 ± 0.02	2.8 ± 0.2	44.1 ± 2.3
▲ (ii)	0.88	0.97 ± 0.10	3.5 ± 0.2	45.9 ± 4.8
◇ (iii)	1.25	1.45 ± 0.05	4.4 ± 0.3	45.3 ± 1.5
● (iv)	1.62	1.85 ± 0.07	5.4 ± 0.3	46.3 ± 2.8
◆ (v)	2.00	2.30 ± 0.27	6.4 ± 0.3	43.1 ± 2.4
□ (vi)	2.38	2.62 ± 0.12	6.6 ± 0.5	44.4 ± 3.4


Cumulative
Percentage
Mortality
(Probit Scale)
98
90
80
70
60
50
40
30
20
10
2
(i)
(ii)
(v)
(iii)
(iv)
(vi)
1
5
10
50
100
t (h)
(Log Scale)

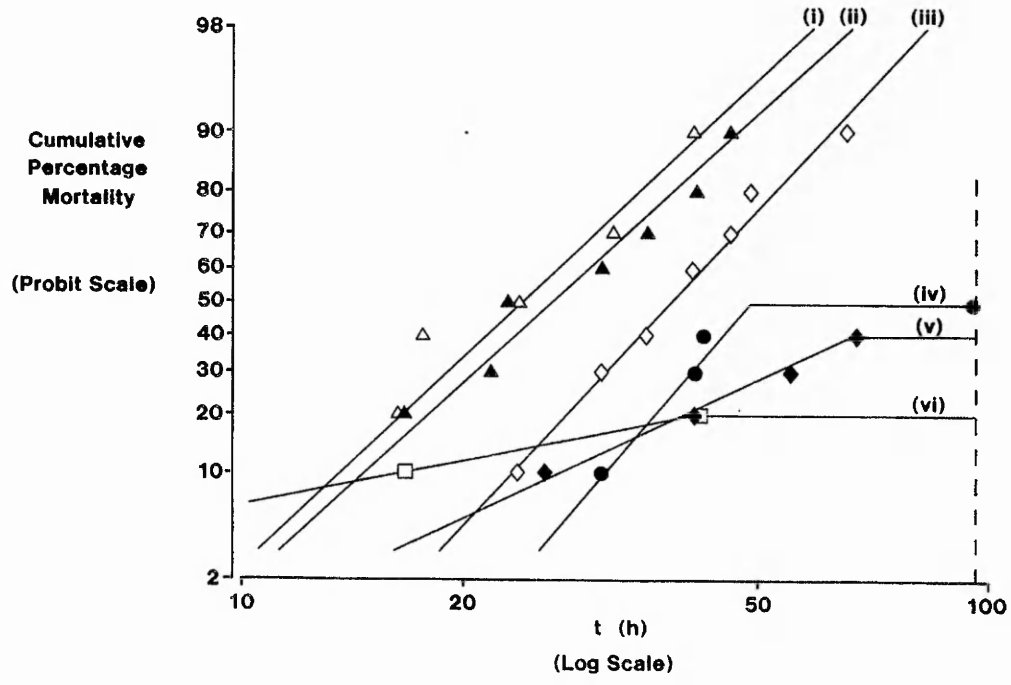
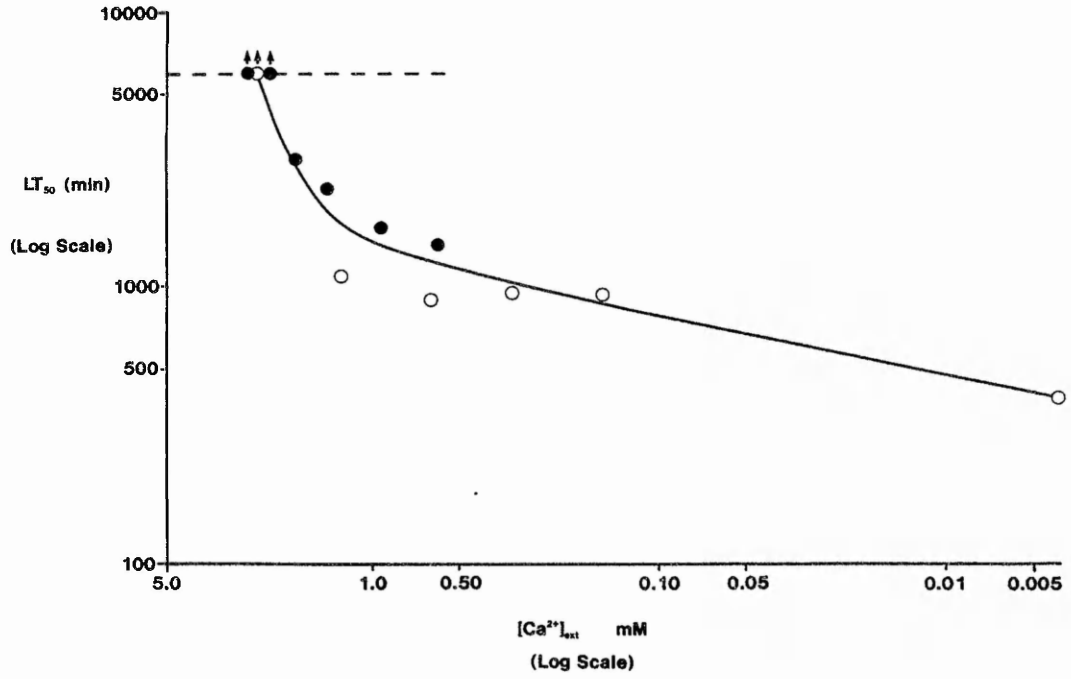

Cumulative
Percentage
Mortality
(Probit Scale)
98
90
80
70
60
50
40
30
20
10
2
(i)
(ii)
(iii)
(iv)
(v)
(vi)
10
20
50
100
t (h)
(Log Scale)

Figure 5

The effect of a range of external [Ca^{2+}]s on the median lethal times of groups of ten fish exposed to a single [Zn^{2+}]. Data were obtained from that displayed in Figures 3 and 4. The broken line indicates the termination of the two experiments. Arrows signify less than 50% mortality on termination of the experiment.

○ Data from Figure 3

● Data from Figure 4


10000
5000
1000
500
100
LT50 (min)
(Log Scale)
5.0
1.0
0.50
0.10
0.05
0.01
0.005
[Ca2+]ext mM
(Log Scale)

2.4 DISCUSSION

Meaningful comparison of the 96 h LC_{50} of 48.8 μM (3.17 mg l^{-1}) obtained for zinc in pH 6 hard water in this study is rendered difficult due to much of the data published concerning zinc toxicity having been obtained from experiments conducted at circumneutral pH (Sinley et al., 1974; Alderdice and Maclean, 1982; Meisner and Quan Hum, 1987). pH is known to have a profound influence on the toxicity of zinc (Everall and Macfarlane, 1984) even at acid pH where little change in zinc speciation occurs (Bradley and Sprague, 1985a; Everall and Macfarlane, 1985; Cusimano et al., 1986). Previous work at this institution (Everall, 1987) yielded a 96 h LC_{50} of 41.4 μM under very similar experimental conditions to those employed here. Bradley and Sprague (1985a) worked on rainbow trout tested in, but not adapted to, pH 5.5 and 7.0 hard and soft waters (390 and 30 mg l^{-1} as $CaCO_3$ respectively). Extrapolation from their graphical data to the hardness and pH used in this study yields an approximate value of 57 μM. Thus, in the few comparisons that are possible the results obtained are similar.

Information available from the literature relating to the modifying influence of water hardness on the toxicity of zinc was reviewed in Chapter 1. From the results discussed it appeared likely that the protective action of water hardness was largely conferred through an

internal "biological" mechanism, one requiring time to be acquired and lost on transfer of fish between waters of differing hardness. In comparison with the conclusions drawn from previous experiments of this type therefore, the results obtained in the transfer experiments presented here were somewhat unexpected. The composition of the acclimation water appeared to have little influence on the toxicity of zinc, toxicity largely appearing to be governed by the actual composition of the water during zinc exposure. Supporting data for this observation are available from other studies however. Wicklund (unpublished observations) noted that the protective effect conferred by water hardness against the toxicity of cadmium to minnows (Phoxinus phoxinus) was lost within a few hours on transfer of fish from hard to soft water. Similarly, the hard to soft water fish transfer effected by Calamari et al. (1980) in their work on cadmium produced an LC_{50} higher than that for soft water acclimated/ soft water exposed fish but still substantially lower than that for hard water acclimated/ hard water exposed fish. Thus, on this basis, in choosing the hard water LC_{50} as the nominal zinc concentration to be used in these experiments it might be expected that hard water acclimated/ soft water exposed fish would die relatively quickly, which indeed they did.

Closer examination of the data of Calamari and co-workers, from a report often cited as a classic

example of the "biological mechanism" of the protective action of water hardness, reveals that this proposal is not strictly accurate. The hardness of the water during cadmium exposure was shown to have a very significant influence on toxicity, an influence not affected by the acclimation history of the fish. Combining this result with that for zinc in the work presented here therefore, suggests that though it is not possible to entirely discount an internal mechanism for the protective action of water hardness, strong evidence is available contradicting the widely held belief that protection requires appreciable time to be acquired and lost. At this point the uptake-site competition hypothesis proposed by Pagenkopf (1983) seems the mechanism more likely to explain the modification of zinc toxicity by water hardness, at least for acutely toxic concentrations.

In section 2.3.1 it was briefly mentioned that fish exposed to the 96 h LC_{50} $[Zn^{2+}]$ in hard water during the transfer experiments exhibited a lower than expected rate of mortality. Two possible conclusions can be drawn from this. Either, the calculated LC_{50} could have been abnormally low, or, acclimation to soft water before zinc exposure may have increased the resistance of the fish to zinc. The fact that mortality in group (ii) fish in the multiple switch experiment was only 20% after 96 h would tend to imply that the former was true as this group was

tested in hard water after a one week acclimation period in hard water. One possible source of error in the calculated LC_{50} can be derived from the mortalities at the completion of the definitive test. Only one external $[Zn^{2+}]$ produced mortality intermediate between 0 and 100%. Although the calculation method of Litchfield and Wilcoxon does allow for the inclusion of 0 and 100% effects the possible errors associated with the calculation are obviously increased. Hence, the 95% confidence limits associated with the LC_{50} are rather wide. It should be noted however, that the lower than expected mortalities in hard water do not detract from the validity of the results which clearly showed that media composition during zinc exposure, rather than acclimation media composition, was the dominant factor influencing zinc toxicity.

Apart from changes in water hardness associated with the water softening process there were large changes in sodium levels which could conceivably have affected the toxicity of zinc. Sodium is generally considered not to exert an effect on metal toxicity however (Pagenkopf, 1983), a proposal supported by studies on cadmium (Carroll *et al.*, 1979). Despite the comparatively large increase of $[Na^{+}]$ in the artificially softened water of this study it was considered that any effect it may have exerted would have been of secondary importance when compared to the effects of water hardness. Indeed, in the

majority of experiments presented in the subsequent chapters of this thesis soft water with calcium or magnesium added as required was used as the experimental water so variations in sodium levels between these waters were small.

Changes in water hardness and pH are usually accompanied by associated changes in alkalinity. Alkalinity has been attributed with the ability to modify zinc toxicity (Holcombe and Andrew, 1978). Below pH 7 however, it was shown not to exert any significant effect relative to water hardness and pH (Bradley and Sprague, 1985a). The alkalinities of the hard and soft waters in this study were similar as it was not a factor affected by the softening process. Thus it can be assumed that alkalinity was unlikely to have exerted any significant effect.

Acidification of all water to pH 6 with sulphuric acid resulted in an associated increase in sulphate concentration. Branchial membranes have recently been shown to be permeable to sulphate (Hobe, 1987) in contrast to earlier work where they were assumed to be impermeable to this anion (Garcia-Romeu and Maetz, 1964; Graham and Wood, 1981). Though the functional significance of this is still highly uncertain its movement is likely to affect the passage of other ions simply through the demands of electroneutrality (Hobe, 1987). What is clear however is that acid type, and hence

presumably the acid anion, does exert an effect on the resulting response of fish to acid stress (Graham and Wood, 1981; McWilliams, 1983). Data are not available regarding the effect of sulphate on zinc toxicity. Studies on cadmium however suggest little or no effect (Carroll et al., 1979). In addition, in an analysis of the chemistry of 719 Norwegian lakes Brown and Sadler (1981) concluded that fishery status was independent of sulphate concentration. This evidence, together with the fact that all experiments in this thesis used water acidified to the same extent suggest that sulphate, like sodium and alkalinity, can be discounted as having any modifying influence on the toxicity of zinc in this work.

In the two experiments where the effect of calcium on zinc toxicity was investigated the chloride concentration of the water varied simultaneously with calcium. No attempt was made to separate any possible effect due to this increased anion concentration but on the evidence of the few studies where this has been attempted chloride seems generally to have little effect on metal toxicity when present at relatively low concentrations. Mercury toxicity provides the only obvious exception to this, with high chloride levels resulting in the formation of the relatively non-toxic $HgCl_3^-$ and $HgCl_4^{2-}$ complexes at the expense of the highly permeable, highly toxic $HgCl_2$ (Walczak et al., 1986). Cadmium uptake in perfused rainbow trout gills was

shown to be significantly reduced by the addition of 120 mM NaCl (Part et al., 1985). Experiments with an iso-osmotic concentration of mannitol showed that this reduction was not related to changes in water transfer. The reduction was attributed to complexation of cadmium by chloride resulting predominantly in the formation of $CdCl^+$ and $CdCl_2$ complexes shown not to be available to the gills. The highest $[Cl^-]$ measured in the work presented here was 6.6 mM, considerably lower than that used by Part and co-workers. This, combined with the fact that the protective action of calcium is evident at sub milli-molar concentrations means that chloride was unlikely to have been of importance in this study.

The results of the two experiments investigating the effect of calcium clearly showed that increased calcium levels reduced zinc toxicity over the wide range of concentrations investigated. As in the soft to hard water transfer experiments this protection was evident after acclimation to water of very low calcium content, a result emphasising the importance of the calcium status of the medium during zinc exposure relative to the previous acclimation history of the fish.

Two possible mechanisms are available to account for the reduced toxicity of zinc with increasing external $[Ca^{2+}]$. Either increased $[Ca^{2+}]$ may cause slower zinc accumulation by fish, or, elevation of external $[Ca^{2+}]$ may necessitate greater accumulation of zinc by the fish

before death results. Bradley and Sprague (1985b) have defined the intrinsic toxicity of zinc as that amount present in the gills at death. Their data on rainbow trout indicated that water hardness altered the uptake and/ or the excretion dynamics of zinc in gill tissue resulting in a slower rate of zinc accumulation. The intrinsic toxicity however, was very similar in fish exposed to zinc in hard and soft waters. Previous work on the brown trout at this institution (Everall, 1987) has also demonstrated reduced zinc uptake in hard water compared to that in soft water.

Calcium is known to reduce the permeability of branchial membranes to both water (Potts and Fleming, 1970; Oduleye, 1975) and electrolytes (Potts and Fleming, 1971; Cuthbert and Maetz, 1972; Eddy, 1975; McWilliams and Potts, 1978). Graham et al. (1982) have pointed out that, with the exception of the work of Oduleye, all of these studies involved acute exposure of the fish to the desired calcium concentration. Following the acclimation of goldfish (Carassius auratus) to deionised water for three weeks for example, subsequent exposure to sodium yielded high net fluxes of sodium (Cuthbert and Maetz, 1972). Calcium caused a reduction of sodium influx within 20 min of its addition, a similar response to that observed on sodium influx when calcium chelating agents were used before the addition of calcium. The similarities of these responses were accounted for on the

basis of the deionised water acclimation process causing the loss of membrane bound calcium, an effect rapidly reversible on addition of calcium to the water. Similarly, after 24 h acclimation of goldfish to deionised water addition of calcium caused an immediate change in the transepithelial potential and associated reductions in sodium and chloride fluxes (Eddy, 1975). Using tritiated water to measure the gill permeability of the plains killifish (Fundulus kansae) Potts and Fleming (1970) showed an almost instantaneous decrease in the rate of exchange of water on addition of calcium.

The reduction of membrane permeability by calcium has frequently been proposed as the "biological mechanism" involved in the reduction of metal toxicity (Bradley and Sprague, 1985b). A prerequisite for this proposal is that the modification of membrane permeability is not a rapid process and occurs over several days. Much of the evidence available from the literature is obviously in direct contradiction to this however, since permeability changes occur rapidly on transfer between media of differing $[Ca^{2+}]$. It may be therefore, that the reduction in zinc toxicity observed following the addition of calcium to the medium in this study was explicable in terms of a rapid modification of membrane permeability.

Although the response of zinc toxicity to external $[Ca^{2+}]$ was dose-dependent it was stated that there was

some evidence of a possible threshold [Ca^{2+}] between 1.0 and 1.5 mM. This apparent threshold [Ca^{2+}] reflected the influence that those calcium concentrations which reduced fish mortality to below 50% at the end of the experiment exerted on the method of presentation of the results. Use of a lower [Zn^{2+}] for example, would probably have resulted in a lower apparent threshold [Ca^{2+}], the lower [Ca^{2+}] being sufficient to reduce mortality due to the new [Zn^{2+}] to below 50%. The possible existence of a true threshold [Ca^{2+}], that is a threshold beyond which increasing external [Ca^{2+}] has no additional effect irrespective of increases in the external [Zn^{2+}], might have been revealed by investigating the effect on fish mortality of a range of external zinc concentrations in addition to the range of calcium concentrations tested. Other studies investigating the effect of varying external [Ca^{2+}] have produced equivocal results. Judy and Davies (1979) reported a dose-dependent response of acute zinc toxicity in fathead minnows in relation to external calcium concentrations up to 3.5 mM. Cadmium toxicity was found to be reduced at [Ca^{2+}]'s up to 3 mM, with no further effect at 10 mM (Part et al., 1985). Some membrane permeability studies have suggested that the greatest effect of calcium on trans-epithelial potentials (TEPs) and sodium fluxes in brown trout occurs at concentrations below 1 mM with no further change above 2 mM (McWilliams and Potts, 1978; McWilliams 1982). In

contrast though, TEP in goldfish was progressively affected by external [Ca^{2+}] at concentrations up to 10 mM (Eddy, 1975). Overall, the general paucity of data concerning the possible existence of a threshold [Ca^{2+}] precludes quantitative comparison between data obtained from the study of membrane permeability and data obtained from the study of acute trace metal toxicity.

In conclusion, it is clear that the elevation of water hardness in general, and specifically external [Ca^{2+}], brought about a rapid reduction in the acute toxicity of zinc to brown trout. Though it is not possible at this stage to be certain as to the mechanism by which this occurred, similarities of the results with those from studies concerned with membrane permeability in relation to external [Ca^{2+}] are clear, and suggest that the reduction of membrane permeability is important in this process. Other workers in this field have supported this same "biological hypothesis" by stating that it is a mechanism acting internally and so requires time to be acquired and lost. The work presented in this chapter has shown that although data concerning the modification of membrane permeability do support a role in protection from metals, the previously undisputed reasoning behind this proposition is no longer valid. Thus, protection is evident very soon, if not immediately, after transfer of fish to media of elevated hardness or [Ca^{2+}]. It should be mentioned however, that

although membrane permeability changes could account for the observed response to zinc so too could competition for uptake sites on the gill surface. At this point it is not possible to ascertain the relative importance of these two processes in determining the acute toxicity of zinc to brown trout.

CHAPTER 3

The modifying influence of calcium and magnesium on the uptake and distribution of zinc.

3.1 INTRODUCTION

The work presented in Chapter 2 examined the modifying influences of water hardness and external $[Ca^{2+}]$ on the acute toxicity of zinc to brown trout and found, as have many previous workers in this field, that both increased water hardness and external $[Ca^{2+}]$ greatly reduced toxicity. In contrast to some reports however, the composition of the acclimation medium that the fish were maintained in before zinc exposure had little, if any effect on the subsequent toxicity of zinc, toxicity being governed mainly by media composition during metal exposure. The primary aim of the experiments reported in this chapter was to investigate the mechanism by which calcium and magnesium modify the uptake and subsequent distribution and excretion of zinc. In doing this it was also intended to clarify the relative importance of these two ions in these processes.

Zinc does not exert its toxic action at sublethal concentrations through the same mechanism as at acutely toxic concentrations (Spry and Wood, 1984, 1985). The impairment of respiratory function frequently reported at acutely toxic concentrations is absent at lower concentrations. For this reason it was decided to investigate the mechanism(s) of zinc toxicity at more environmentally relevant sublethal concentrations. From earlier work completed at this institution (Everall,

1987) therefore, a zinc concentration known to be sublethal under the experimental conditions employed was chosen for use in all of the experiments described here. The concentration chosen (0.77 μM, 0.05 mg l^{-1}) was also known to be environmentally relevant as regards zinc concentrations found in polluted natural waters (Forstner and Prosi, 1979; Vymazal, 1985).

The γ-ray emitting radioisotope ^{65}Zn was used as a biological tracer of zinc uptake in this investigation. The use of radioactive isotopes as biological tracers is widespread in many areas of the biological sciences and stems from the fact that all isotopes of any given element, whether radioactive or not, differ mainly in nuclear mass, their electronic constitution and hence their valency electrons being identical. Thus, their chemical properties are virtually indistinguishable so suitable radioactive isotopes of any given element can be used as biological tracers for the more abundant stable species. The addition of ^{65}Zn to stable zinc in the medium in the present study, therefore, allowed the measurement of zinc uptake by the fish without recourse to intrusive surgical techniques. Through the measurement of total zinc concentrations it was intended to simultaneously measure the influx, efflux and net flux of zinc, and thereby determine the overall turnover of zinc by the fish during the various experiments.

The percentage distribution and relative

accumulation of zinc amongst the various body tissues of the fish following the sublethal zinc exposure was determined by the measurement of ^{65}Zn accumulated within these tissues. As potential indicators of ionoregulatory disturbances the concentrations of the major plasma ions were also determined. Combination of these data with those provided by measurements of zinc fluxes was intended to elucidate the mechanisms by which zinc exerts its toxic action, is detoxified and excreted.

Through the completion of a number of different series of experiments, all of similar design, the modifying influence of external $[Ca^{2+}]$ and $[Mg^{2+}]$ on the sublethal toxicity of zinc was investigated. As a consequence of the somewhat unexpected nature of some of the results reported in the previous chapter both the influence of the previous acclimation history of the fish, and the influence of the external divalent ion concentration during exposure to zinc were investigated.

3.2 MATERIALS AND METHODS

3.2.1 Fish acclimation

Groups of 10 fish of weight 50-130 g, fork length 16-22 cm were placed in a c.400 l acclimation tank supplied with 0.8 l min^{-1} dechlorinated mains water at 15 ± 1°C as outlined in Chapter 2. All water was maintained at pH 6 as before. With the exception of a single experiment all flux experiments using ^{65}Zn were undertaken in softened water with calcium or magnesium added as appropriate. The composition of the softened water before the addition of calcium or magnesium was as outlined in Table 2. Where required, calcium or magnesium were dosed with the incoming water as concentrated reagent grade solutions of the chloride through a single channel peristaltic pump into a mixing chamber, where a siphon arrangement delivered c.1.0 l volumes to the acclimation tank. As in the acute zinc toxicity experiments at a range of external $[Ca^{2+}]$ a one week acclimation period was employed with transfer of fish to the flux apparatus at the end of this time. The fish were not fed during acclimation and testing.

3.2.2 Flux apparatus and experimental protocol

Ten identical flux chambers were used with a single

fish placed in each. Each chamber consisted of an opaque polythene container of approximately 1500 ml volume fitted with a loop of polythene air tubing and with a hole cut in the lid to allow sample withdrawal and pH measurement, and a stoppered overflow pipe to allow drainage to the desired volume for closed circuit operation. As in earlier work all materials in contact with the experimental media were chosen for their low zinc adsorptive properties. The 10 chambers were placed in two radioactivity containment trays which during experimentation were filled with water maintained at 15 ± 1°C by continual recirculation through a cooling unit.

Irrespective of composition all water used in the flux experiments was vigorously aerated and maintained at pH 6 for at least 24 h before use to ensure complete decarbonation. Failure to do this resulted in pH rapidly rising outside desired limits when aerated during experimentation. Nitrogenous excretion by the fish also caused a rise in pH during experiments so a few drops of 0.1 M H_2SO_4 were added manually at approximately 1 h intervals throughout all experiments. The pH fluctuated by a maximum of ± 0.3 units around the desired level of 6.0.

The fish were introduced into the flux chambers 3 h before the start of an experiment. Water composition during this time was as supplied during the one week acclimation period. Water of the desired composition for

experimentation was introduced 30 minutes before the start of a flux period with approximately 7 l allowed to flow through each flux chamber to ensure complete replacement of acclimation water. The chambers were then partially drained to give an experimental volume of 1100 ± 100 ml and switched to closed circuit.

All work presented in this chapter was undertaken with an aliquot of a concentrated zinc sulphate solution added to the experimental water to give a nominal starting $[Zn^{2+}]$ of 0.77 μM (0.05 mg l^{-1}). Accompanying the addition of the stable zinc an aliquot of ^{65}Zn (as $ZnCl_2$, Amersham International) was added to give a nominal starting activity of 185 kBq (5 μCi) per chamber. The system was then allowed to equilibrate for 10 minutes with aeration and fish movement ensuring adequate mixing by the end of this time. 2 ml aliquots were removed by auto-pipette at intervals and dispensed into polystyrene tubes for radioactivity measurement by γ-counting in a Packard 500 auto-gamma scintillation counter fitted with a 3 x 3" thallium-activated sodium iodide crystal of through-hole design. Through the use of a multi channel analyser the ^{65}Zn photopeak was identified to lie between 980 and 1250 keV. To avoid counting errors due to low energy Compton electrons all samples were counted using these limits. Under these conditions a counting efficiency of 7-8% was obtained for a 2 ml volume of a standard of known activity.

During initial experiments the 2 ml samples taken for analysis of radioactivity were subsequently analysed for stable zinc by standard methods of AAS. Results obtained proved to be unreliable, probably due to contamination by the sample tubes themselves. In later experiments therefore, samples were collected specifically for stable zinc measurement. 5 ml aliquots were dispensed by auto-pipette into polystyrene tubes containing 50 µl of 3.5 M nitric acid. All tubes had previously been washed in 5% Decon 90 then 20% nitric acid with a final thorough rinse in two changes of distilled water. The calcium and magnesium content of experimental waters was also analysed at the start and end of experiments on appropriately diluted samples containing 0.25% lanthanum oxide.

At the end of each experiment the fish were killed by overdose of anaesthetic (MS222, Sandoz Ltd.) and susequently weighed before being blood sampled by caudal venipuncture using a lithium heparinised syringe fitted with a 23 gauge needle. The blood was dispensed into a sodium heparinised Eppendorf tube. A 30 µl sub-sample was put into a sodium heparinised micro-capillary tube for haematocrit determination after centrifugation at 12000 xG for 5 minutes in a Hawksley micro-haematocrit centrifuge. The main blood sample was centrifuged at 8800 xG for 5 minutes in an Eppendorf centrifuge (model no. 5413) and 100 µl of the plasma placed in a polystyrene

tube for γ-counting. The remaining plasma was stored at -20°C in a non heparinised Eppendorf tube for subsequent plasma ion analysis. The cell fraction was placed in a pre-weighed γ-counting tube for radioactivity measurement as were excised gills, spleen, gall bladder, liver, alimentary canal and kidney. The gills were rinsed in distilled water and blotted dry to remove any loosely bound ^{65}Zn before counting. The remaining carcasses were stored in individual bags at -20°C and within two weeks were homogenized in a Waring blender with 40 ml of distilled water added. Duplicate 2-3 g sub-samples of the homogenate were γ-counted in pre-weighed sample tubes.

The volume of water in the flux chambers at the completion of the experiments was determined for subsequent use in flux calculations.

3.2.3 Plasma ion analysis

Plasma $[Ca^{2+}]$ was measured in 50 µl aliquots on a Corning model 940 calcium analyser, a technique involving the quenching of calcein fluorescence by titration of calcium ions with the chelating agent EGTA.

Plasma $[Cl^{-}]$ was also measured on undiluted plasma samples, in this instance 20 µl samples on a Corning model 920 chloride titrator. The use of a Hamilton syringe fitted with Chaney adaptor ensured accurate and reproducible dispensing.

Plasma [Na^+] was determined on Hamilton syringe and Chaney adaptor dispensed 10 µl samples diluted 1:500 with double- distilled water. A Perkin Elmer model 1100 AAS set up on flame emission mode was used for these determinations.

In all cases samples were analysed in duplicate or triplicate with previously analysed samples reanalysed concurrently to ensure continuity of results.

3.2.4 Calculation of zinc influx

The following equation, modified from equation 5 of Kirschner (1970), was used to calculate the influx of zinc.

$$J_{in} = \frac{(\ln Q_{out(0)} - \ln Q_{out(t)})}{t \cdot W} \cdot Q_{out}$$

where $Q_{out(0)}$ and $Q_{out(t)}$ represent the total amount of radioactivity (c min^{-1}) in the medium at the start and end of the experiment, Q_{out} represents the mean total quantity of zinc (µmol) in the water during the experiment, t is the duration of the experiment in hours and W is the fish weight in kg. J_{in} was thus derived as µmol kg^{-1} h^{-1}.

Influx calculated by this method assumes that disappearance of isotope from the water is fully

accounted for by entry into the fish and that the backflux of isotope from the fish to the water remains negligible throughout the duration of the experiment. The first assumption was partially investigated using control experiments run under the standard conditions outlined but with the omission of fish from the system. Thus the extent of isotope adsorption onto the experimental system was evaluated. The second assumption was not specifically investigated in this work, but for reasons discussed later was considered not to be of significance in most of the experiments using ^{65}Zn.

3.2.5 Statistical procedures

A number of statistical procedures were employed in the analysis of data provided by the flux experiments.

Influx data itself was first subjected to Bartlett's Test for Homogeneity of Variance (Snedecor and Cochran, 1967). In most experiments a significant departure from the null hypothesis was indicated so all influx data were therefore analysed by the Kruskal-Wallis Test (Conover, 1980), the non-parametric alternative to analysis of variance. Where significant differences were indicated average ranks were compared as outlined in Conover (1980).

Tissue and plasma samples analysed by γ-spectrometry yielded data in the form of counts per wet weight or

volume of sample respectively. To allow comparison of the relative zinc uptake of individual tissues this data was adjusted to a standard weight of tissue (1 g) or volume of plasma (1 ml) and a correction was made to allow for small differences in mean external specific activity between treatments. The formulae used in these calculations are detailed below.

$$\frac{\text{Tissue c min}^{-1}\ \text{g}^{-1}\ \text{or Plasma c min}^{-1}\ \text{ml}^{-1}}{\text{Specific activity}}$$

$$\text{where Specific Activity} = \frac{\bar{x}\ \text{external}\ ^{65}\text{Zn}\ (\text{c min}^{-1}\ \text{ml}^{-1})}{\bar{x}\ \text{external}\ [\text{Zn}^{2+}]\ (\text{nmol ml}^{-1})}$$

The resulting data were then expressed as nmol of zinc accumulated per g of tissue or per ml of plasma.

Due to radioactive count data having a natural tendency towards the Poisson distribution the resulting corrected data were analysed by the Kruskal-Wallis Test with average ranks compared where a significant difference was indicated.

The relative distribution of zinc throughout the fish was calculated from the weight of individual tissues and their respective radioactive counts. Plasma and packed cell volumes were estimated for each fish from data obtained for rainbow trout using ^{51}Cr-labelled erythrocytes (Gingerich et al., 1987). The resulting zinc

distributions were analysed by one-way analysis of variance, a significant departure from the null hypothesis allowing subsequent comparison of mean values against the mean value obtained in the experiment conducted at the lowest divalent ion concentration.

Plasma ion data were analysed for homogeneity of variance by Bartlett's Test. Analyses of variance or Kruskal-Wallis tests were then employed as appropriate with subsequent comparisons of means or average ranks.

3.2.6 Experimental design

In total, five series of experiments were completed investigating zinc uptake and distribution, the plasma ionoregulatory disturbances associated with sublethal zinc exposure, and modification of these by external calcium and magnesium. Details of these five series are summarised in Table 6.

The first series was conducted using a 30 h flux period. Fish were acclimated for one week to water of the minimum external $[Ca^{2+}]$ and $[Mg^{2+}]$ achievable from the softening process. At the end of this time they were transferred into the flux chambers and, after 3 h, exposed to 0.77 μM zinc in water of the same low $[Ca^{2+}]$ (one experiment) or at one of three higher $[Ca^{2+}]$s (three experiments).

The second series represented a single experiment

Table 6

Protocols employed in the five series of ^{65}Zn flux experiments

Series	Acclimation Media Composition	Flux Period (h)	Divalent Ion Investigated	Nominal Concentrations Investigated (mM)
1	Low Ca and Mg	30	Ca	0, 0.5, 1.0, 1.5
2	Hard Water	30	Ca and Mg	Hard Water
3	Low Ca and Mg	6	Ca	0, .06, .12, .25, .5, 1.0, 1.5
4	Low Mg, Test Ca	6	Ca	0, .06, .12, .25, .5, 1.0, 1.5
5	Low Ca and Mg	6	Mg	0, .06, .2, .5, 1.0

conducted over 30 h in hard water in an attempt to reproduce results obtained by Everall (1987) in the previous work on brown trout at this institution.

A third series of experiments similar in design to those undertaken in the first series was next completed using a 6 h flux period in place of the former 30 h. Initially the same four external [Ca^{2+}]s were tested. The remaining three tests at 0.06, 0.12 and 0.25 mM calcium were conducted approximately five months later.

The fourth series was of similar design to the third with the exception that the one week acclimation period involved acclimation to the external [Ca^{2+}] to be used in the subsequent test rather than to the low [Ca^{2+}] used during acclimation in series three. In this way it was hoped to elucidate any effect of calcium acclimation on sublethal zinc exposure as the work at acute zinc levels had suggested little protection arising from acclimation to water hardness.

The fifth and final series examined the specific effect of magnesium on sublethal zinc exposure. As in the third series fish were held in low [Ca^{2+}] and [Mg^{2+}] for one week before zinc exposure, on this occasion with magnesium added in place of calcium. Magnesium concentrations were chosen to allow direct comparison on an equimolar basis with the effect of calcium.

As in the third series the experiments at 0.06, 0.12 and 0.25 mM [Ca^{2+}] in the fourth series, and 0.06 and

0.20 mM [Mg^{2+}] in the fifth series were completed approximately five months after the remainder. This was not due to any deliberate planning but rather because, only after completion of the initial block of experiments did it become apparent that calcium and magnesium exerted greatest effect at concentrations below those initially tested.

3.3 RESULTS

3.3.1 Zinc influx

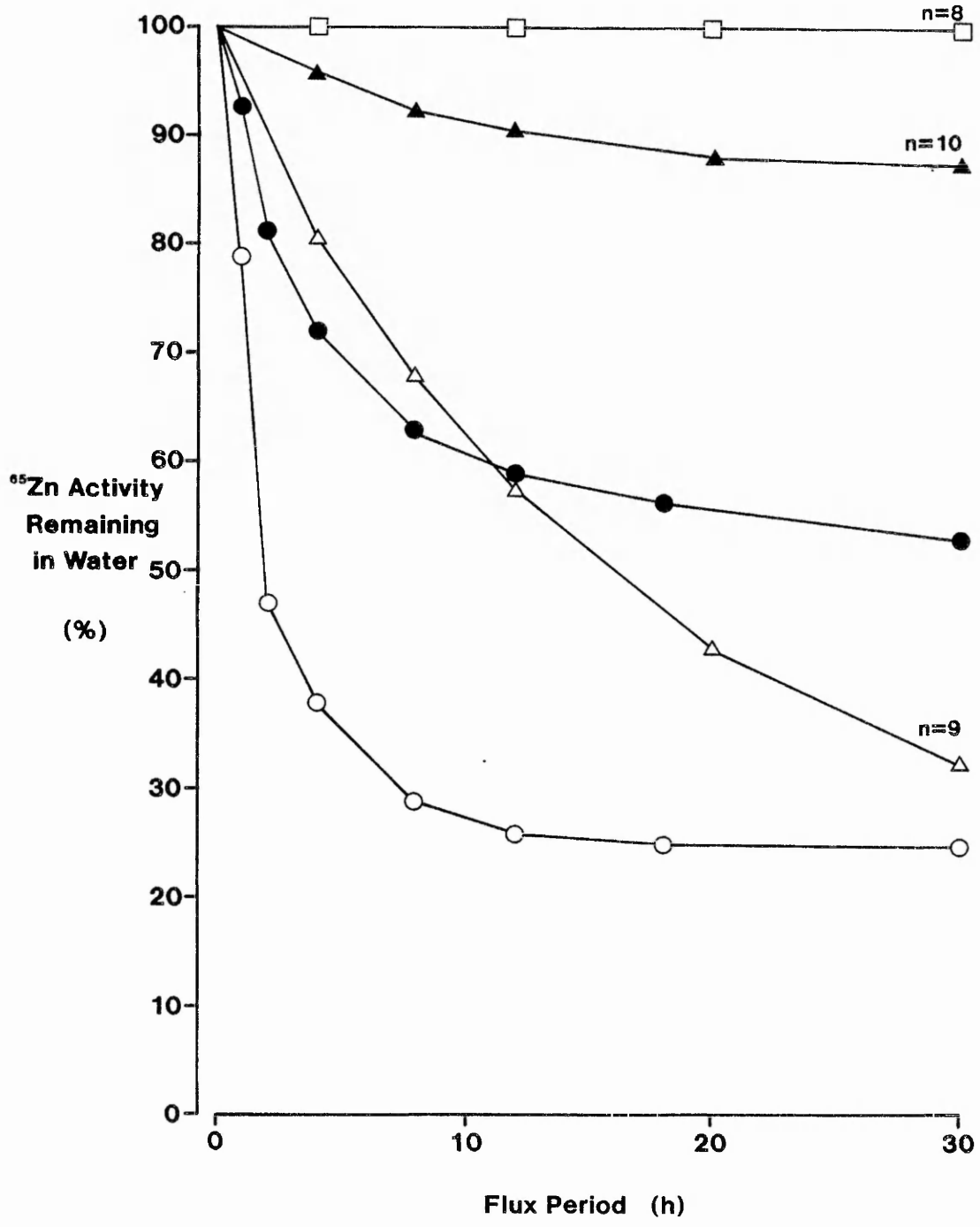
Three control experiments were completed under standard experimental conditions but with fish omitted from the system to evaluate the extent of ^{65}Zn adsorption onto the experimental apparatus. The divalent ion status of the water for these experiments was chosen to represent the extremes of experiments conducted with fish present, namely, hard water, very low divalent ion concentration, and 1.5 mM $[Ca^{2+}]$. Illustrated in Figure 6 is the result from the test conducted at low external divalent ion concentration, the situation where most adsorption might have been expected to occur. From this figure it is apparent that external ^{65}Zn activity did not decline in the absence of fish indicating that adsorption of zinc onto the flux apparatus itself was not a problem in this work.

Slight variations in the volume of medium in the flux chambers and in the ^{65}Zn activity added to the chambers at the start of a flux period produced corresponding variations in initial specific activities. For ease of comparison therefore, all curves illustrating the disappearance of ^{65}Zn with time are presented on the basis of percentage activity remaining in the water. In addition, each plotted curve represents the mean of all

Figure 6

Curves of ^{65}Zn disappearance from the media during experiments in this study (▲: hard water, △: soft water) and that of Everall (1987) (●: hard water, ○: soft water). Also shown (□) is the result of the control experiment conducted at low external [Ca^{2+}] and [Mg^{2+}] (0.017 and 0.004 mM respectively). Due to small variations in the media volume and the ^{65}Zn activity added at the start of these experiments all results of this type are plotted on the basis of percent activity remaining in the water with time.

Curves reproduced from the work of Everall represent plots from "typical fish", whereas those from this study represent the mean response determined from all fish within an individual flux experiment, the number of fish being denoted by n.


n=8
n=10
n=9
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
65Zn Activity Remaining in Water (%)
Flux Period (h)

individual fish within each particular flux experiment so in this respect can be considered to represent the mean response.

Earlier work at this institution (Everall, 1987) had produced distinctive shaped curves of ^{65}Zn disappearance from the media during exposure of brown trout to zinc in hard and artificially softened water. Curves representing the response noted by Everall for "typical" fish are illustrated in Figure 6 together with the curves obtained under comparable conditions in the present study. Of the latter, the hard water curve is that obtained from the single experiment of the second series of ^{65}Zn flux experiments and the soft water curve is that obtained in the experiment conducted at the lowest external $[Ca^{2+}]$ in the first series. Both studies clearly revealed reduced zinc uptake in hard water in comparison to soft water. Marked differences in the shape of the uptake curves were apparent however, the significance of which will be discussed later.

Similarly calculated curves of ^{65}Zn disappearance during the 30 h flux experiments comprising the first series are shown in Figure 7. From this figure it is evident that the external $[Ca^{2+}]$ strongly influenced the disappearance of zinc from the water, with this effect apparently greatest below 0.46 mM calcium, the uptake of zinc increasing markedly below this concentration.

Figure 8 illustrates the ^{65}Zn disappearance curves

Figure 7

Curves of ^{65}Zn disappearance from the media during the first series of ^{65}Zn flux experiments. All points represent the mean (± se) of 9 or 10 fish. Figures in parentheses represent the external $[Zn^{2+}]$ (μM) at the start of the flux period.

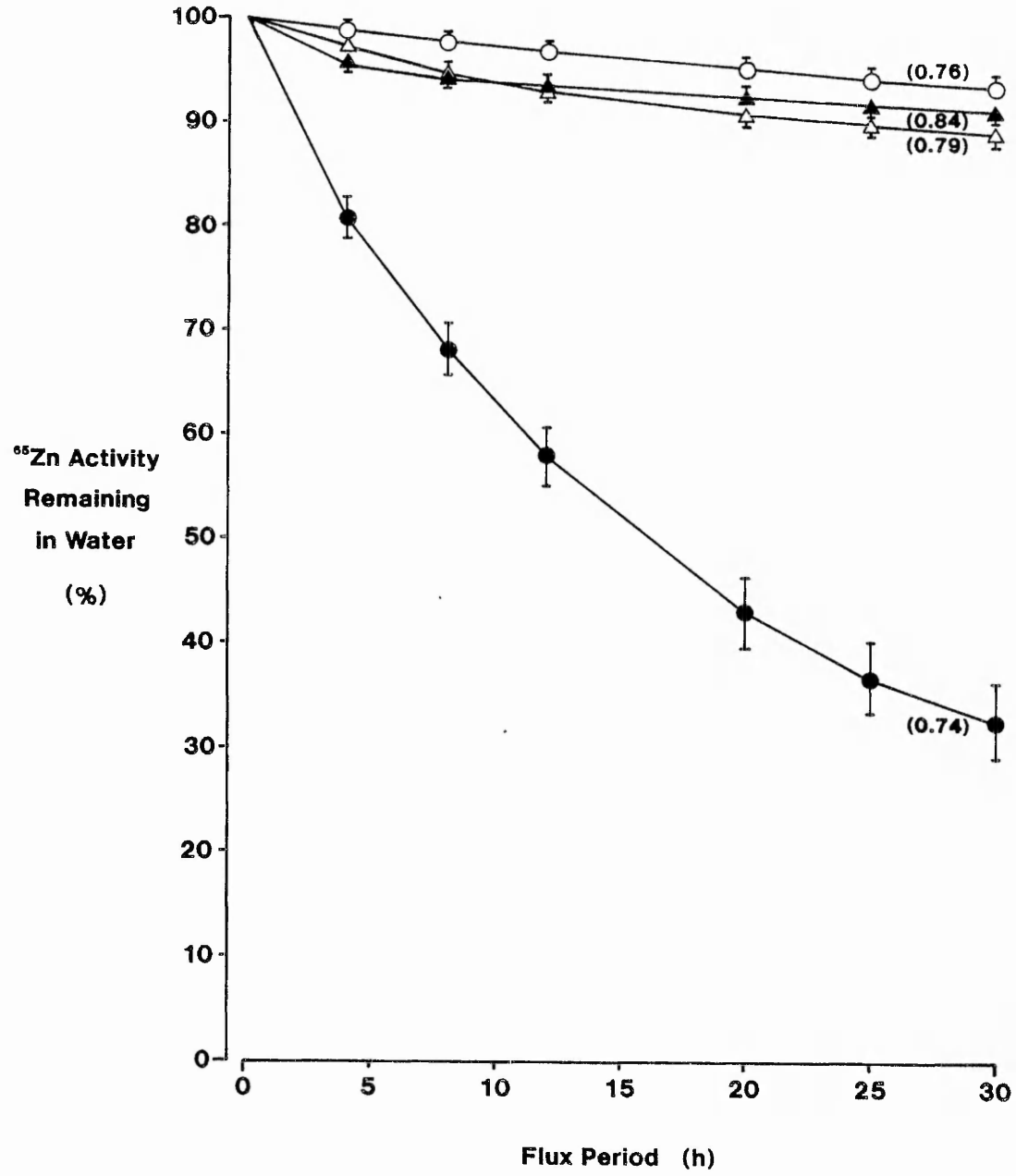
$[Ca^{2+}]$ during experiment (mM)

● 0.009

△ 0.46

▲ 0.92

○ 1.40


100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
(0.76)
(0.84)
(0.79)
(0.74)
65Zn Activity Remaining in Water (%)
Flux Period (h)

obtained during similar experiments completed over a 6 h flux period (the third series). Again increased external $[Ca^{2+}]$ decreased the rate of disappearance of zinc, although over this reduced flux period there was no obvious large change below 0.46 mM. Also shown in Figure 8 are stable zinc concentrations measured at 1.5 h intervals throughout the duration of the tests. Declining ^{65}Zn activities were not accompanied by parallel changes in total $[Zn^{2+}]$. Indeed, in those experiments at 0.42 and 0.97 mM calcium, total external $[Zn^{2+}]$ rose consistently throughout the tests. Similar results were also obtained in the remaining experiments of the fourth and fifth series, illustrated in Figures 9 and 10. Possible reasons for, and the significance of these observations will be discussed later.

The zinc influxes calculated for all 6 and 30 h flux experiments of series 1-5 are listed in Table 7 together with the mean external $[Ca^{2+}]$, $[Mg^{2+}]$ and $[Cl^{-}]$ present during each experiment. The influx values are plotted against external divalent ion concentration in Figure 11. The results of statistical analyses performed within each series of experiments are given in Table 8. From Figure 11 it is clear that over both 6 and 30 h increasing external calcium and magnesium reduced the influx of zinc into brown trout. Influx values measured over 30 h were lower than those measured in similar external $[Ca^{2+}]$ over 6 h. This suggested the possible presence of a rapid

Figure 8

Curves of ^{65}Zn disappearance and total external $[Zn^{2+}]$ during the third series of ^{65}Zn flux experiments. Points from both sets of data represent the mean of 9 or 10 fish. For the sake of clarity standard errors have been omitted.

$[Ca^{2+}]$ during experiment (mM)

● 0.002

○ 0.056

◆ 0.14

◇ 0.22

▲ 0.42

△ 0.97

■ 1.38

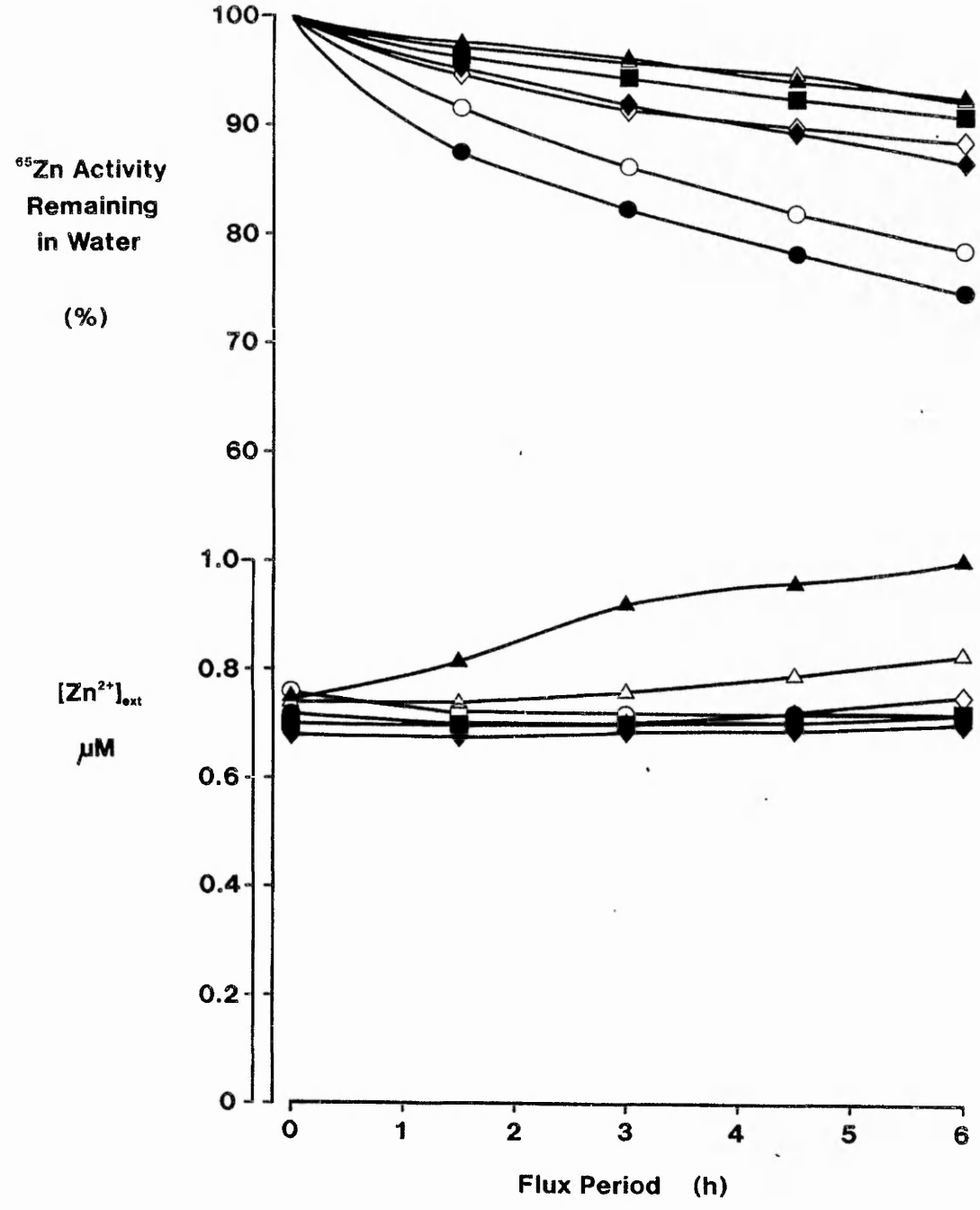

^{65}Zn Activity Remaining in Water (%)
100
90
80
70
60
$[Zn^{2+}]_{ext}$ µM
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
Flux Period (h)

Figure 9

Curves of ^{65}Zn disappearance and total external $[Zn^{2+}]$ during the fourth series of ^{65}Zn flux experiments. Points from both sets of data represent the mean of 9 or 10 fish. For the sake of clarity standard errors have been omitted.

$[Ca^{2+}]$ during experiment (mM)

● 0.063

○ 0.15

◆ 0.23

◇ 0.45

▲ 0.96

△ 1.27

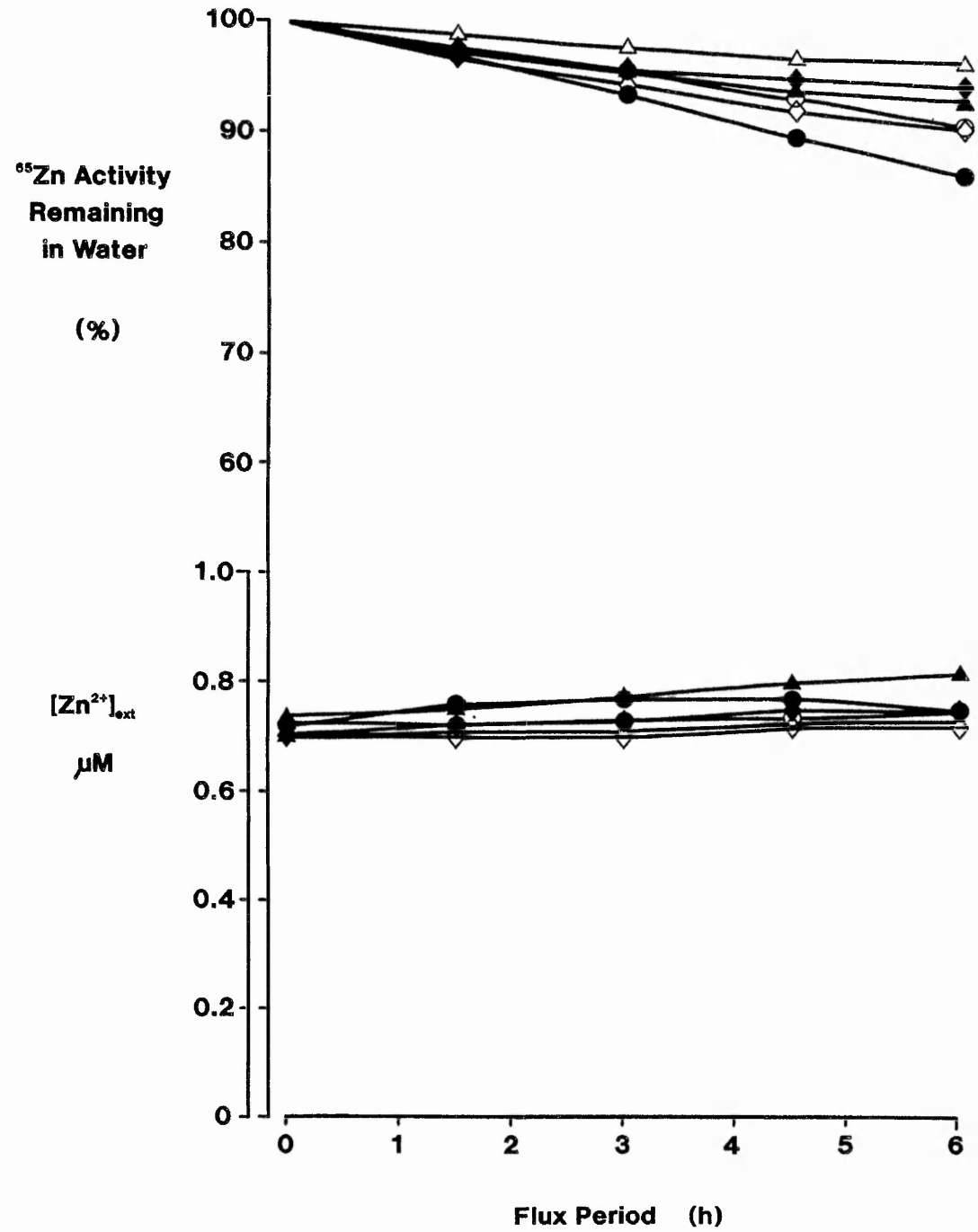

100
90
80
70
60
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
65Zn Activity Remaining in Water (%)
[Zn2+]ext µM
Flux Period (h)

Figure 10

Curves of ^{65}Zn disappearance and total external $[Zn^{2+}]$ during the fifth series of ^{65}Zn flux experiments. Points from both sets of data represent the mean of 9 or 10 fish. For the sake of clarity standard errors have been omitted.

$[Mg^{2+}]$ during experiment (mM)

● 0.058

○ 0.22

▲ 0.47

△ 0.99

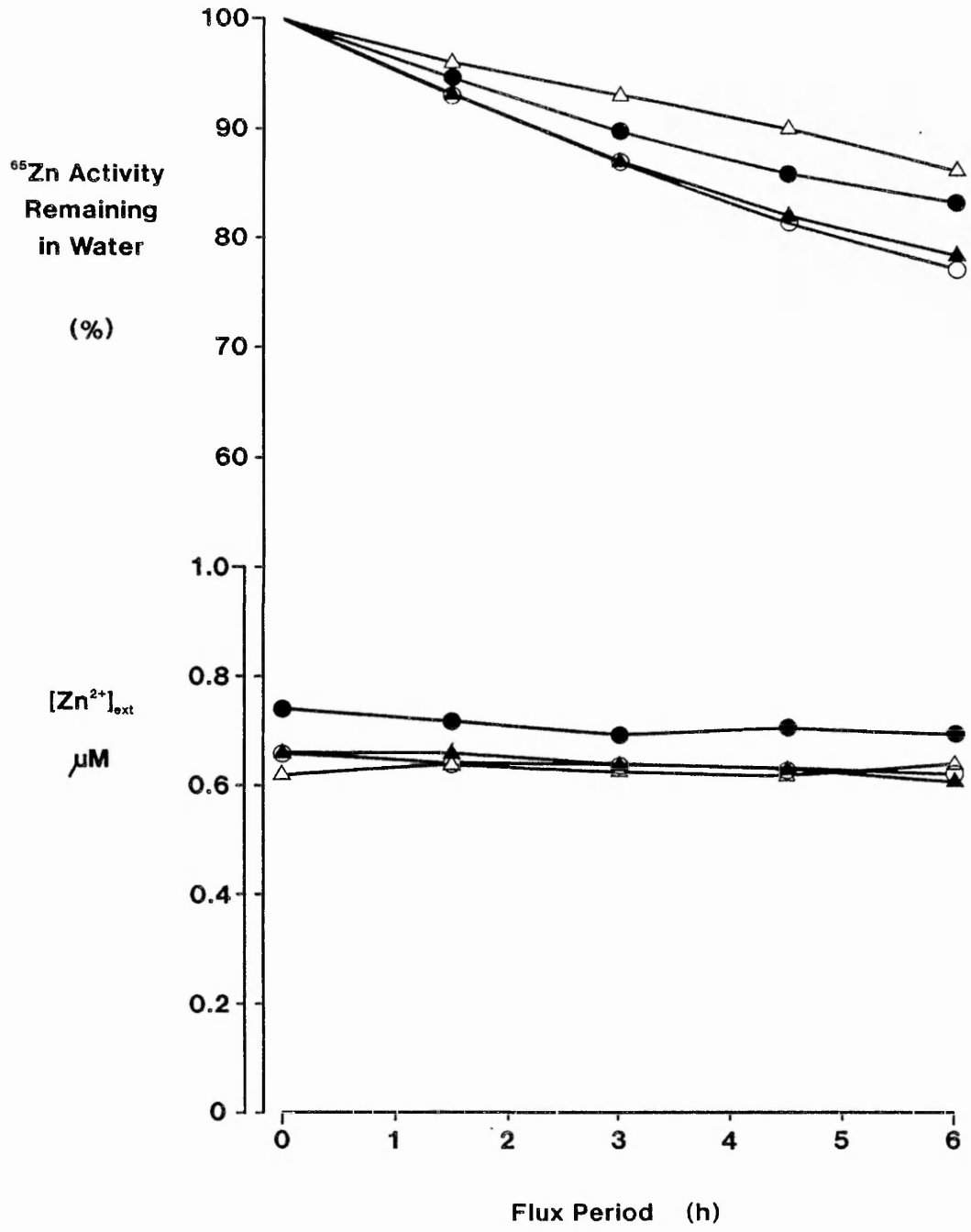

100
90
80
70
60
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
65Zn Activity Remaining in Water
(%)
[Zn2+]ext
μM
Flux Period (h)

Table 7

Zinc influx rates, external $[Ca^{2+}]$, $[Mg^{2+}]$ and $[Cl^{-}]$ during the five series of zinc flux experiments.

Influx rates are $\bar{x} \pm$ se (n = 9 or 10)
Ion concentrations are $\bar{x} \pm$ range (n = 9 or 10)

	Influx (μ mol kg^{-1} h^{-1})	$[Ca^{2+}]$ mM	$[Mg^{2+}]$ mM	$[Cl^{-}]$ mM
Series 1	0.393 ± 0.021	0.009 ± 0.001	<0.025	1.7
	0.062 ± 0.012	0.46 ± 0.04	<0.025	2.6
	0.058 ± 0.010	0.92 ± 0.04	<0.025	3.6
	0.030 ± 0.003	1.40 ± 0.08	<0.025	4.5
Series 2	0.046 ± 0.005	1.65 ± 0.03	0.40 ± 0.02	1.5
Series 3	0.456 ± 0.036	0.002 ± 0.0001	<0.010	1.6
	0.337 ± 0.027	0.056 ± 0.003	<0.020	1.5
	0.152 ± 0.008	0.14 ± 0.01	<0.015	1.8
	0.133 ± 0.014	0.22 ± 0.03	<0.015	2.0
	0.172 ± 0.021	0.42 ± 0.01	<0.015	2.3
	0.166 ± 0.019	0.97 ± 0.01	<0.020	3.3
	0.124 + 0.006	1.38 + 0.03	<0.020	4.2
Series 4	0.271 ± 0.025	0.063 ± 0.001	<0.005	1.5
	0.150 ± 0.013	0.15 ± 0.03	<0.005	1.8
	0.095 ± 0.012	0.23 ± 0.01	<0.005	2.0
	0.126 ± 0.016	0.45 ± 0.02	<0.010	2.4
	0.105 ± 0.009	0.96 ± 0.02	<0.010	3.4
	0.097 ± 0.023	1.27 ± 0.03	<0.010	4.0
Series 5	0.380 ± 0.033	<0.005	0.058 ± 0.001	1.4
	0.312 ± 0.023	<0.010	0.22 ± 0.01	1.8
	0.373 ± 0.024	<0.010	0.47 ± 0.02	2.5
	0.232 ± 0.020	<0.010	0.99 ± 0.03	3.8

Figure 11

Rates of zinc influx calculated for the 6 and 30 h ^{65}Zn flux experiments of series 1, 3, 4 and 5. Each point represents the $\bar{x} \pm$ se of 9 or 10 fish.

◆ Series 1: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 30 h in a range of external $[Ca^{2+}]$s.

● Series 3: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 6 h in a range of external $[Ca^{2+}]$s.

○ Series 4: Acclimated to media of similar $[Ca^{2+}]$ to that employed in the subsequent 6 h test.

◇ Series 5: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 6 h in a range of external $[Mg^{2+}]$s.

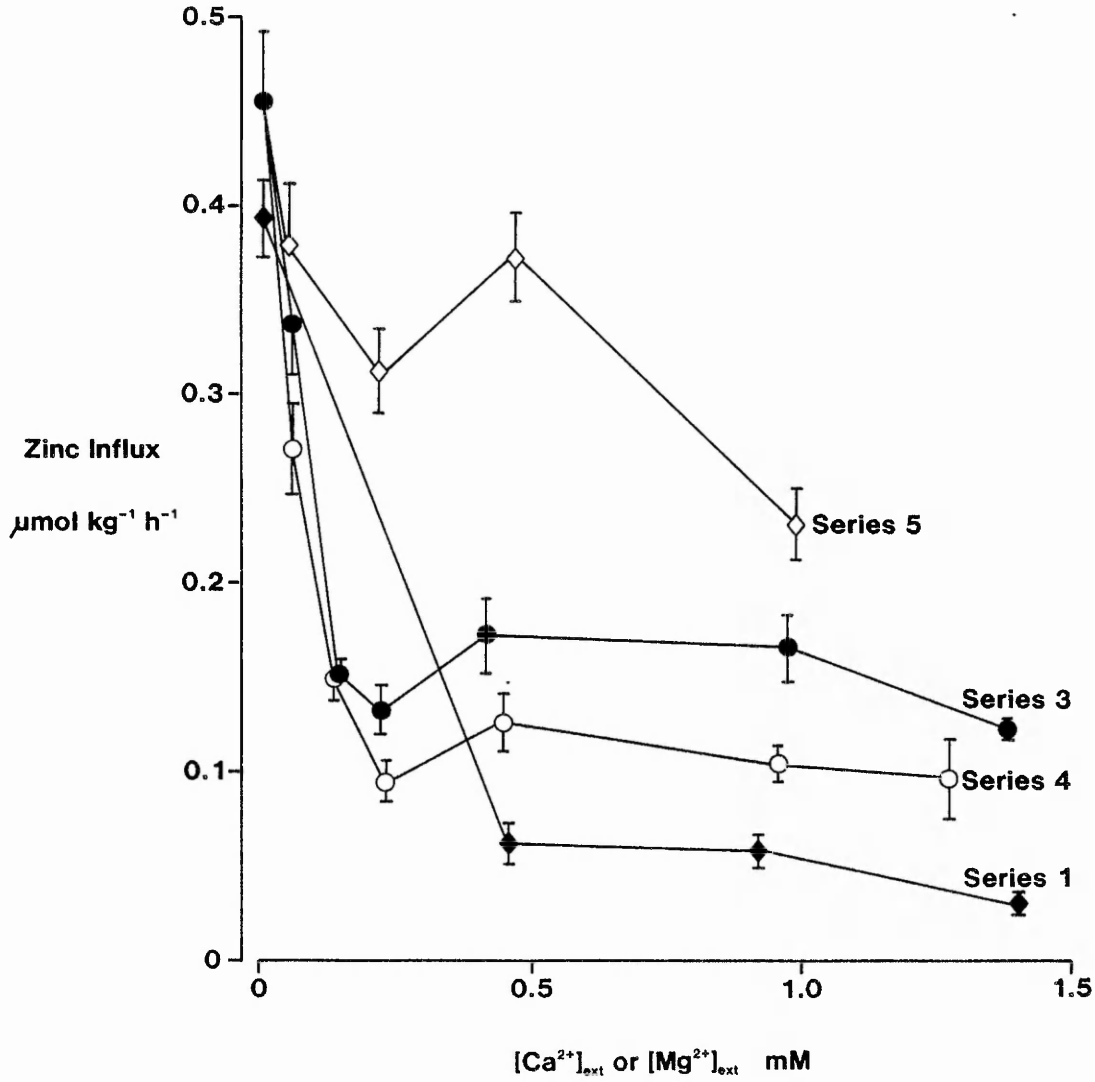

0.5
0.4
0.3
0.2
0.1
0
Zinc Influx
μmol kg⁻¹ h⁻¹
Series 5
Series 3
Series 4
Series 1
0
0.5
1.0
1.5
[Ca2+]ext or [Mg2+]ext mM

Table 8

The results of the statistical analyses performed within each series of experiments examining the modifying influence of external calcium and magnesium concentration on the influx of zinc.

NS Not Significant * $P < 0.01$ ** $P < 0.01$ *** $P < 0.001$

Series 1	$[Ca^{2+}]$	0.46	0.92	1.40			
	0.009	***	***	***			
	0.46		NS	***			
	0.92			***			
Series 3	$[Ca^{2+}]$	0.056	0.14	0.22	0.42	0.97	1.38
	0.002	NS	***	***	***	***	***
	0.056		***	***	***	***	***
	0.14			NS	NS	NS	*
	0.22				*	NS	NS
	0.42					NS	**
	0.97						**
Series 4	$[Ca^{2+}]$	0.063	0.15	0.23	0.45	0.96	1.27
	0.002	NS	***	***	***	***	***
	0.063		***	***	***	***	***
	0.15			**	NS	**	**
	0.23				NS	NS	NS
	0.45					NS	NS
	0.96						NS
Series 5	$[Mg^{2+}]$	0.058	0.22	0.47	0.99		
	0.002	NS	***	NS	***		
	0.058		NS	NS	***		
	0.22			NS	*		
	0.47				***		

initial adsorption of zinc onto the surface of the fish for this would tend to exert a greater effect on influxes measured over 6 h than those over 30 h. Influxes measured over 30 h clearly showed that calcium exerted its greatest effect in reducing the influx of zinc at 0.46 mM. Flux measurements over 6 h employed a number of concentrations below this level and revealed the greatest effect to be at 0.15 mM calcium. The statistical analyses in Table 8 suggest that, at least in the 6 h experiments, increasing the external $[Ca^{2+}]$ beyond 0.15-0.23 mM had relatively little additional effect on reducing the influx of zinc as the comparison of means of the rates of influx at these higher concentrations produced relatively few significant differences.

Table 9 lists the results of the statistical analyses examining the effect of acclimation of fish to the test $[Ca^{2+}]$ before exposure to zinc and the effect of replacing calcium with magnesium as the external divalent ion. Combining these results with Figure 11 it is clear that the protective effect of magnesium was not so great as that of calcium when compared on an equimolar basis. Even at an external $[Mg^{2+}]$ of 1 mM zinc influx was not reduced as much as at a $[Ca^{2+}]$ of 0.14 mM. It is also evident that although acclimation to the test $[Ca^{2+}]$ for one week before zinc exposure did confer some additional protection against the influx of zinc this effect was of

Table 9

The results of the statistical analyses investigating the effect on zinc influx of using magnesium in place of calcium as the external divalent ion and of acclimating the fish to the test calcium concentration before zinc exposure.

NS Not Significant * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

The effect of magnesium relative to calcium:

[Divalent Ion] mM		
Magnesium	Calcium	
0.058	0.056	NS
0.22	0.22	***
0.47	0.42	***
0.99	0.97	**

The effect of calcium acclimation before zinc exposure:

$[Ca^{2+}]$ mM		
Test $[Ca^{2+}]$ Acclimated	Low $[Ca^{2+}]$ Acclimated	
0.063	0.056	NS
0.15	0.14	NS
0.23	0.22	NS
0.45	0.42	*
0.96	0.97	**
1.27	1.38	NS

secondary importance in comparison to the actual external $[Ca^{2+}]$ during zinc exposure. Thus, zinc influx was reduced in test $[Ca^{2+}]$-acclimated fish relative to low $[Ca^{2+}]$-acclimated fish at all the external $[Ca^{2+}]$s tested. These reductions were only of sufficient magnitude to be of significance at 0.45 and 0.96 mM calcium however.

3.3.2 The distribution of zinc

Figures 12 to 15 illustrate the relative distributions of ^{65}Zn among the various body tissues at the conclusion of the 6 and 30 h flux experiments. Percentage distributions were calculated from the measurements taken of total tissue weight and ^{65}Zn activity per unit weight of each tissue. Most zinc was found to be located in the gills, plasma and carcass. In those experiments where calcium was the divalent ion under investigation increased external $[Ca^{2+}]$ resulted in an increase in the ^{65}Zn content of the gills at the expense of internal tissues such as the plasma, gut, liver and kidney. This effect on zinc distribution was not seen when magnesium was used in place of calcium. Magnesium appeared to have little effect on zinc distribution, only counts from blood cells, relatively unimportant as regards the overall distribution, showed a significant reduction of zinc content with increased

Figure 12

The percentage distribution of ^{65}Zn amongst the tissues of brown trout following the first series of flux experiments. Within each tissue, distributions are plotted in order of ascending test $[Ca^{2+}]$. Where significant the results of the statistical comparisons performed against fish exposed to zinc in the lowest external $[Ca^{2+}]$ are also illustrated.

** $P < 0.01$

Series 1: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 30 h in a range of external $[Ca^{2+}]$s.

$[Ca^{2+}]$s (mM): 0.009, 0.46, 0.92, 1.40

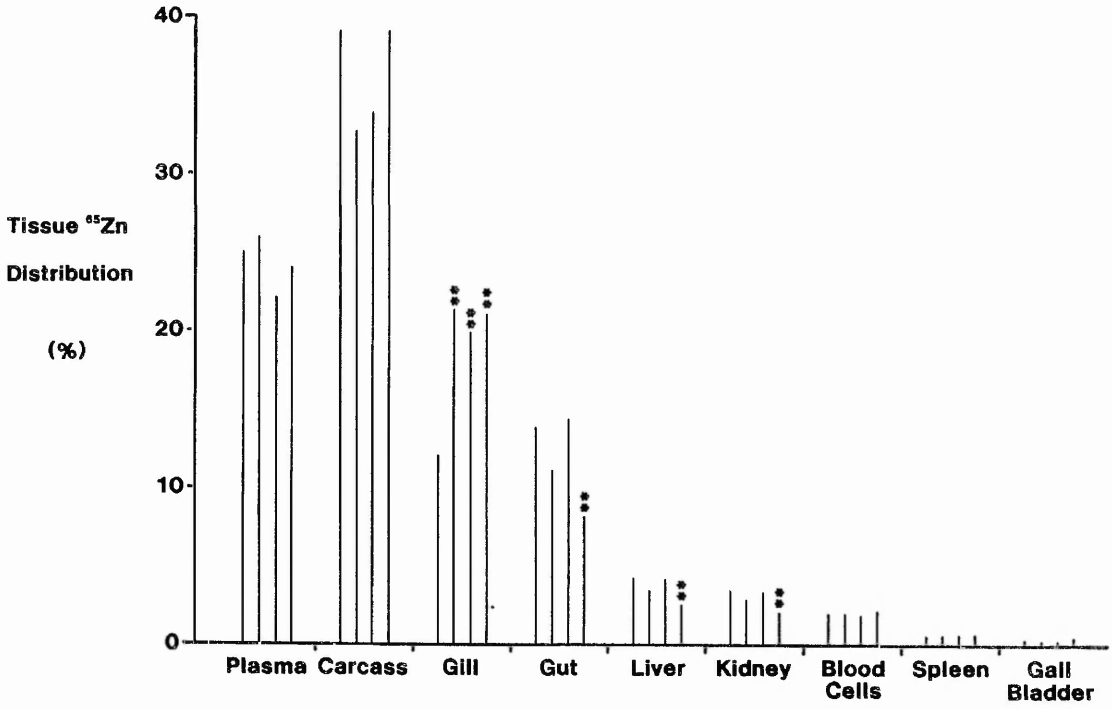

Tissue ^{65}Zn
Distribution
(%)
40
30
20
10
0
Plasma
Carcass
Gill
Gut
Liver
Kidney
Blood Cells
Spleen
Gall Bladder

Figure 13

The percentage distribution of ^{65}Zn amongst the tissues of brown trout following the third series of flux experiments. Within each tissue, distributions are plotted in order of ascending test $[Ca^{2+}]$. Where significant the results of the statistical comparisons performed against fish exposed to zinc in the lowest external $[Ca^{2+}]$ are also illustrated.

* $P < 0.05$ ** $P < 0.01$

Series 3: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 6 h in a range of external $[Ca^{2+}]$s.
$[Ca^{2+}]$s (mM): 0.002, 0.056, 0.14, 0.22, 0.42, 0.97, 1.38

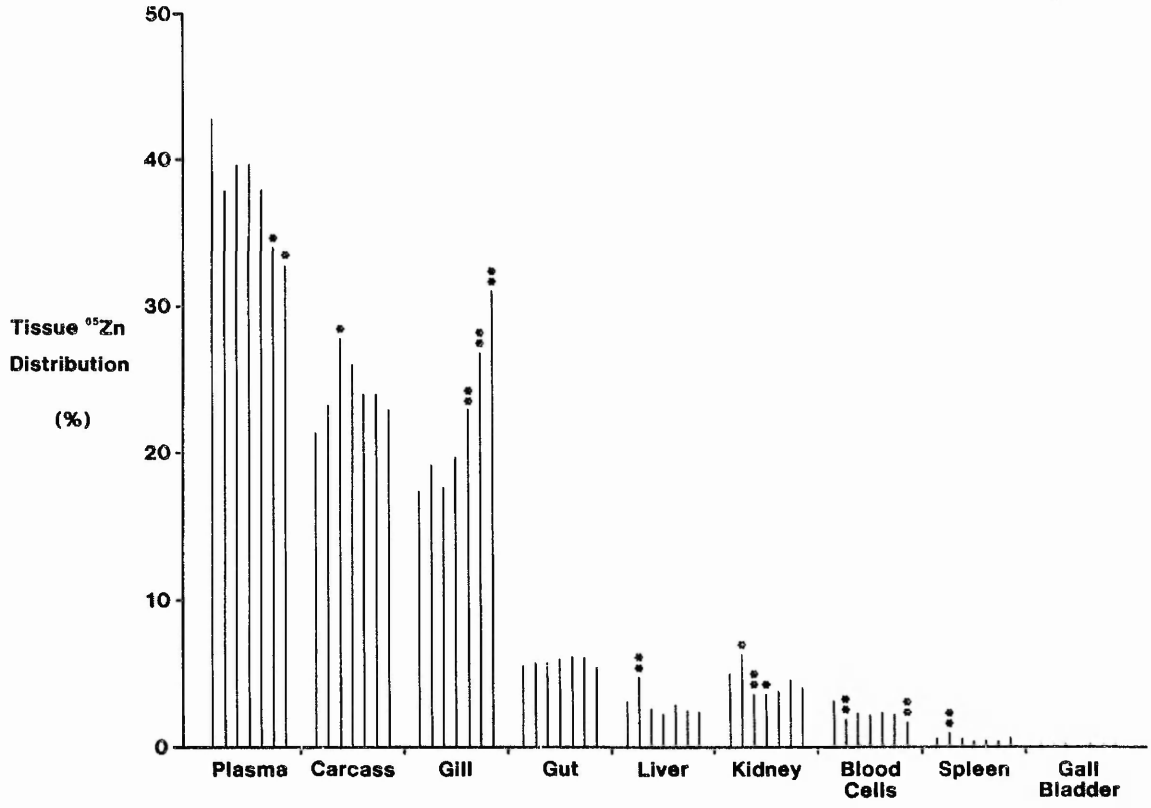

Tissue ^{65}Zn Distribution (%)
50
40
30
20
10
0
Plasma
Carcass
Gill
Gut
Liver
Kidney
Blood Cells
Spleen
Gall Bladder

Figure 14

The percentage distribution of ^{65}Zn amongst the tissues of brown trout following the fourth series of flux experiments. Within each tissue, distributions are plotted in order of ascending test $[Ca^{2+}]$. Where significant the results of the statistical comparisons performed against fish exposed to zinc in the lowest external $[Ca^{2+}]$ are also illustrated.

* $P < 0.05$ ** $P < 0.01$

Series 4: Acclimated to media of similar $[Ca^{2+}]$ to that employed in the subsequent 6 h test.

$[Ca^{2+}]$s (mM): 0.002, 0.063, 0.15, 0.23, 0.45, 0.96, 1.27

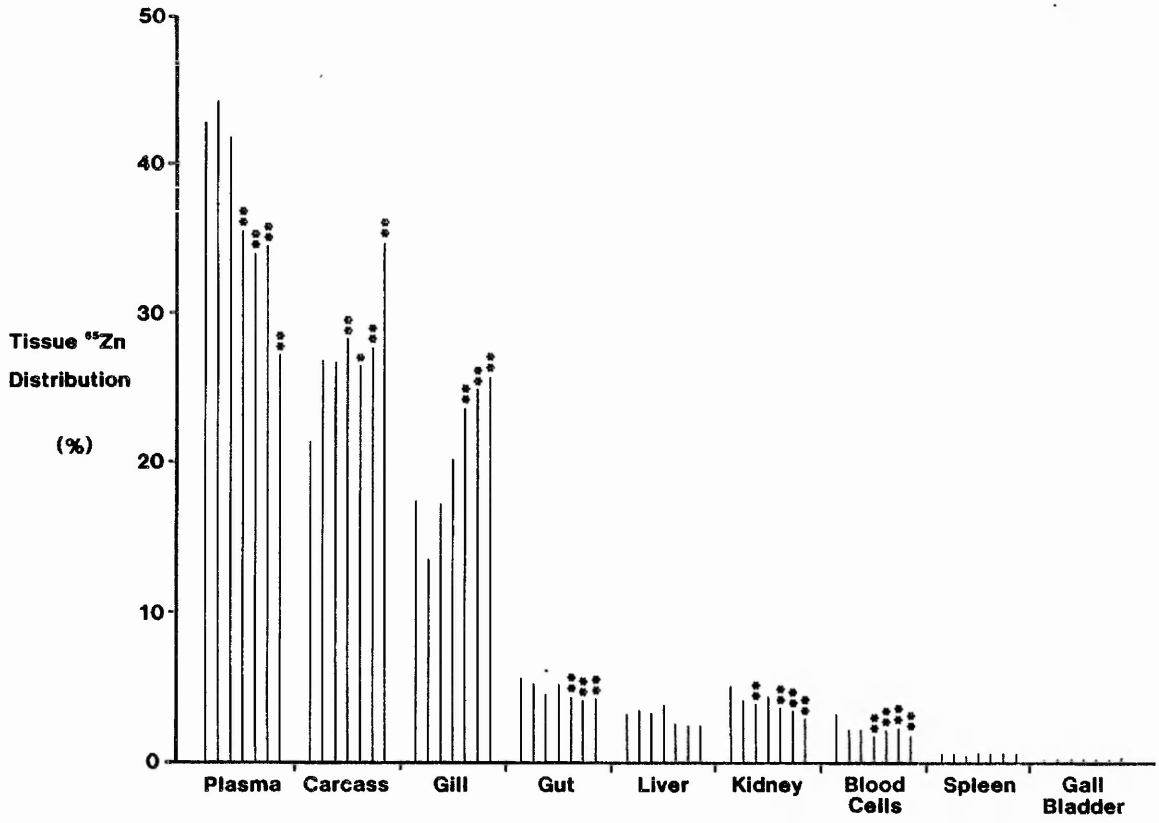

50
40
30
20
10
0
Tissue ^{65}Zn Distribution (%)
Plasma
Carcass
Gill
Gut
Liver
Kidney
Blood Cells
Spleen
Gall Bladder

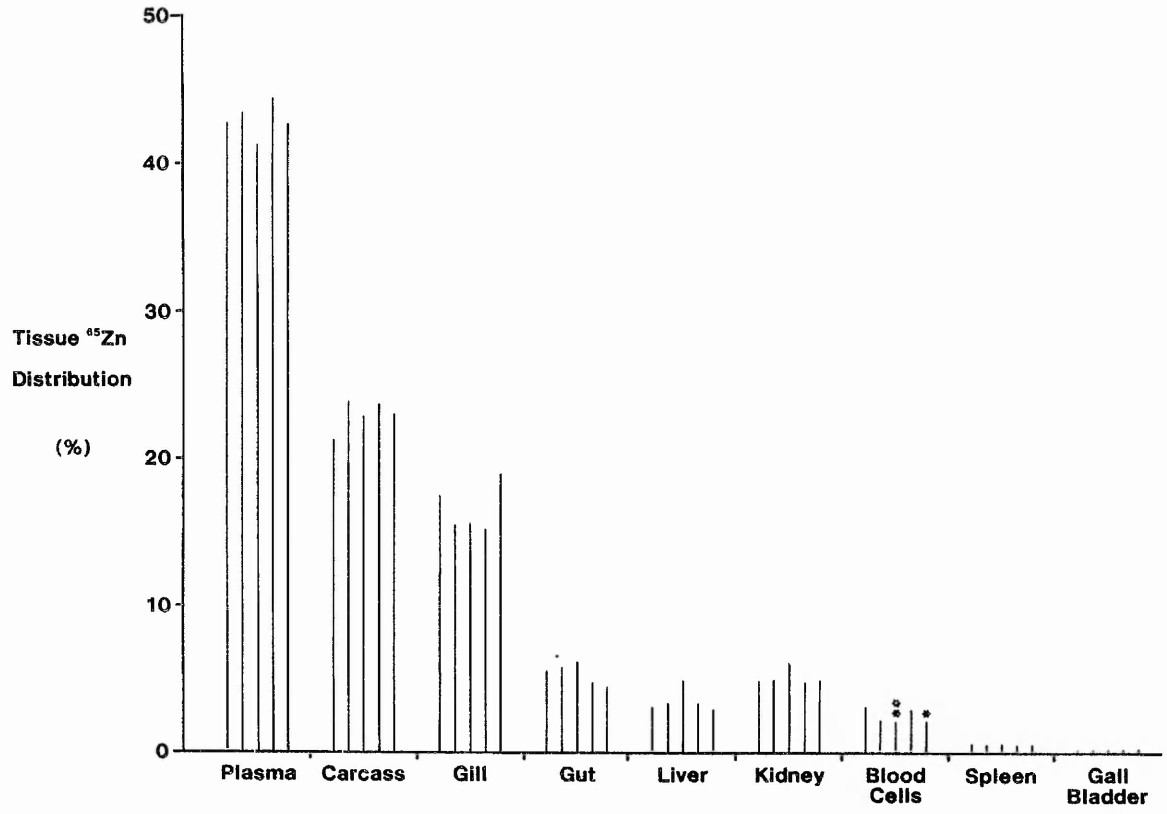
Figure 15

The percentage distribution of ^{65}Zn amongst the tissues of brown trout following the fifth series of flux experiments. Within each tissue, distributions are plotted in order of ascending test $[Mg^{2+}]$. Where significant the results of the statistical comparisons performed against fish exposed to zinc in the lowest external $[Mg^{2+}]$ are also illustrated.

* $P < 0.05$ ** $P < 0.01$

Series 5: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 6 h in a range of external $[Mg^{2+}]$s.

$[Mg^{2+}]$s (mM): <0.010, 0.058, 0.22, 0.47, 0.99


50
40
30
20
10
0
Tissue ^{65}Zn
Distribution
(%)
Plasma
Carcass
Gill
Gut
Liver
Kidney
Blood Cells
Spleen
Gall Bladder

external $[Mg^{2+}]$.

Figures 16 to 19 illustrate the accumulation of zinc by the plasma (nmol ml^{-1}) and body tissues (nmol g^{-1}) during the various ^{65}Zn flux experiments. The presentation of results on this basis revealed that the plasma, gill, kidney, liver, spleen and gut all accumulated significant quantities of zinc, whereas the carcass contained relatively little, its high percentage distribution figure for ^{65}Zn resulting from its large weight in comparison to that of other tissues. Trends observed in zinc influx in relation to external $[Ca^{2+}]$ and $[Mg^{2+}]$ are reflected in the appearance of ^{65}Zn in the internal tissues. Thus, over 30 h, fish exposed to zinc in 0.46 mM calcium and above had a considerably lower internal burden of ^{65}Zn than those exposed in 0.009 mM calcium.

The ^{65}Zn content of plasma, gill, kidney and liver following the three series of 6 h flux experiments are presented together in Figure 20. To allow direct comparison of the three treatments only results from the four divalent ion concentrations common to all three series of experiments are shown. For all four tissues it is clear that zinc uptake was affected less by magnesium than by calcium at all concentrations tested except 0.06 mM. Acclimation to calcium before zinc exposure reduced zinc uptake when compared to fish acclimated to low $[Ca^{2+}]$ although as with magnesium, this effect was not

Figure 16

Zinc uptake in the various body tissues at the conclusion of the first series of flux experiments. Within each tissue results are plotted in order of ascending test $[Ca^{2+}]$. All results represent the $\bar{x} \pm$ se of 9 or 10 fish. The results of the statistical comparisons performed against fish exposed to zinc in the lowest external $[Ca^{2+}]$ are also illustrated. Arrows denote significance at the same level of the results from all higher calcium concentrations.

*** $p < 0.001$

Series 1: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 30 h in a range of external $[Ca^{2+}]$s.

$[Ca^{2+}]$s (mM): 0.009, 0.46, 0.92, 1.40

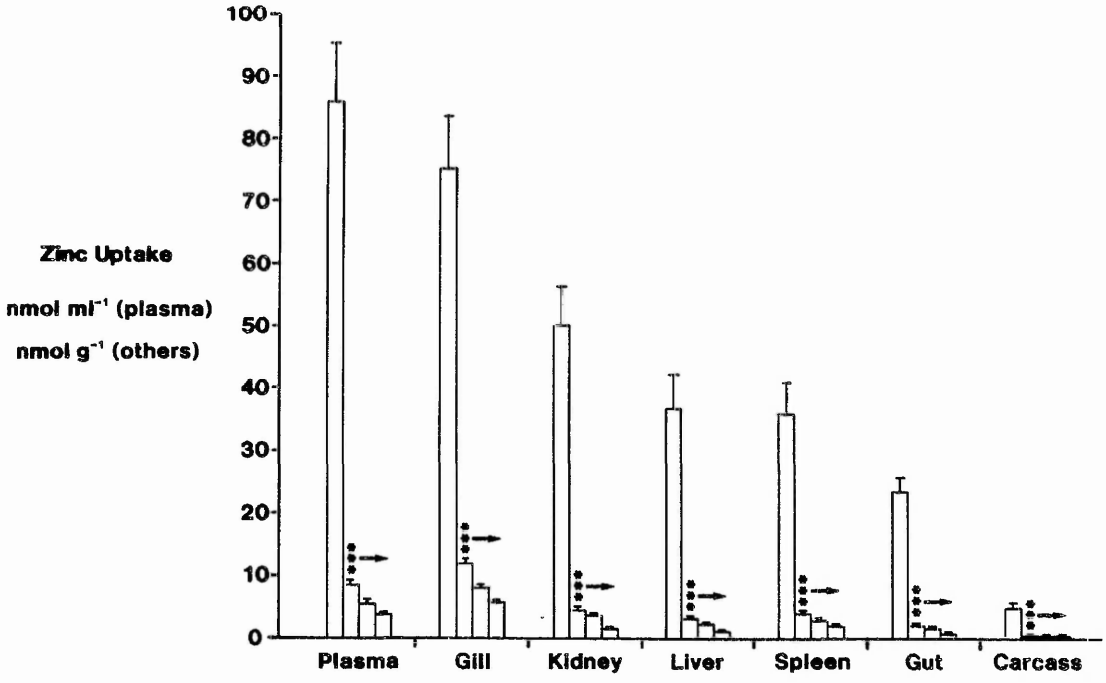

Zinc Uptake
nmol ml⁻¹ (plasma)
nmol g⁻¹ (others)
100
90
80
70
60
50
40
30
20
10
0
Plasma
Gill
Kidney
Liver
Spleen
Gut
Carcass

Figure 17

Zinc uptake in the various body tissues at the conclusion of the third series of flux experiments. Within each tissue results are plotted in order of ascending test $[Ca^{2+}]$. All results represent the $\bar{x} \pm$ se of 9 or 10 fish. The results of the statistical comparisons performed against fish exposed to zinc in the lowest external $[Ca^{2+}]$ are also illustrated. Arrows denote significance at the same level of all higher calcium concentrations.

NS Not significant * $P < 0.05$ *** $P < 0.001$

Series 3: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 6 h in a range of external $[Ca^{2+}]$s.
$[Ca^{2+}]$s (mM): 0.002, 0.056, 0.14, 0.22, 0.42, 0.97, 1.38

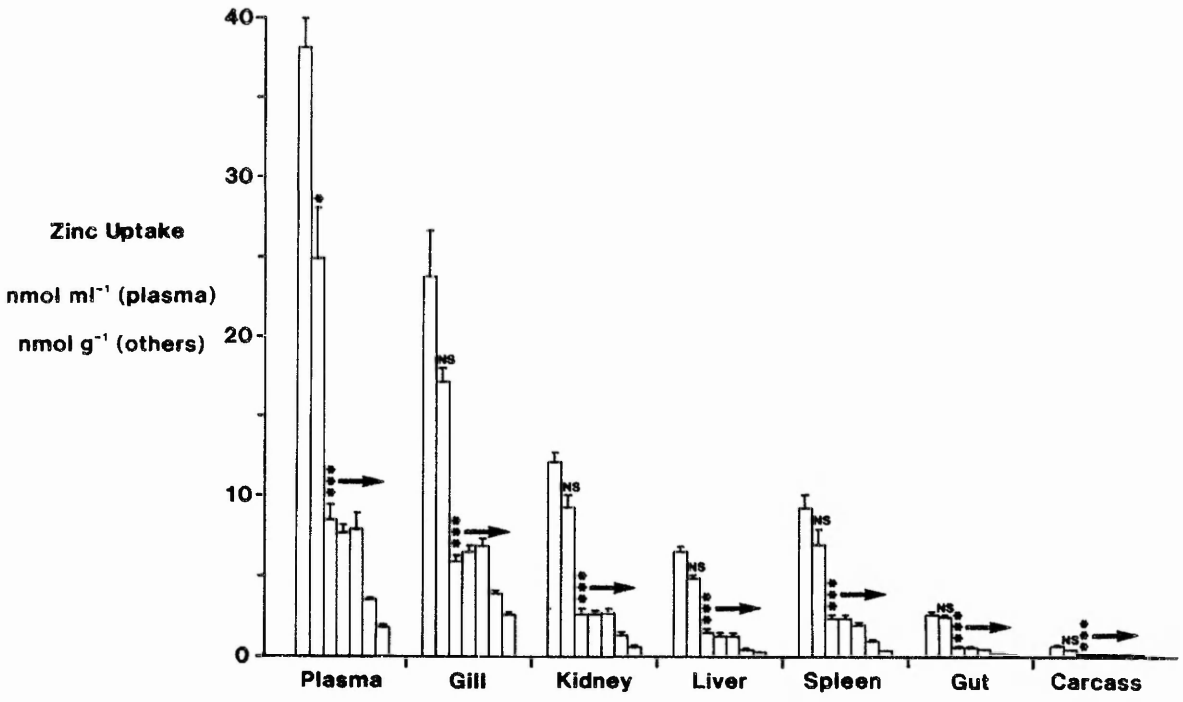

Zinc Uptake
nmol ml⁻¹ (plasma)
nmol g⁻¹ (others)
40
30
20
10
0
NS
Plasma
Gill
Kidney
Liver
Spleen
Gut
Carcass

Figure 18

Zinc uptake in the various body tissues at the conclusion of the fourth series of flux experiments. Within each tissue results are plotted in order of ascending test $[Ca^{2+}]$. All results represent the $\bar{x} \pm$ se of 9 or 10 fish. The results of the statistical comparisons performed against fish exposed to zinc in the lowest external $[Ca^{2+}]$ are also illustrated. Arrows denote significance at the same level of all higher calcium concentrations.

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Series 4: Acclimated to media of similar $[Ca^{2+}]$ to that employed in the subsequent 6 h test.

$[Ca^{2+}]$s (mM): 0.002, 0.063, 0.15, 0.23, 0.45, 0.96, 1.27

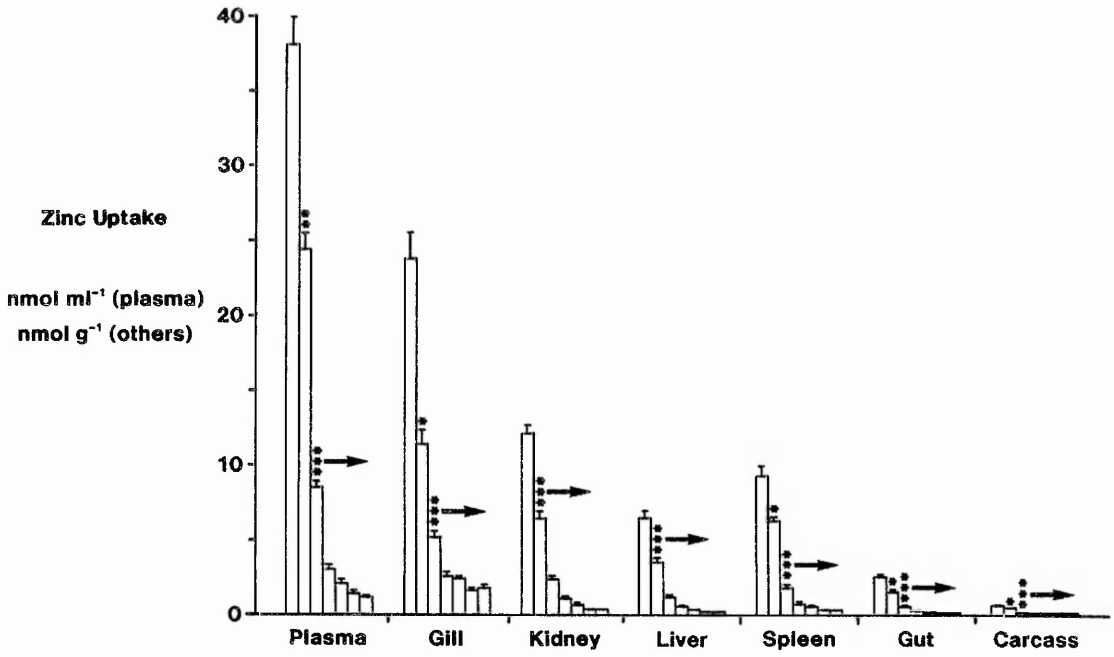

Zinc Uptake
nmol ml⁻¹ (plasma)
nmol g⁻¹ (others)
40
30
20
10
0
Plasma
Gill
Kidney
Liver
Spleen
Gut
Carcass

Figure 19

Zinc uptake in the various body tissues at the conclusion of the fifth series of flux experiments. Within each tissue results are plotted in order of ascending test $[Mg^{2+}]$. All results represent the $\bar{x} \pm$ se of 9 or 10 fish. The results of the statistical comparisons performed against fish exposed to zinc in the lowest external $[Mg^{2+}]$ are also illustrated.

NS Not significant

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Series 5: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested for 6 h in a range of external $[Mg^{2+}]$s.

$[Mg^{2+}]$s (mM): <0.010, 0.058, 0.22, 0.47, 0.99

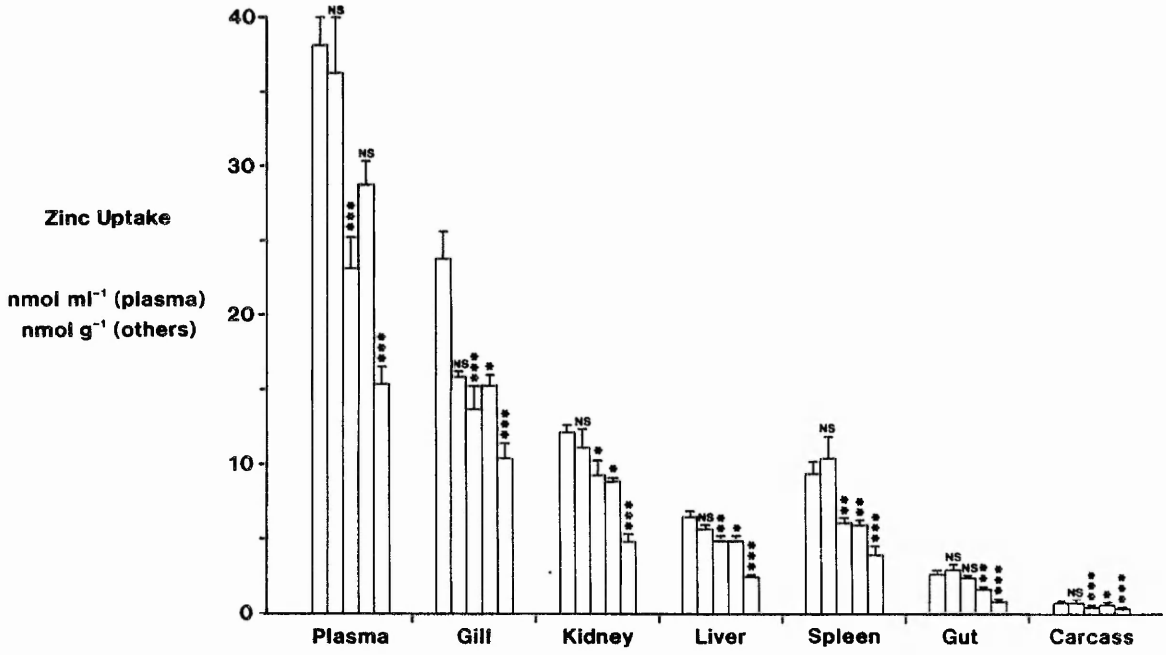

Zinc Uptake
nmol ml⁻¹ (plasma)
nmol g⁻¹ (others)
40
30
20
10
0
Plasma
Gill
Kidney
Liver
Spleen
Gut
Carcass

Figure 20

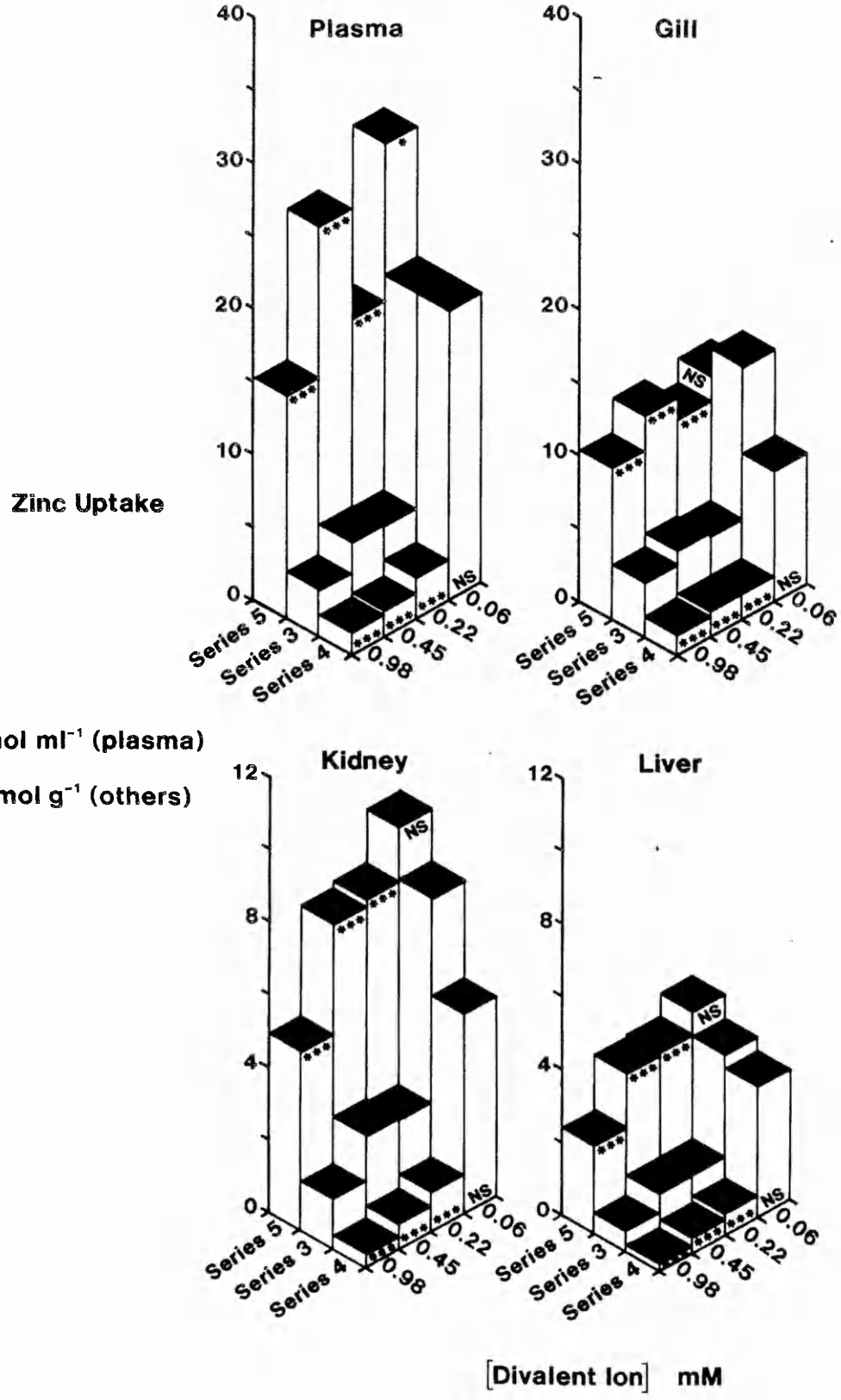
Zinc uptake by selected tissues during the three series of 6 h flux experiments. Only data from the four divalent ion concentrations common to all three series are shown. The results of the statistical comparisons performed at each concentration against fish acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$ before exposure to zinc in a higher external $[Ca^{2+}]$ (series 3 experiments) are also illustrated.

NS Not significant * $P < 0.05$ *** $P < 0.001$

Series 3: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested in a range of external $[Ca^{2+}]$s.

Series 4: Acclimated and tested in the same external $[Ca^{2+}]$.

Series 5: Acclimated to low external $[Ca^{2+}]$ and $[Mg^{2+}]$, tested in a range of external $[Mg^{2+}]$s.


Plasma
Gill
Kidney
Liver
Zinc Uptake
nmol ml⁻¹ (plasma)
nmol g⁻¹ (others)
40
30
20
10
0
12
8
4
Series 5
Series 3
Series 4
0.06
0.22
0.45
0.98
NS
***
*
[Divalent Ion] mM

significant at 0.06 mM.

The trends observed therefore, in the zinc influx results were confirmed by the pattern of ^{65}Zn distribution in a variety of fish tissues. This suggests that if zinc adsorption to the surface of the fish had occurred, thereby causing an over-estimate of zinc influx, it was not of sufficient magnitude to mask the overall trends in the modification of zinc uptake by external calcium and magnesium.

3.3.3 The effect of zinc on ionoregulation

Haematocrit and plasma ion concentrations determined in fish at the end of the 30 h fluxes are shown in Figure 21. Those for the 6 h experiments are listed in Table 10 with Figure 22 showing the results from the four comparable concentrations in these three series of experiments. From these results it is evident that haematocrit was unaffected by any of the treatments employed. Plasma $[Cl^-]$ too was relatively unaffected despite the elevation of ambient $[Cl^-]$ when external $[Ca^{2+}]$ was increased. Over 30 h plasma $[Na^+]$ was significantly higher in those fish exposed to zinc in elevated $[Ca^{2+}]$. These results may also have been repeated over 6 h but unfortunately results were not easily comparable due to plasma $[Na^+]$ in the 0.06 and 0.22 mM groups being significantly higher than the

Figure 21

Haematocrit and plasma ion concentrations measured in blood samples taken from fish at the conclusion of the first series of flux experiments. All values represent the $\bar{x} \pm$ se obtained from 9 or 10 fish. Statistical comparisons were performed against the results obtained from fish exposed to zinc in the lowest external $[Ca^{2+}]$.

NS Not significant * $P < 0.05$ ** $P < 0.01$

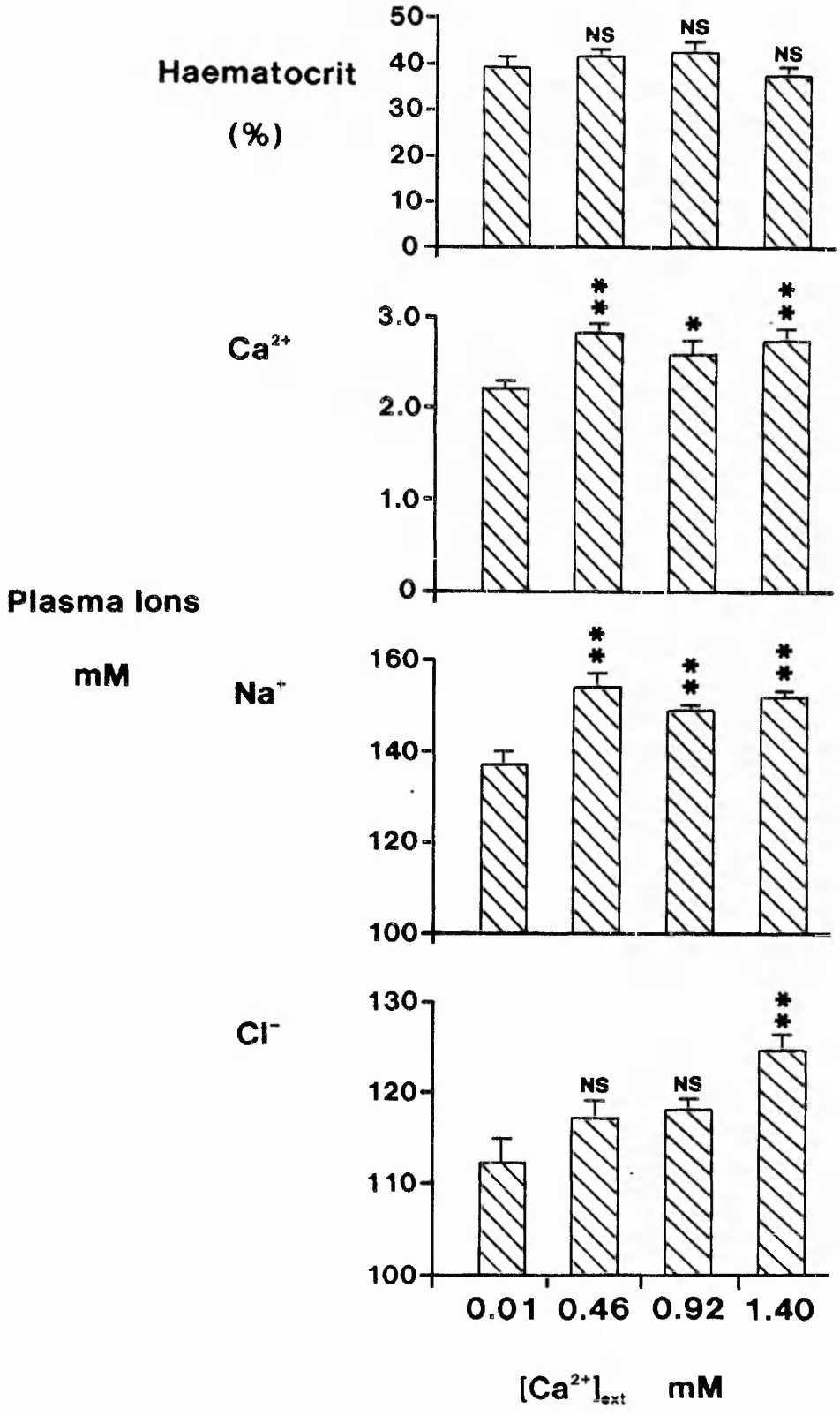

Haematocrit
(%)
50
40
30
20
10
0
NS
NS
NS
Plasma Ions
mM
Ca2+
3.0
2.0
1.0
0
Na+
160
140
120
100
Cl-
130
120
110
100
NS
NS
0.01
0.46
0.92
1.40
[Ca2+]ext mM

Table 10

Haematocrit and plasma ion concentrations in fish at the end of the 6 h flux experiments.

Within each series the results are listed in order of ascending test divalent ion concentration.

All values $\bar{x}$ ± se (n = 9 or 10)

	$[Ca^{2+}]$ mM	$[Cl^{-}]$ mM	$[Na^{+}]$ mM	H'crit %
Series 3	1.93 ± 0.07	112.6 ± 2.8	137.1 ± 2.2	40.4 ± 1.3
	2.58 ± 0.13	113.4 ± 1.2	161.6 ± 3.7	39.4 ± 1.8
	2.46 ± 0.08	115.6 ± 1.6	165.8 ± 2.6	44.1 ± 1.4
	2.46 ± 0.16	117.5 ± 3.4	164.7 ± 4.9	40.1 ± 2.6
	3.08 ± 0.11	114.3 ± 2.2	144.7 ± 2.2	42.7 ± 0.9
	2.82 ± 0.08	115.9 ± 1.0	141.6 ± 1.4	42.9 ± 0.8
	2.94 ± 0.11	113.6 ± 1.2	143.3 ± 1.5	43.1 ± 1.9
Series 4	2.54 ± 0.05	114.1 ± 2.5	158.2 ± 5.0	37.8 ± 1.8
	2.44 ± 0.05	116.7 ± 1.7	149.2 ± 1.6	37.8 ± 1.7
	2.51 ± 0.09	124.3 ± 1.4	165.3 ± 3.3	33.6 ± 1.7
	2.57 ± 0.05	111.6 ± 1.4	136.2 ± 1.2	39.8 ± 2.2
	2.85 ± 0.07	116.8 ± 1.3	141.4 ± 2.3	42.6 ± 1.5
	2.74 ± 0.04	117.4 ± 1.1	144.9 ± 1.1	43.2 ± 2.4
Series 5	2.26 ± 0.10	106.3 ± 1.9	149.8 ± 1.2	41.3 ± 2.1
	2.40 ± 0.14	108.8 ± 0.9	152.6 ± 1.4	34.6 ± 1.4
	2.19 ± 0.07	115.1 ± 1.3	141.4 ± 2.6	37.9 ± 2.0
	2.16 ± 0.08	111.1 ± 1.1	144.8 ± 1.3	37.8 ± 1.1

Figure 22

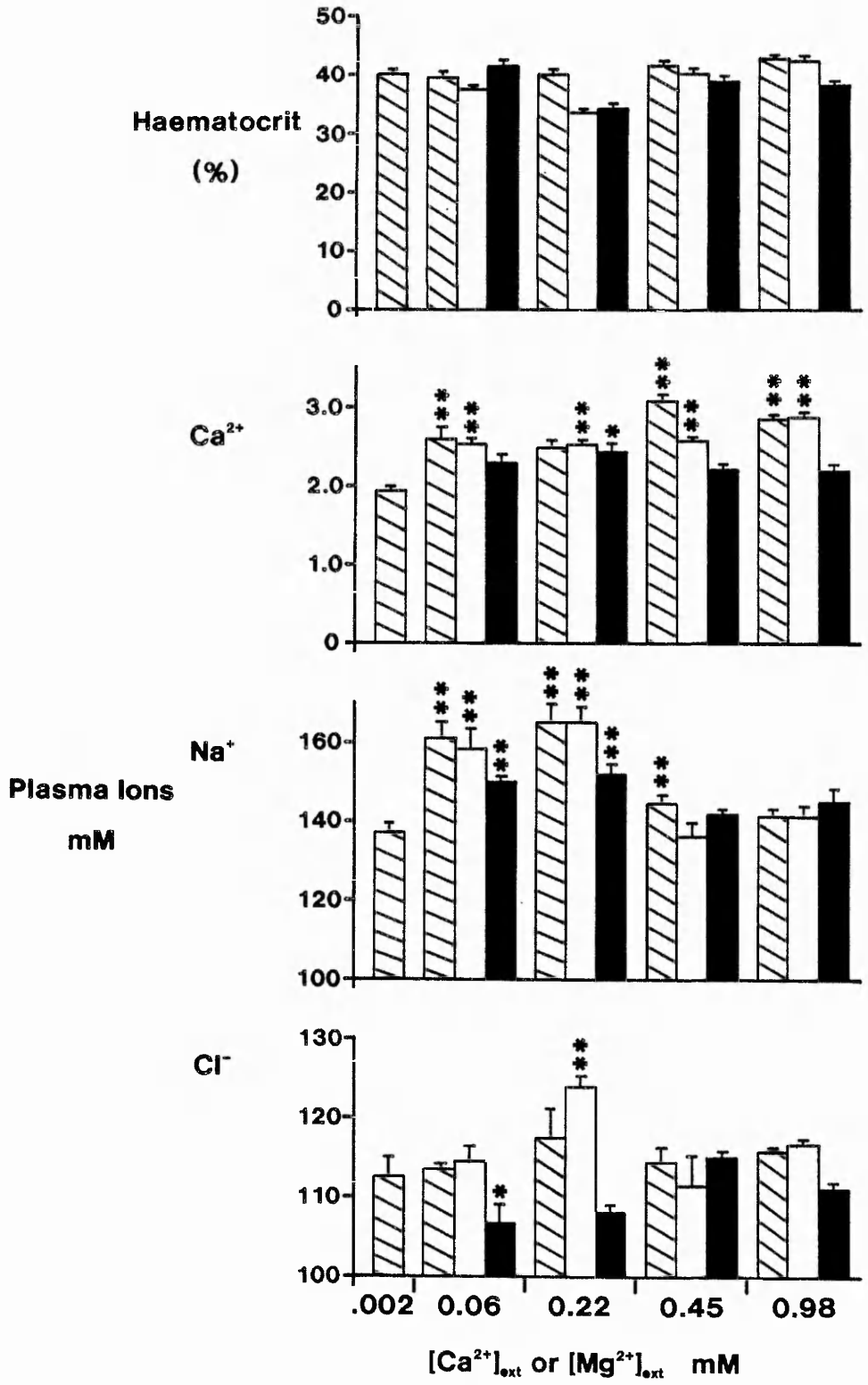
Haematocrit and plasma ion concentrations measured in blood samples taken from fish at the conclusion of the three series of 6 h flux experiments. All values represent the $\bar{x}$ ± se obtained from 9 or 10 fish. Only those results for the four comparable divalent ion concentrations are shown. Also illustrated are the results of the statistical comparisons performed against the results from fish exposed to zinc in the lowest external $[Ca^{2+}]$.

* $P < 0.05$ ** $P < 0.01$

Series 3

Series 4

Series 5


Haematocrit
(%)
50
40
30
20
10
0
Ca2+
3.0
2.0
1.0
0
Plasma Ions
mM
Na+
160
140
120
100
Cl-
130
120
110
100
.002
0.06
0.22
0.45
0.98
[Ca2+]ext or [Mg2+]ext mM

others. As stated earlier these experiments were completed a number of months after the others and on a different batch of fish so a possible seasonal effect cannot be excluded. The 6 h experiments at 0.45 and 0.98 mM calcium were completed at the same time as that at 0.002 mM however and do not reveal any significant effect on plasma $[Na^+]$ suggesting that zinc had relatively little effect on sodium balance.

Plasma calcium was strongly affected by sublethal zinc exposure. Over both 6 and 30 h plasma $[Ca^{2+}]$ in fish exposed to zinc in the lowest external $[Ca^{2+}]$ was lower than that in fish exposed in all higher external calcium concentrations, an effect which was not observed when magnesium was used in place of calcium as the external divalent ion.

3.4 DISCUSSION

3.4.1 Calculation of zinc influx

Much of the discussion which follows is based on the results of zinc influx calculated in media of differing composition. As stated in section 3.2.4 the equation of Kirschner (1970) used for the calculation of influx assumes that ^{65}Zn lost from the media is fully accounted for by that appearing in the fish. The control experiments conducted in the absence of fish indicated no adsorption of zinc to the flux apparatus so discounting one possible source of error in this calculation.

^{65}Zn disappearance from the water during the 30 h flux experiments was generally fastest at the start of a flux period with gradual slowing as the experiment progressed. The influx calculation method of Kirschner (1970) assumes that the disappearance of isotope from the medium during the flux period is linear. The levelling off of the uptake curve observed in the 30 h flux experiments of this study may have been indicative of two possible sources of error in the calculation of influx, namely, the adsorption of zinc onto the surface of the fish or, the backflux of ^{65}Zn from the fish to the water.

Comparison of the curves of isotopic zinc disappearance in this study with those of Everall (1987) revealed marked differences in both the shape of the

curve and in the magnitude of the fluxes in experiments conducted under basically similar conditions. Curves obtained by Everall (1987) were typically much steeper over the first 8 h and shallower over the remainder than those obtained in this study. Similar results to those of Everall were obtained by Joyner (1961) and were explained on the basis of zinc adsorbing to the mucus on the fish surface before true absorption, by diffusion, into the body fluids. Enhanced secretion of mucus by salmonids in the presence of elevated cadmium and lead (Varanasi and Markey, 1978) and zinc (Eddy and Fraser, 1982) concentrations has been reported. In addition, rainbow trout mucus has been shown to have a high binding affinity for cadmium and mercury (Part and Lock, 1983). Part and Svanberg (1981) found evidence of a threshold $[Cd^{2+}]$ below which little cadmium was transferred through the gills but above which transfer increased one-hundred fold. Part and Lock (1983) suggested that only above this threshold was the cadmium binding capacity of the mucus exhausted and free cadmium ions available for entry through the gills. Thus, the rapid uptake phase observed by Everall may have represented the binding of zinc to mucus, with the subsequent saturation of binding sites allowing the true influx of zinc to become apparent. The absence of this steep uptake phase in this study tends to suggest that if zinc was adsorbing to the fish mucus the process proceeded comparatively slowly with the

saturation of mucus binding sites reached gradually rather than abruptly. Indeed, comparison of influx results from experiments conducted at similar external $[Ca^{2+}]$ revealed significantly lower influxes measured over 30 h than those measured over 6 h. A surface adsorption effect that was greatest at the start and gradually declined with time would have tended to produce this result. It was likely therefore that zinc did adsorb to mucus or some other surface site before entry into the fish but it was not thought that this detracted from the validity of the results obtained. The main effect would have been to raise the calculated influxes somewhat above their true value particularly in those experiments conducted over 6 h. The overall trends observed on zinc influx in relation to changing external $[Ca^{2+}]$ and $[Mg^{2+}]$ were not considered to have been greatly affected by this phenomenon. This hypothesis was supported by the ^{65}Zn content of the numerous tissues sampled, where the trends observed in relation to external divalent ion concentrations largely confirmed those determined from zinc influxes.

The third possible source of error in the calculation of influx has already been mentioned briefly, namely the backflux of ^{65}Zn from the fish to the water. For backflux to be small and negligible the specific activity inside an animal should remain low in comparison to that in the surrounding medium. To gauge internal

specific activity a measure of the size of the internal exchangeable pool of the ion in question is required. Numerous data are available from the literature reporting total body zinc content of fish in both normal and polluted waters (Giesy and Wiener, 1977; Murphy et al., 1978; Lowe et al., 1985). None give any indication of the size of the exchangeable zinc pool however. An indication of the magnitude of backflux could possibly have been gained by holding fish in zinc-free water at the end of a flux period and monitoring the reappearance of ^{65}Zn in the medium. Rapid reappearance of ^{65}Zn possibly indicative of backflux has been noted by Joyner (1961) and Everall (1987). Pentreath (1973) however, has suggested that this effect may be due to disproportionately high surface or gut ^{65}Zn contamination resulting from the relatively short exposure times employed, as his work on plaice (Pleuronectes platessa), over longer periods, did not produce this effect. On the basis of information available from the literature therefore, it was not considered possible to estimate the extent of ^{65}Zn backflux. It does seem reasonable to assume however, that over the 6 h flux periods which formed the majority of the experiments of this work backflux would be low. Graphs of ^{65}Zn disappearance do support this view to some extent as the curvature observed over 6 h was not as marked as that recorded over 30 h.

Declining ^{65}Zn activity in the water during the 6 h experiments was found not to be accompanied by similar falls in total $[Zn^{2+}]$. Indeed, in some experiments total external $[Zn^{2+}]$ was actually found to increase through the duration of the experiment. The control experiments conducted in the absence of fish had revealed no release of zinc from the experimental apparatus (as well as the absence of adsorption that they were primarily designed to look for). Thus, it was considered that the only possible sources of the additional zinc appearing in the system were the fish themselves. The comparable experiments conducted by Everall had not revealed this effect. The fish used in the two studies were generally obtained from the same supplier although a second supplier was used by Everall when problems with disease were encountered by the first. Similarly, the chemistry of the holding water was not greatly different, both having a relatively constant low background level of zinc. Without more data explanation of this difference in results between these two studies is difficult. Further experiments investigating the effect of long-term acclimation of fish to waters devoid of zinc, and to food of low zinc content would be useful in clarifying the situation. At this point it is not possible to do more than speculate on the possible reasons for the somewhat unexpected results obtained. It is possible though, that what was being observed was merely the efflux component

of normal zinc turnover or the initiation of an excretory mechanism of some description. Irrespective of the mechanism involved however, it was considered that because of the uncertainties concerning the nature of the total [Zn^{2+}] changes the calculation of zinc effluxes and net fluxes from these data would be of little use in comparison to the results obtained for the influx of zinc in a range of external conditions.

With regard to the calculation of zinc influx the zinc concentration changes, though somewhat unexpected, were approximately linear. Thus, it was considered that the mean [Zn^{2+}] measured throughout a flux period was adequate for use in the calculation of zinc influx. In addition, it was also thought that any adsorption of zinc onto the surface of the fish altered only the magnitude of zinc influx and did not significantly affect the overall trends observed on altering flux duration, external [Ca^{2+}], external [Mg^{2+}] or acclimation media. With these assumptions in mind a number of conclusions from the influx results can be drawn and discussed.

3.4.2 The influx and distribution of zinc

The flux of zinc into brown trout was shown to be strongly dependent on external [Ca^{2+}], increasing [Ca^{2+}] reducing influx over both 6 and 30 h periods. In those experiments over 30 h this effect was shown to be

greatest at 0.46 mM calcium, a range of concentrations below this revealing the greatest effect at 0.15 mM in experiments over 6 h. In some respects therefore, these results at a sublethal [Zn^{2+}] agree with those obtained at the acutely toxic [Zn^{2+}] of 48.8 μM, in that increased external [Ca^{2+}] reduced both the acute toxicity of zinc and its rate of influx into the fish. These effects of calcium were still evident even after a one week acclimation period in water of very low divalent ion content. The distinct threshold evident at 0.15 mM in the sublethal work was not apparent in the acute work however. It represents a concentration below which small changes in external [Ca^{2+}] exerted a relatively large effect on zinc influx. Brown (1968) showed that the degree of protection conferred by water hardness against metal toxicity varied with the log of the water hardness, indicating that at low levels of water hardness small variations are likely to have a comparatively larger effect on metal toxicity than a similar change at higher water hardness. Acidified natural waters are typically characterised by their low divalent ion content and are often subject to elevated concentrations of heavy metals (NRCC, 1981). In a study of 700 acidified lakes in southern Norway Wright and Snekvik (1978) stated that calcium concentration was the major determinant of fishery status. In addition, an external [Ca^{2+}] as low as 50 μM has been shown to prolong the survival time of fish

populations at low pH (Brown, 1981, 1982), and as low as 25 μM to reduce the toxicity of aluminium to brown trout (Brown, 1983). Thus, the protective action of calcium in acidified waters is clear and appears to operate even at very low concentrations.

The results of the two series of 6 h flux experiments investigating the influence of calcium on the influx of zinc supported the results presented in Chapter 2 whereby the acute toxicity of zinc was shown to be ameliorated by calcium in the medium at the time of exposure, rather than through acclimation of the fish to hard water or elevated external $[Ca^{2+}]$ before zinc exposure. Thus, acclimation to the test $[Ca^{2+}]$ for one week before zinc exposure did result in a reduction of zinc influx relative to that in fish acclimated to low external $[Ca^{2+}]$, but only at 0.45 and 0.96 mM calcium was this reduction of great enough magnitude to be of significance. Overall, across the range of calcium concentrations examined acclimation history had relatively little influence on the influx of zinc compared to the actual external $[Ca^{2+}]$ during metal exposure.

Like calcium, magnesium was also shown to reduce the influx of zinc, though on an equimolar basis its effect was not so great. Similar results to this have been obtained in studies investigating the toxicity of cadmium though generally at more acutely toxic concentrations

than those employed here (Carroll et al., 1979; Part et al., 1985). Work on membrane permeability has also revealed that magnesium is generally of lesser importance than calcium. Magnesium was found not to influence sodium fluxes in goldfish acclimated to deionised water for example (Cuthbert and Maetz, 1972), though in contrast to this the branchial water permeability of sea-water acclimated eels was increased only on the removal of both calcium and magnesium from the water, the removal of calcium alone not producing this effect (Isaia and Masoni, 1976). Both calcium and magnesium were shown to reduce the osmotic water permeability of tilapia (Sarotherodon mossambicus) but with magnesium being considerably less effective and only producing a significant reduction at concentrations above 35 mM (Wendelaar Bonga et al., 1983). Both ions also reduced the sodium transport-dependent oxygen consumption of toad bladder, magnesium again being less effective (Cuthbert and Wong, 1971). Magnesium was also able to replace calcium in some of its effects on ion transport across the gills of mullet (Mugil capito) (Pic and Maetz, 1975).

Calcium is thought to reduce the permeability of branchial membranes through the formation of reversible cross-links with proteins and other cell membrane structures thereby reducing the permeability of paracellular pathways through the epithelial membrane (Williams, 1976; Oschman, 1978). It has been suggested

however, that the main site of action is the cell membrane itself and not the intercellular junctions (Steen and Stray-Pederson, 1975). In this cross-linking ability magnesium is considered to be less effective than calcium due to its lower ability to bind to the irregular geometry of biological molecules (Williams, 1976).

In Chapter 2 it was stated that membrane permeability data available from the literature were in general accord with the acute toxicity of zinc noted in this study. From the work reported in this chapter it is evident that the influx of zinc during exposure to a sublethal concentration is also in general agreement with much of the data concerning membrane permeability changes in relation to changes in external $[Ca^{2+}]$ and $[Mg^{2+}]$. A similar conclusion was reached by Part *et al*. (1985) working on the uptake of cadmium by perfused gills of rainbow trout. They demonstrated that the retention of cadmium in the gills was dependent only on external $[Cd^{2+}]$, external $[Ca^{2+}]$ having no effect. Calcium, and to a lesser extent magnesium, were found to exert their modifying influence through alteration of the rate of cadmium transfer from the gill into the perfusion medium, a result interpreted as evidence of a modification of membrane permeability. As the $[Ca^{2+}]$ of the medium had no influence on cadmium accumulation by the gill competition between the divalent ions for uptake sites on the gill surface was deemed unimportant. In the present study

analysis of the distribution of ^{65}Zn amongst the various fish tissues revealed that as the external $[Ca^{2+}]$ was increased the proportion of zinc contained within the gill tissue also increased, this increase being reflected in a corresponding reduction of the internal zinc burden. This effect probably represents a reduction in the transfer of zinc from the gills to the blood, a situation similar to that observed for cadmium by Part *et al*. (1985). It was not apparent when magnesium was used in place of calcium however. On the basis of Part and co-workers results it is likely that a $[Mg^{2+}]$ four to five times as high as that of calcium would have been required to produce a similar effect.

The transfer of calcium from the gills to the blood is thought to occur through the activity of Ca^{2+}-ATPases located on the basolateral membrane of the epithelial cells (Flik *et al*., 1985b). Although strong evidence has already been presented for the hypothesis that membrane permeability modification is the mechanism through which calcium and magnesium modify the influx of zinc, possible competition between these ions at the basolateral calcium transporter cannot be ruled out. Such a proposal was tentatively put forward by Reader (1986) for cadmium uptake by brown trout. Similarly, in a study of calcium uptake by the freshwater amphipod *Gammarus pulex* cadmium was found to substitute in place of calcium, apparently through "accidental active uptake". The inhibition of

calcium influx by cadmium was far from equimolar however, suggesting possible non-competitive inhibition (Wright, 1980). In this same study zinc too was found to inhibit calcium influx, though on a molar basis its effect was not so great as that of cadmium. The possible interaction of zinc with homeostatic mechanisms involved in the maintenance of normal calcium balance is examined further in Chapter 4.

Analysis of the appearance of ^{65}Zn in the various fish tissues sampled revealed greatest accumulation of zinc by the gills, plasma and carcass. The accumulation of zinc, per unit weight or volume of tissue, was greatest in the gills and plasma, with lesser quantities in the kidney, liver, spleen and gut. Insignificant quantities were accumulated by the gall bladder and blood cells. Carcass accumulation was also generally low, the high percent distribution figure for this tissue being a direct result of its large mass in comparison to other tissues.

Similar distributions of zinc throughout various fish tissues have been reported by other workers. Stable or radio-isotopic zinc accumulation was generally greatest in the gill, kidney and liver of sticklebacks (Gasterosteus aculeatus) (Matthiesen and Brafield, 1977), rainbow trout (Lloyd, 1960), brook trout (Holcombe et al., 1979), carp (Cyprinus carpio) (Saiki and Mori, 1955) and bullhead (Ictalurus nebulosus) (Joyner 1961). Cadmium

too has been found to exhibit a similar pattern of distribution (Thomas et al., 1985; Olsson and Hogstrand, 1987).

The main body surface of teleosts is considered to be virtually impermeable to ions, the low counts obtained for carcass samples (including the skin) in this, and other studies (Slater, 1961; Matthiesen and Brafield, 1977) tending to confirm this with regard to its permeability to zinc. Drinking too was not thought to contribute significantly to zinc uptake in this work. Rainbow trout held in freshwater have been demonstrated to exhibit a low drinking rate (Bath and Eddy, 1979), further decreased following the addition of zinc (Lovegrove and Eddy, 1982). In addition, zinc accumulation in the gut of bullheads was found not to be significantly affected by plugging of the oesophagus (Joyner, 1961). Direct uptake of zinc from food has been demonstrated on a number of occasions. (Nakatini, 1966; Patrik and Loutit, 1978; Singh and Ferns, 1978; Wekell et al., 1983; Hardy and Shearer, 1985; Hardy et al., 1987). Fish used in the present study were starved for a week before experimentation however, and excision of the alimentary canal for γ-counting revealed that this period was adequate for its complete evacuation. Zinc uptake via the alimentary canal was therefore thought to be of little importance during flux periods in this study. It was therefore, likely that the gills were the

major site of zinc uptake, a similar conclusion to that reached by other workers (Matthiesen and Brafield, 1977; Bradley and Sprague, 1985b), and one that is perhaps not surprising in view of the extreme specialisation of the gills for gas and ion exchange through their relatively large surface area and short blood-water diffusion distance (Hughes, 1980).

The large proportion of ^{65}Zn found in gill and plasma, relative to other tissues was considered to be an artifact arising from the relatively short duration of the experiments. Thus, turnover and translocation of zinc from the gills to internal sites of deposition was still in progress at the conclusion of experiments. The cell fraction of the blood was found to contain little ^{65}Zn, a similar situation to that found in rainbow trout held in normal conditions (Bettger et al., 1987) and also following acute zinc exposure (Spry and Wood, 1984). It was likely that zinc contained in the plasma was at least partly available for exchange with internal tissues. Of the latter zinc was contained predominantly in the liver, kidney, spleen and alimentary canal.

Metallothioneins and other metal binding proteins have frequently been linked with a possible role in metal detoxification in many different animals (review: Kagi and Nordberg, 1979). In fish, metal binding to metallothionein or other proteins has been demonstrated for zinc (Bradley et al., 1985; Pierson, 1985), cadmium

(Thomas et al., 1985; Olsson and Hogstrand, 1987), copper (Lauren and McDonald, 1987) and numerous other heavy metals. The liver and kidney are generally considered to be the organs of primary importance in this storage/ detoxification role (Pierson, 1985; Roch and McCarter, 1986; Olsson and Hogstrand, 1987; Olsson et al., 1988), with translocation of these metals from the site of uptake to these organs having been clearly demonstrated for zinc (Holcombe et al., 1979) and cadmium (Karlsson-Norrgren and Runn, 1985; Olsson and Hogstrand, 1987; Wicklund et al., 1988). Previous work at this institution (Everall, 1987) implicated melano-macrophage aggregates present in the spleen, kidney and, to a lesser extent, liver with a possible storage/ detoxification role for zinc. These aggregates were thought to contain sulphydryl-rich proteins characteristic of metallothionein. No experimental evidence was provided on this point however.

In conclusion therefore, despite the short term nature of the experiments in this study, a pattern of distribution of zinc throughout the internal organs similar to that reported by other workers has been found. It is not appropriate though to speculate on possible biochemical storage or detoxification processes involved in the turnover of zinc in the fish.

3.4.3 The effect of zinc on ionoregulation

The modification of zinc uptake by external $[Ca^{2+}]$ and $[Mg^{2+}]$ was accompanied by plasma ionoregulatory disturbances, a pronounced hypocalcaemia being evident in those fish exposed to zinc in the lowest external $[Ca^{2+}]$ in comparison to those exposed to zinc at all higher calcium levels. Plasma $[Ca^{2+}]$ is a parameter generally unaffected by changes in external $[Ca^{2+}]$ (review: Pang et al., 1980). A similar response to that observed in this work was, however, reported in rainbow trout following acute exposure to zinc in natural soft water (Spry and Wood, 1985), though in artificial soft water the effect was obscured by hypocalcaemia in non zinc-exposed control groups (Spry and Wood, 1984). In contrast to these results, another study by the same group revealed no effect of dietary or waterborne zinc on plasma calcium and whole body calcium content of rainbow trout (Spry et al., 1988). As in the discussion of zinc influx few comparative data are available from the literature regarding the effect of zinc on ionoregulation, cadmium having received more attention. Plasma calcium levels were found not to be a reliable indicator of branchial calcium flux disturbances observed during short-term acute exposure to cadmium (Reid and McDonald, 1988). In contrast to this however, pronounced hypocalcaemia is a frequently reported response of fish to cadmium, having been demonstrated for rainbow trout (Roch and Maly, 1979;

Giles, 1984; Haux et al., 1985), Atlantic salmon (Rombough and Garside, 1984) and flounder (Platichthys flesus) (Larsson et al., 1976, 1981). On the basis of previously observed cadmium induced pathological changes in kidney structure (Gardner and Yevich, 1970) Roch and Maly (1979) proposed that decreased calcium reabsorption from the kidney during cadmium exposure was the major cause of this hypocalcaemia. Giles (1984) however, observed a slight enhancement of renal calcium reabsorption in response to lowered plasma $[Ca^{2+}]$ and proposed that cadmium impaired the calcium uptake mechanism, a proposition supported by recent studies on calcium uptake through the perfused rainbow trout head (Verbost et al., 1987).

Replacement of calcium with magnesium in the external medium in this study did not produce the same response in plasma $[Ca^{2+}]$. It seems likely therefore, that the previously discussed hypocalcaemia in fish exposed to zinc in the lowest external $[Ca^{2+}]$ relative to those exposed in higher concentrations was at least partly a response to the external $[Ca^{2+}]$ and not a response due to the mobilisation of internal calcium reserves.

Plasma $[Na^{+}]$ and $[Cl^{-}]$ were comparable with those measured by McWilliams (1980) in brown trout after a one week acclimation period to water of pH 6. The effect of zinc on these ions was less clear than that observed on

plasma [Ca^{2+}]. After the 30 h flux experiments the concentrations of both ions were significantly lower in those fish exposed to zinc in the lowest external [Ca^{2+}]. Over 6 h though, both were relatively unaffected by any of the treatments employed, the results for sodium being complicated by the elevated levels measured in fish from those experiments completed a number of months after the others. Both plasma [Na^{+}] and [Cl^{-}] were unaffected by sublethal zinc exposure in the work of Everall (1987), although more acute zinc concentrations resulted in the significant depression of both, a result in marked contrast to similar work conducted on rainbow trout where acute zinc exposure was found to have little effect on plasma [Na^{+}] and [Cl^{-}] (Spry and Wood, 1985). Zinc-induced changes in sodium and chloride regulation may be a result of zinc-induced changes in branchial permeability, possibly through increased branchial diffusional ion losses via the paracellular channels (Marshall, 1985), or by the inhibition of ATPases present in branchial membranes. The latter has been demonstrated *in vitro* (Watson and Beamish, 1981), although a 30 day *in vivo* zinc exposure had a stimulatory effect on ATPase activity (Watson and Beamish, 1980). Chloride transport across the isolated opercular epithelium of *Fundulus heteroclitus* was inhibited by zinc (Crespo and Karnaky, 1983), the authors suggesting that inhibition of Na^{+},K^{+}-ATPase located on the basolateral membrane of chloride

cells was the cause of this response. On the evidence currently available, disturbances of normal sodium or chloride balance are likely to be of secondary importance in comparison to the disturbance of calcium balance.

In conclusion therefore, it is proposed that the sublethal toxicity of zinc to teleosts is intimately associated with the disruption of homeostatic mechanisms involved in the maintenance of normal calcium balance. This proposal is the subject of further investigation in the next chapter. The disruption is profoundly modified by the divalent ion status of the water actually during metal exposure, the composition of the acclimation water having relatively little effect. Of the two major water hardness ions calcium is of particular importance, a large reduction in the influx of zinc being evident at very low levels of calcium, levels which are environmentally relevant as regards soft waters vulnerable to acidification. Calcium probably modifies the uptake of zinc through alterations in membrane permeability, previous acclimation history having little influence on this process.

CHAPTER 4

The interaction of zinc with calcium balance and the effects of lanthanum on the uptake of calcium and zinc.

4.1 INTRODUCTION

In Chapter 3 it was proposed that the sublethal toxicity of zinc to brown trout is intimately associated with the disruption of the homeostatic mechanisms involved in the maintenance of normal calcium balance. The primary purpose of the work presented in this chapter was to examine further the interaction of zinc and calcium dynamics, on this occasion through the investigation of calcium turnover using ^{45}Ca as a biological tracer of calcium. Initially therefore, the turnover of calcium was examined in the absence of zinc to gain a picture of the "normal" situation, particularly in relation to the acclimation history of the fish. The effects of a range of external zinc concentrations on this calcium exchange were then studied. Both the dose-dependency and the reversibility of any zinc-induced modification of the turnover of calcium were investigated.

The last experiments reported in this chapter are concerned with an investigation of the comparative effects of lanthanum on the influxes of calcium and zinc. It was thought that these experiments would be of particular interest. Lettvin *et al*. (1964) predicted that lanthanum, due to its ionic radius being similar to that of calcium but its charge density being much greater, would bind to specific calcium binding sites with a

greater tenacity than calcium. Since this prediction was made many workers have made use of lanthanum in the examination of the chemistry of calcium in biological systems (van Breeman and McNaughton, 1970; review: Weiss, 1974). As regards its use in fish studies, it has recently been applied to an investigation of calcium transport across the branchial membranes of rainbow trout (Perry and Flik, 1988), and in an investigation of cadmium-induced inhibition of calcium influx in rainbow trout (Verbost et al., 1987). Through the use of lanthanum in the present study it was aimed to produce data for zinc comparable to that currently available for cadmium. It was considered that this data would be of significant value in understanding the mechanisms of sublethal toxic action of zinc and possibly other heavy metals also.

In addition to the measurement of calcium fluxes, haematocrit and plasma ion concentrations were also measured with the aim of determining the extent of any disturbance of normal ion balance.

^{45}Ca, the radioisotope used in these experiments is a β-emitting isotope and as such is measured using the technique of liquid scintillation counting. Samples of tissue analysed for radioactive content by this technique require solubilizing before analysis. Due to problems associated with the solubilization of large numbers of tissue samples it was not possible to repeat with ^{45}Ca

all of the work completed with ^{65}Zn whereby a number of internal organs were excised and their radioactive content measured. As a representative of internal tissues in general therefore, plasma was chosen for analysis as it did not require solubilizing before counting for radioactivity.

4.2 MATERIALS AND METHODS

4.2.1 Fish acclimation

The acclimation procedure used for fish in the ^{45}Ca flux experiments was essentially similar to that outlined in section 3.2.1 and used in the ^{65}Zn experiments. Fish size, acclimation period, water chemistry and water dosing procedures were all similar.

4.2.2 Flux apparatus and experimental protocol

The flux apparatus used in the experiments described in this chapter was as outlined in section 3.2.2. As before all water was decarbonated before use and maintained at pH 6 ± 0.3 during experiments by the manual addition of 0.1 M H_2SO_4. After the one week acclimation period the fish were transferred to the flux apparatus and maintained for 3 h in water of the same composition as that supplied during acclimation. Water of the desired composition for experimentation was gradually introduced over the final half-hour of this 3 h period. The chambers were then partially drained to the desired volume of 1100 ± 100 ml.

A number of different techniques were used to investigate the turnover of calcium in brown trout, both

in relation to the acclimation history of the fish, and in relation to the external [Zn^{2+}]. In later experiments the comparative effect of lanthanum on the influx of calcium and zinc was also investigated. Full details of individual experiments are given later in this section. A number of standard procedures were applicable to most or all of the experiments however, and are reported below. When required, stable zinc was added as an aliquot of a concentrated solution of $ZnSO_4 . 7H_2O$ at the start of the flux period. ^{45}Ca (obtained as $CaCl_2$ from Amersham International) or, in a single experiment, ^{65}Zn was also added at the start to give a nominal activity of 222 kBq (6 μCi) or 185 kBq (5 μCi) per chamber respectively. When required, lanthanum was also added from a stock solution of $LaCl_3$. A 10 minute equilibration period was then allowed before withdrawal of the first sample by auto-pipette. Throughout the duration of each flux period samples were regularly collected for measurement of radioactivity, total [Ca^{2+}] and [Zn^{2+}], the latter two being analysed as described in section 3.2.2. It was not possible to measure the concentration of lanthanum added but on the basis of results obtained when calcium or zinc were added from stock solutions it was considered that actual concentrations were within ±10 % of nominal values. The ^{45}Ca activity of the media was measured on 0.5 ml volumes of the experimental water dispensed into 4.5 ml of scintillation fluid (Optiphase 'Safe', LKB

Scintillation Products) contained in 5 ml scintillation vial inserts held within 25 ml glass scintillation vials. Samples were counted for radioactivity on a Packard A300CD liquid scintillation counter. To minimise counting errors samples were counted for sufficient time to accrue 10,000 counts. The resulting count per minute data were corrected to disintegrations per minute (d min^{-1}) from a quench curve generated using 5 ml aliquots of scintillation fluid containing known quantities of radioactivity to which had been added increasing volumes of the quenching agent trichloromethane. All samples were counted between 8 and 257 keV, under such conditions a counting efficiency of approximately 76 % being obtained.

At the end of the experiment fish were killed by MS222 overdose and weighed. The final volume of medium in the flux chambers was also determined. When required a blood sample was collected by caudal venipuncture as described in section 3.2.2. Following centrifugation a 100 µl (50 µl in later experiments) aliquot of plasma was dispensed into 5 ml of scintillation fluid for the measurement of radioactivity. From a quench curve generated using increasing volumes of plasma added to a known activity of ^{45}Ca in 5 ml of scintillation fluid it was found that the degree of quench occurring with plasma was not significantly different from that obtained with water. Plasma samples were therefore counted using the same d min^{-1}-correction programme as that used for water

samples. The remaining plasma was stored at -20°C for the subsequent determination of $[Ca^{2+}]$, $[Na^{+}]$ and $[Cl^{-}]$ as outlined in section 3.2.3.

On those occasions when plasma samples were analysed for radioactivity content, samples of homogenized fish were similarly analysed. The entire carcass was homogenized in a Waring blender with 40 ml of distilled water added to aid homogenization. Quadruplicate sub-samples of the homogenate were placed carefully in the bottom of a scintillation vial and accurately weighed to the nearest mg. Sample weight was always less than 250 mg. 1 ml of tissue solubilizer (Soluene 350, Canberra-Packard) was added to each vial and all were stoppered and incubated overnight at 37°C. 15 ml of scintillation fluid was then added to each, all were vigorously shaken and again left overnight, this time to allow the decay of chemiluminescence. The resulting solubilized tissue samples were then counted for radioactivity using a d min^{-1}-correction programme generated from the degree of quench measured from a large range of weights of digested tissue. From this quench curve it had been demonstrated that tissue weights of less than 250 mg did not significantly affect counting efficiency despite variation in the degree of quench.

4.2.3 Flux calculation

Through the measurement of changes in ^{45}Ca activity and stable $[Ca^{2+}]$ with time it was possible to simultaneously measure the influx and net flux of calcium. Knowing these, efflux could then be determined thereby allowing determination of the overall calcium balance of the fish under various external conditions.

As in the work using ^{65}Zn a modified version of equation 5 of Kirschner (1970) was used to calculate influx. Thus

$$J_{in} = \frac{(\ln Q_{out(o)} - \ln Q_{out(t)})}{t \cdot W} \cdot Q_{out}$$

where $Q_{out(o)}$ and $Q_{out(t)}$ represent the total quantity of radioactivity (d min^{-1}) in the medium at the start and end of the experiment, Q_{out} represents the mean total quantity of calcium (μmol) in the medium during the experiment, t is the duration of the experiment in h and W is the fish weight in kg.

Net fluxes were calculated from changes in stable $[Ca^{2+}]$. Thus

$$J_{net} = \frac{n_o - n_t}{t \cdot W}$$

where n_o and n_t represent the total quantity of calcium (μmol) in the medium at the start and end of the flux

period.

Efflux was then derived as

$$J_{out} = J_{net} - J_{in}$$

Calculation of the data in this way yielded rates of influx, efflux and net flux in the form of μmol kg^{-1} h^{-1}.

The same assumptions as those outlined in section 3.2.4 for zinc were applicable in the calculation of calcium influx. As was the case with the ^{65}Zn fluxes a control experiment was completed using water of very low $[Ca^{2+}]$ labelled with ^{45}Ca and with fish omitted to evaluate the extent of isotope adsorption onto the experimental apparatus. The possibility of radioisotope backflux from the fish to the medium was also investigated and will be discussed later.

4.2.4 Experimental design

Four series of experiments were completed investigating the turnover of calcium in relation to fish acclimation history and external $[Zn^{2+}]$.

The first series involved a total of five 4 h flux experiments, examining the modifying influence of acclimation medium composition on calcium balance. Groups of 10 fish were acclimated for one week to water of either very low $[Ca^{2+}]$ (3 groups) or of a higher $[Ca^{2+}]$

(2 groups). Details of media composition during acclimation and testing are shown in Table 11. Duplicate water samples were collected at 1 h intervals throughout the flux period for measurement of radioactivity. Total $[Ca^{2+}]$ was also measured on samples collected each hour whilst $[Zn^{2+}]$ was measured at the start and end of the flux period. No zinc was added so only background concentrations were present in the flux media. At the completion of these experiments blood samples were collected and the carcasses homogenized.

The second series was similarly completed over a 4 h flux period and involved four experiments investigating the modifying influence of external zinc on calcium balance. Details of acclimation and test water composition are listed in Table 12. Again blood and carcass samples were collected at the end of the experiment.

The third series consisted of a single experiment investigating whether the effect that zinc had previously been demonstrated to have on calcium balance was reversible. 10 fish were acclimated to low $[Ca^{2+}]$ for one week and were then allowed to settle in the flux chambers for 3 h. All chambers were then flushed with water of 0.125 mM calcium and zinc was added to give a nominal concentration of 3.85 μM (0.25 mg l^{-1}). To five chambers ^{45}Ca was added and the calcium fluxes determined over a 3 h period. At the end of this time these five

Table 11

External calcium concentration during acclimation and testing of fish in the first series of calcium flux experiments.

All concentrations are $\bar{x}$ ± range (mM)

Experiment	$[Ca^{2+}]$ during acclimation	$[Ca^{2+}]$ at start of test
I	0.012 ± 0.010	0.020 ± 0.001
II	0.26 ± 0.01	0.25 ± 0.01
III	0.002 ± 0.001	0.25 ± 0.01
IV	0.49 ± 0.02	0.49 ± 0.01
V	0.002 ± 0.001	0.48 ± 0.01

Table 12

External calcium concentration during acclimation and external calcium and zinc concentrations during testing of fish in the second series of calcium flux experiments.

All concentrations are $\bar{x}$ ± range

Experiment	$[Ca^{2+}]$ during acclimation mM	$[Ca^{2+}]$ at start of test mM	$[Zn^{2+}]$ at start of test µM
I	0.002 ± 0.001	0.141 ± 0.005	0.10 ± 0.01
II	0.003 ± 0.002	0.143 ± 0.003	0.66 ± 0.04
III	0.003 ± 0.002	0.136 ± 0.002	4.06 ± 0.18
IV	0.002 ± 0.001	0.136 ± 0.003	15.43 ± 1.12

chambers were flushed with clean 0.125 mM calcium water over a 20 min period, a further aliquot of ^{45}Ca added and the fish subjected to a second 3 h flux period, this time without zinc present. The remaining 5 fish were allowed to complete the initial 3 h in 3.85 μM zinc (with no ^{45}Ca added) and were then transferred back to the low $[Ca^{2+}]$ acclimation tank and held there for a further 48 h. They were then moved back to the flux apparatus and tested for 3 h in water containing 0.125 mM calcium labelled with 6 μCi ^{45}Ca. Both groups were blood sampled and the carcasses homogenized. Actual $[Ca^{2+}]$ and $[Zn^{2+}]$ of the media for these 3 fluxes are shown in Table 13.

The fourth series of experiments specifically investigated the comparative effects of lanthanum on the influxes of calcium and zinc. It comprised 3 experiments all of which used fish acclimated to low $[Ca^{2+}]$ for one week. The first of these was a pilot experiment to establish the external $[La^{3+}]$ to be used in the other two. Following the 3 h fish recovery period all chambers were flushed with water of 0.125 mM calcium and ^{45}Ca added. The fish were then fluxed for 2 h to establish the "normal" rate of influx. An aliquot of the stock lanthanum solution was then added to each chamber such that chambers 1 and 2 received a volume calculated to give a concentration of 0.001 μM; 3 and 4 a volume to give 0.01 μM; 5 and 6, 0.1 μM; 7 and 8, 1.0 μM; and 9, 10 μM (only 9 fish were available). After a further 15 mins

Table 13

External calcium and zinc concentrations during the testing of fish in the third series of calcium flux experiments

Groups I and II represent the same group of 5 fish tested initially in the presence of 3.49 μM zinc and subsequently after the removal of the same. Group III represents the group of 5 fish exposed for 3 h to 3.85 μM zinc (in the absence of radioactive calcium) and allowed to recover in "clean" water for 48 h prior to the measurement of calcium flux in the absence of zinc.

All concentrations are $\bar{x}$ ± range

Group	$[Ca^{2+}]$ during acclimation mM	$[Ca^{2+}]$ at start of test mM	$[Zn^{2+}]$ at start of test μM
I	0.003 ± 0.003	0.131 ± 0.001	3.49 ± 0.11
II	0.003 ± 0.003	0.136 ± 0.001	0.11 ± 0.02
III	0.003 ± 0.003	0.126 ± 0.001	0.11 ± 0.02

a second 2 h flux was completed. From the results of this experiment it was decided that a $[La^{3+}]$ of 10 μM was sufficient to produce the desired effect, this being the concentration used in the two subsequent experiments. The first of these involved the testing of 10 low calcium acclimated fish in 0.125 mM calcium labelled with ^{45}Ca. To quantify the effect of lanthanum on calcium fluxes an aliquot calculated to give a concentration of 10 μM was added to five of these chambers, calcium fluxes in the remaining five chambers representing those of a control group. The second was of similar design but with zinc (0.77 μM ± 5 μCi ^{65}Zn) added in place of ^{45}Ca. It was thereby aimed to similarly quantify the influence of lanthanum on the uptake of zinc by fish. Flux duration in both experiments was 4 h, the flux period being started 15 min after the addition of lanthanum. Plasma and carcasses were analysed as outlined earlier (^{65}Zn by γ-counting as described in chapter 3).

In section 4.2.3 it was briefly mentioned that an attempt was made to estimate the magnitude of isotope backflux that may have occurred during the ^{45}Ca flux experiments of this work. One of the measurements necessary to allow the calculation of this is the calcium space of the fish. The protocol employed in the attempt to measure this in this study is outlined below. The theory behind calcium space measurement and the calculation of backflux is fully discussed later in this

chapter.

Two experiments were completed on calcium space measurements. The first used 5 fish that were long-term acclimated to hard water of ambient pH and temperature and was conducted in this same medium. It was done mainly to gain experience in the techniques involved but it was also hoped that if it was successful the results obtained could be compared with those calcium spaces measured in fish acclimated and tested in media of different composition. The 5 fish were transferred straight from the stock holding-tanks into anaesthetic (1:10,000 MS222, unbuffered) and then injected intra-peritoneally with approximately 370 kBq (10 μCi) of ^{45}Ca in 200 μl of distilled water. The fish were allowed a few minutes in clean aerated water for recovery and then transferred to the flux apparatus. Syringes were weighed before and after injection to determine accurately the injection volume and hence the total injected ^{45}Ca activity. Over the next 10 h water samples were collected to monitor the appearance of isotope in the water. After 10 h the fish were removed, anaesthetised and blood sampled (by the standard procedure), approximately 0.2 ml being collected. They were then allowed a few minutes to recover before being returned to the flux apparatus which, in the meantime, had been flushed with clean water. They were left for a further 14 h and then killed by MS222 overdose and blood sampled again. From the data

obtained calcium spaces (ml) were determined according to the formula

$$\frac{^{45}Ca \text{ injected (d min}^{-1}) - {}^{45}Ca \text{ lost to water (d min}^{-1})}{Cp \cdot W}$$

where Cp represents the radioactivity per ml of plasma (d min^{-1} ml^{-1}) and W is the fish weight in kg.

The second of these experiments used 5 fish acclimated to, and tested in, pH 6, 15°C, low [Ca^{2+}] water. The protocol was as just outlined with the exception that all fish were killed and blood sampled after the 10 h period, the experiment not being extended to 24 h.

4.2.5 Statistical procedures

As in the ^{65}Zn experiments a number of statistical tests were employed in the analysis of the data provided by the work of this chapter.

Bartlett's Test for Homogeneity of Variance revealed a significant departure from the null hypothesis for much of the flux data. All flux data were therefore analysed using the Kruskal-Wallis test with subsequent comparison of average ranks when a significant departure from the null hypothesis was indicated.

Plasma and homogenized carcass samples counted for radioactivity yielded data in the form of d min^{-1} per wet weight of tissue or volume of plasma. All tissue count data were therefore normalised to a standard weight or volume and corrected for variation of external specific activity between treatments. Thus

$$\frac{\text{Tissue d min}^{-1}\ \text{g}^{-1}\ \text{or plasma d min}^{-1}\ \text{ml}^{-1}}{\text{Specific activity (d min}^{-1}\ \text{nmol}^{-1})}$$

where Specific activity = $\dfrac{\bar{x}\ \text{external}\ ^{45}\text{Ca(d min}^{-1}\ \text{ml}^{-1})}{\bar{x}\ \text{external}\ [Ca^{2+}]\ (\text{nmol ml}^{-1})}$

Similar data provided by the ^{65}Zn experiments discussed earlier were statistically analysed using the Kruskal-Wallis test. Before this particular test had been decided on $\log_{10}$ transformation of the data had been attempted in order to correct to a normal distribution the natural tendency of data in the form of radioactive counts towards the Poisson distribution. This transformation had proved unreliable so the non-parametric statistical test was employed. The same transformation when applied to data from the ^{45}Ca experiments however, did correct them to a normal distribution, this apparent anomaly being a result of the latter data exhibiting greater homogeneity of variance than the former. Transformed data from the ^{45}Ca

experiments were therefore analysed by the more powerful one-way analysis of variance, with subsequent comparison of sample means where necessary.

Plasma ion data were similarly analysed by one-way analysis of variance and sample means were compared.

4.3 RESULTS

4.3.1 Normal calcium exchange

Shown in Figure 23 are typical curves of ^{45}Ca disappearance from the various media during the first series of flux experiments. As in the ^{65}Zn work presented in the previous chapter each plot represents the mean response of all fish used in an individual experiment. Again the results are presented in the form of percentage radioactivity remaining in the medium with time. Also illustrated in this figure is the result of the control experiment conducted at low external $[Ca^{2+}]$ in the absence of fish, adsorption of isotope onto the apparatus being demonstrated not to be a problem in this study. Though only results to 4 h are shown there was in fact no detectable sequestration of ^{45}Ca by the flux apparatus over a 24 h period.

As might have been expected simply on the basis of specific activity, the greatest decline in ^{45}Ca activity occurred in that test conducted at very low external $[Ca^{2+}]$. Similarly, ^{45}Ca disappearance was greater in those tests at 0.25 mM calcium than in those at 0.50 mM. Comparison of the two experiments conducted at 0.25 mM and the two at 0.50 mM suggests that in both cases the disappearance of radioactivity was greater following the acclimation of fish to low external calcium relative to

Figure 23

Curves of isotope disappearance from the media during the first series of ^{45}Ca flux experiments. The result of the control experiment completed in water of very low divalent ion status is also shown. Each plot represents the mean of 10 fish. For the sake of clarity only the largest standard errors are shown.

The treatments assigned to each group are summarised below, being explained in greater detail in the text and in Table 11.

	Acclimation medium	Test medium
I	Low	Low
II	Medium	Medium
III	Low	Medium
IV	High	High
V	Low	High

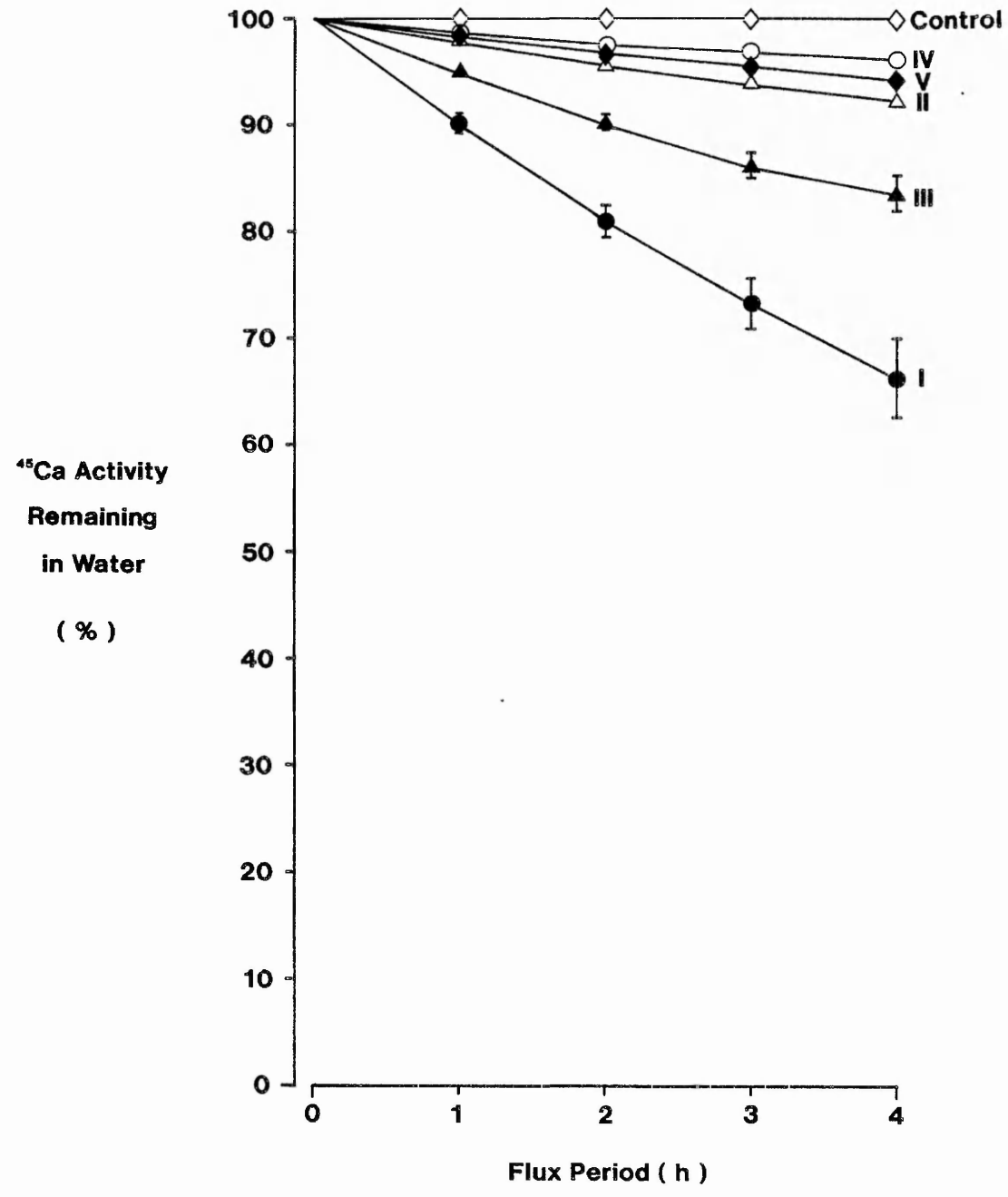
where Low = 0 mM nominal $[Ca^{2+}]$

Medium = 0.25 mM nominal $[Ca^{2+}]$

High = 0.50 mM nominal $[Ca^{2+}]$

Mean total $[Ca^{2+}]$s (mM) at the start and end of the experiments are listed below.

	Start	End
I	0.021	0.017
II	0.256	0.242
III	0.249	0.216
IV	0.498	0.483
V	0.484	0.466
Control	0.005	0.005


100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Control
IV
V
II
III
I
45Ca Activity
Remaining
in Water
(%)
Flux Period (h)

those fish acclimated to the test [Ca^{2+}]. Examination of the shape of the uptake curves reveals a slight curvature similar to that observed in the 6 h ^{65}Zn fluxes. Adsorption of isotope onto the surface of the fish or backflux of isotope from the fish to the water could have produced such effects. The relative importance of these effects will be discussed later.

Calcium influx, efflux and net fluxes for the experiments of series 1 are shown in Figure 24 together with the results of the statistical analyses comparing the various treatments. From this Figure it is clear that all groups were in a state of net positive calcium balance, even that group tested at the lowest external [Ca^{2+}]. This latter group did however exhibit the lowest rate of influx, presumably a reflection of the lack of substrate available for uptake as those fish acclimated to low calcium but tested in a higher concentration (III and V) exhibited considerably faster rates of influx. Indeed, influx in these fish was also considerably greater than that measured for fish tested in the same [Ca^{2+}] as that supplied during acclimation (II and IV), suggesting a possible enhancement of the calcium uptake mechanism during acclimation to low levels of calcium.

The efflux of calcium was affected less than influx by acclimation to low external [Ca^{2+}] though there did appear to be a slight enhancement of efflux in those fish acclimated to low calcium before testing in a higher

Figure 24

Calcium influx, efflux and net flux (shaded area) during the first series of ^{45}Ca flux experiments. All values represent the $\bar{x}$ ± se of 10 fish. An abbreviated key to the treatments is given in Figure 23. The statistical comparisons of all possible pairs of means are also shown.

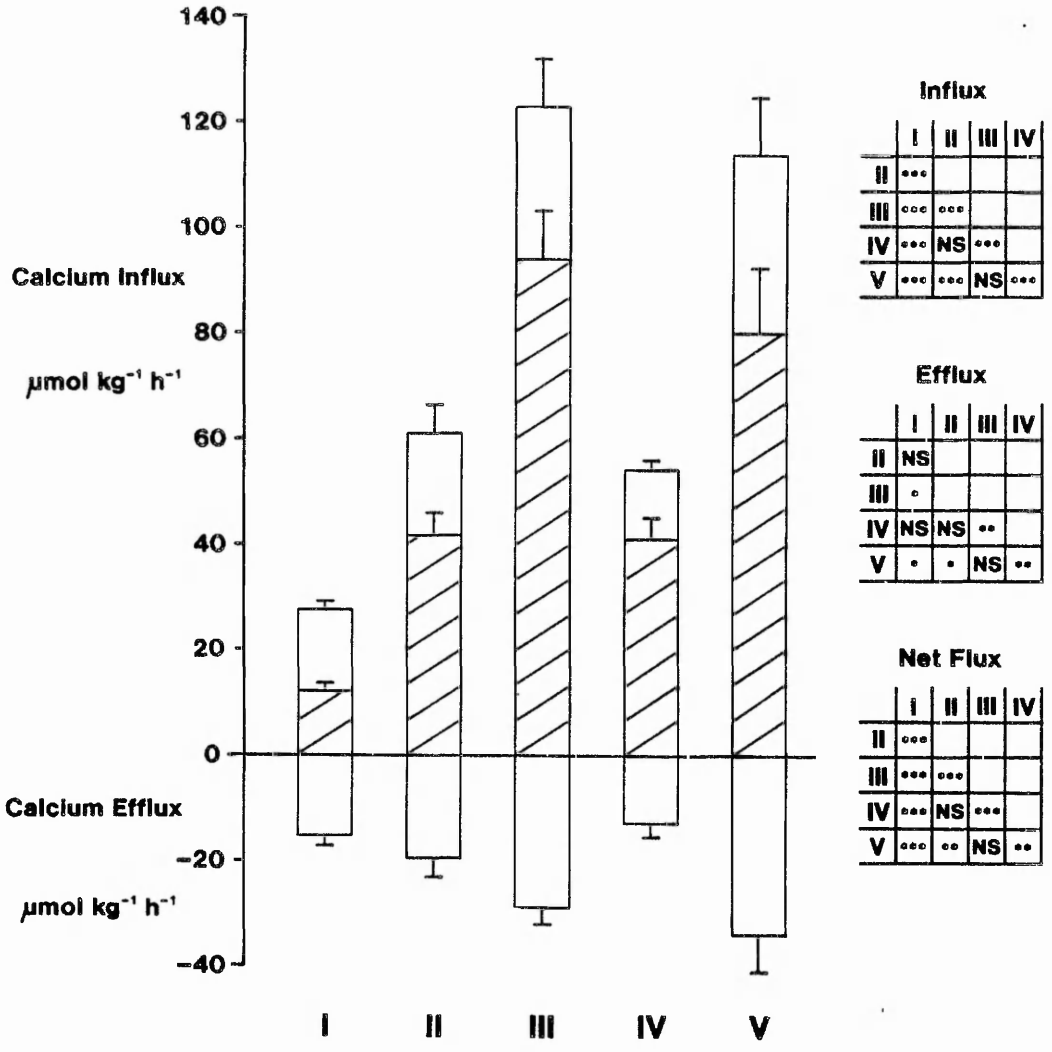
NS Not significant * P <0.05 ** P < 0.01 *** P < 0.001

	Acclimation medium	Test medium
I	Low	Low
II	Medium	Medium
III	Low	Medium
IV	High	High
V	Low	High

where Low = 0 mM nominal $[Ca^{2+}]$

Medium = 0.25 mM nominal $[Ca^{2+}]$

High = 0.50 mM nominal $[Ca^{2+}]$.


140
120
100
80
60
40
20
0
−20
−40
Calcium Influx
µmol kg⁻¹ h⁻¹
Calcium Efflux
µmol kg⁻¹ h⁻¹
I
II
III
IV
V
Influx
Efflux
Net Flux
NS

concentration.

The calcium uptake values of the plasma and homogenized carcass samples for this particular series of experiments are shown in Figure 25. The overall trends observed reflected those obtained for the influx of calcium indicating that these latter trends were real rather than due to a surface adsorption effect. This is particularly true of the data provided by the plasma samples as ^{45}Ca appearing in plasma must have physically entered the fish rather than having been simply adsorbed to its surface.

Haematocrit and plasma ion concentrations are shown in Figure 26. The former was unaffected by any of the treatments. Plasma $[Ca^{2+}]$ was raised in those fish tested in elevated external $[Ca^{2+}]$ following acclimation to low calcium. Thus, the enhancement of calcium influx observed in these fish was accompanied by hypercalcaemia, though this was probably of a transient nature as it was not apparent in fish acclimated to the same external $[Ca^{2+}]$ for the week before testing. Plasma $[Na^{+}]$ was also found to be affected by some of the treatments employed, being raised in those fish acclimated to the two higher calcium concentrations. This could not be attributed to variation of external $[Na^{+}]$ as the latter remained unchanged throughout all experiments. The changes observed were likely a result of the external $[Ca^{2+}]$ during acclimation. Plasma $[Cl^{-}]$ was affected in a similar way

Figure 25

Calcium uptake by the plasma and whole fish at the conclusion of the first series of ^{45}Ca flux experiments. All values represent the $\bar{x}$ ± se of 10 fish. The statistical comparisons of all possible pairs of means are also shown.

NS Not significant * $P < 0.05$ ** $P < 0.01$

	Acclimation medium	Test medium
I	Low	Low
II	Medium	Medium
III	Low	Medium
IV	High	High
V	Low	High

where Low = 0 mM nominal $[Ca^{2+}]$

Medium = 0.25 mM nominal $[Ca^{2+}]$

High = 0.50 mM nominal $[Ca^{2+}]$.

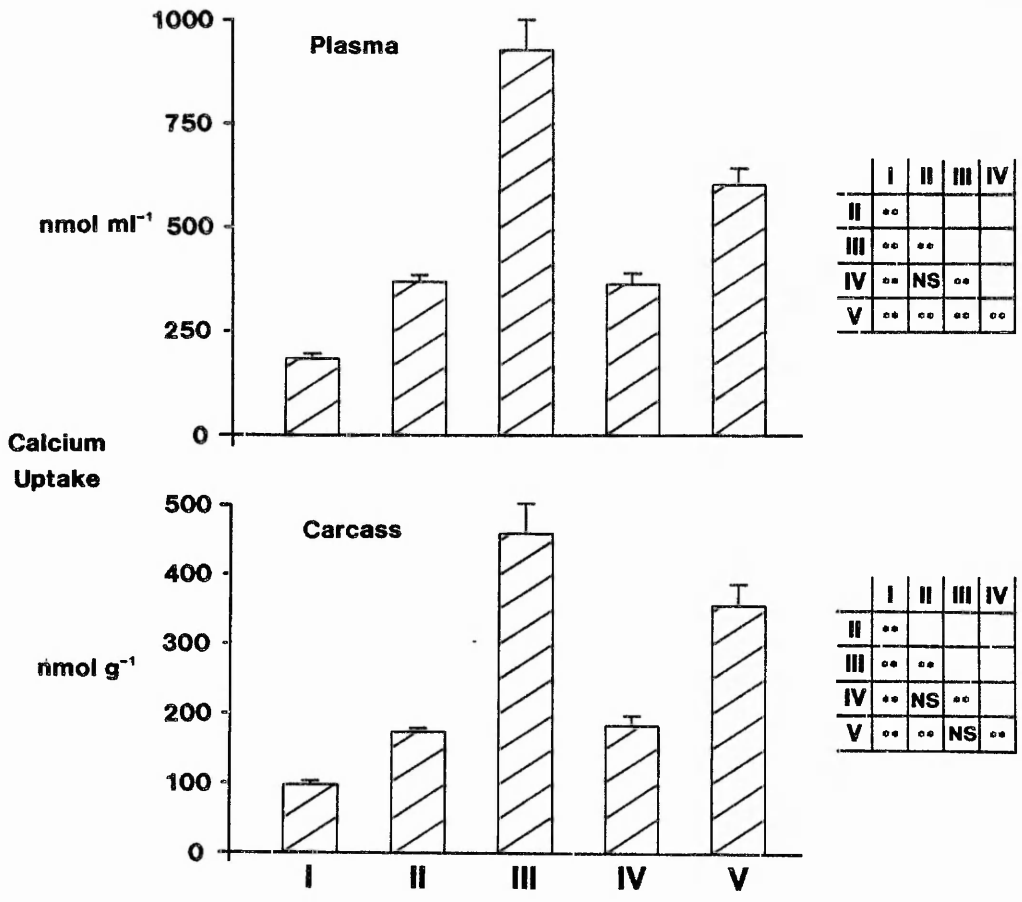

Plasma
1000
750
500
250
0
nmol ml⁻¹
Calcium Uptake
Carcass
500
400
300
200
100
0
nmol g⁻¹
I
II
III
IV
V
I II III IV
II **
III ** **
IV ** NS **
V ** ** ** **
I II III IV
II **
III ** **
IV ** NS **
V ** ** NS **

Figure 26

Haematocrit and plasma ion concentrations measured in blood samples taken from fish at the end of the first series of ^{45}Ca flux experiments. All values represent the $\bar{x} \pm$ se of 10 fish. The statistical comparisons of all possible pairs of means are also shown.

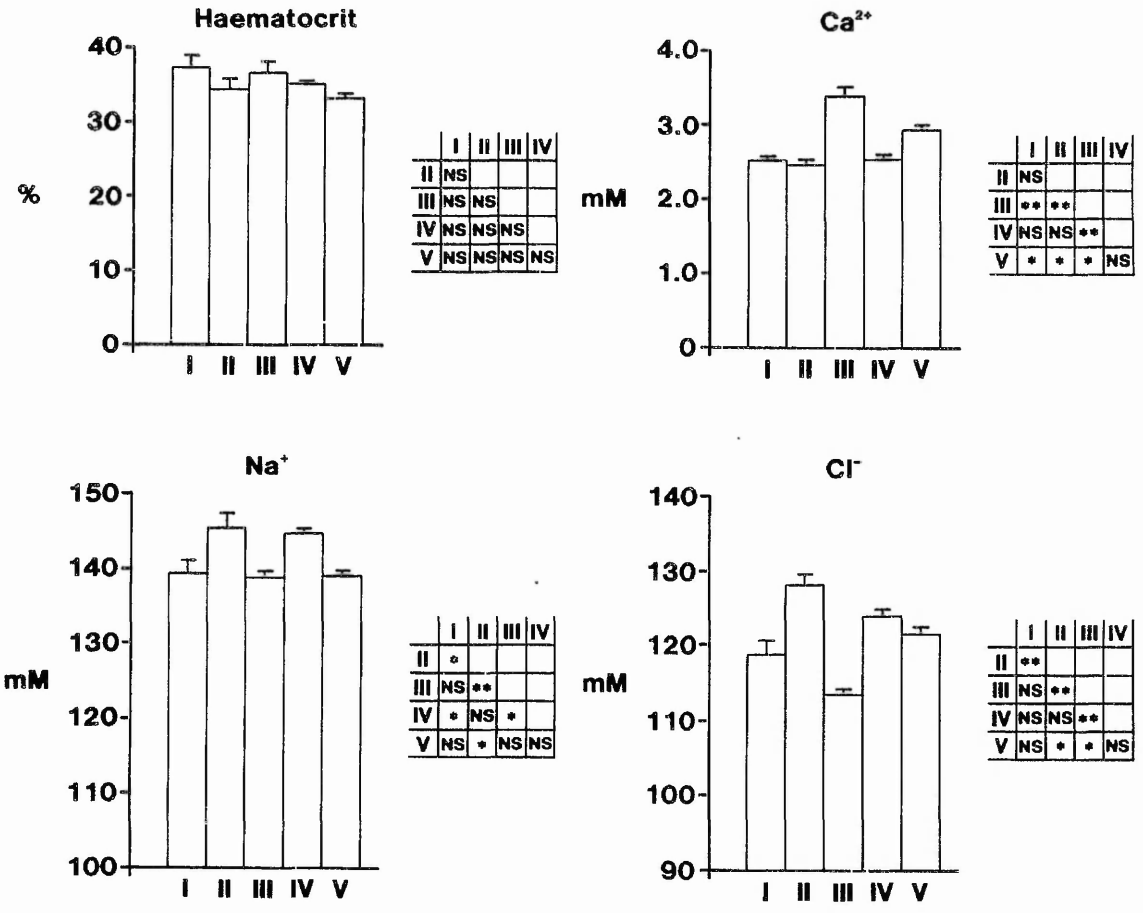
NS Not significant * $P < 0.05$ ** $P < 0.01$

	Acclimation medium	Test medium
I	Low	Low
II	Medium	Medium
III	Low	Medium
IV	High	High
V	Low	High

where Low = 0 mM nominal $[Ca^{2+}]$

Medium = 0.25 mM nominal $[Ca^{2+}]$

High = 0.50 mM nominal $[Ca^{2+}]$.


Haematocrit
%
40
30
20
10
0
I II III IV V
I II III IV
II NS
III NS NS
IV NS NS NS
V NS NS NS NS
Ca2+
mM
4.0
3.0
2.0
1.0
0
I II III IV V
I II III IV
II NS
III ** **
IV NS NS **
V * * * NS
Na+
mM
150
140
130
120
110
100
I II III IV V
I II III IV
II *
III NS **
IV * NS *
V NS * NS NS
Cl-
mM
140
130
120
110
100
90
I II III IV V
I II III IV
II **
III NS **
IV NS NS **
V NS * * NS

as sodium though generally in a less clear manner.

4.3.2 The effect of zinc on calcium balance

^{45}Ca uptake curves, calcium fluxes, tissue and plasma calcium uptake and plasma ion concentrations for the second series of experiments are shown in Figures 27, 28, 29 and 30 respectively. From Figure 27 it is evident that increasing the concentration of zinc reduced the rate of disappearance of ^{45}Ca from the media. Figure 28 clearly shows that this reduction in the uptake of isotope represented a reduction of calcium influx, increasing external zinc causing this reduction in a dose-dependent manner. Efflux however, was not affected by any external $[Zn^{2+}]$ tested so that overall the net flux of calcium was also greatly depressed. This depression was such that at the highest external $[Zn^{2+}]$ of 15.4 μM (1.00 mg l^{-1}) the fish were in a state of negative calcium balance. As was the case in the first series of experiments the plasma and carcass radioactivity data (Figure 29) confirmed the results of the fluxes indicating again that the trends noted were real. Of the plasma parameters measured (Figure 30) $[Ca^{2+}]$ was found to be the one most affected by the addition of zinc to the water, increasing $[Zn^{2+}]$ causing a marked hypocalcaemia. Neither haematocrit nor sodium were affected by zinc though chloride was slightly but

Figure 27

Curves of isotope disappearance from the media during the ^{45}Ca flux experiments conducted at a range of external zinc concentrations. All groups were acclimated to low external $[Ca^{2+}]$ before testing in 0.125 mM nominal $[Ca^{2+}]$ (see Table 12 for actual concentrations).

Test	$[Zn^{2+}]$ (μM)
I	0.10
II	0.66
III	4.06
IV	15.43

Each plot represents the mean of 10 fish. Standard errors were too small for inclusion.

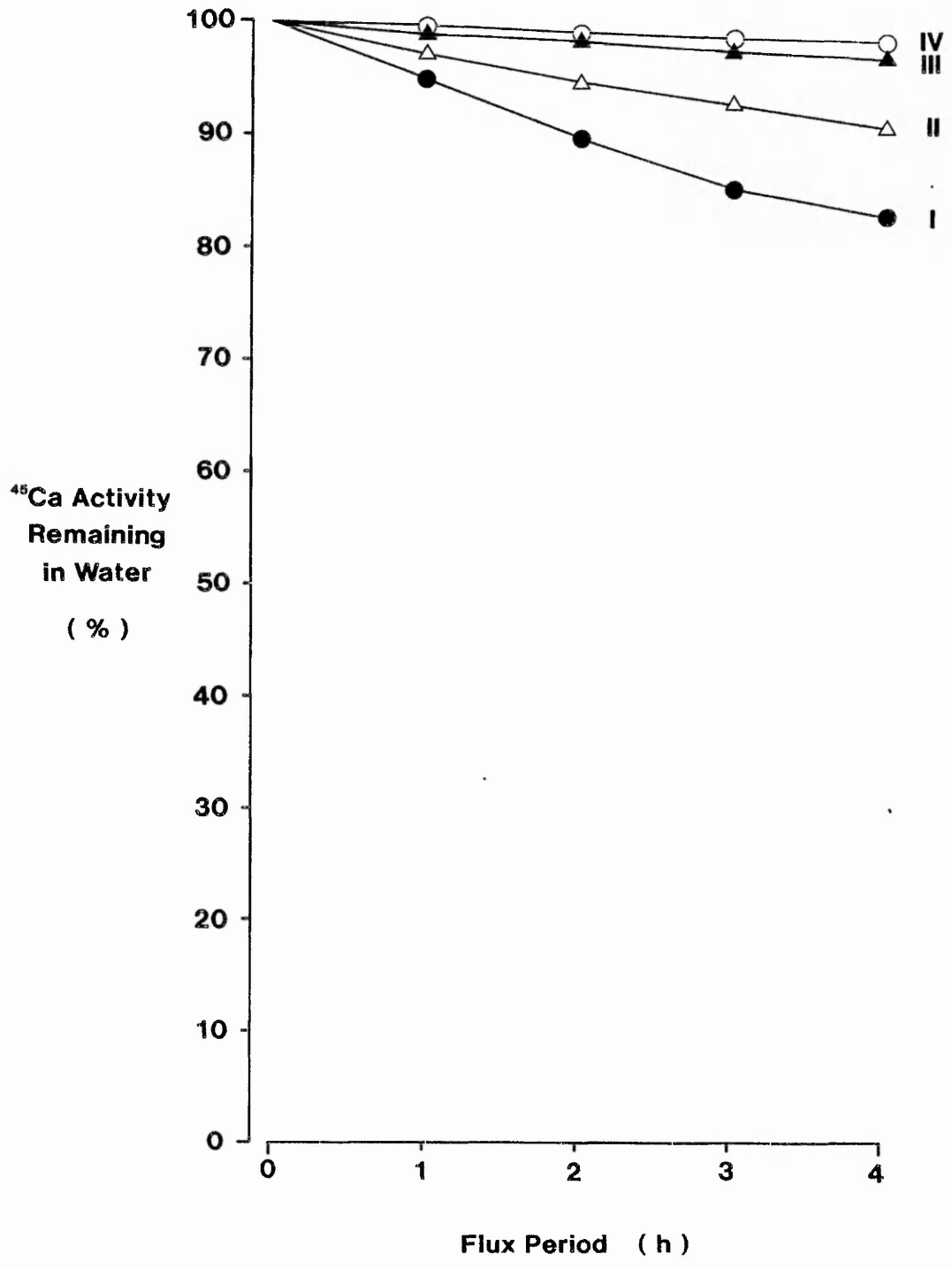

100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
IV
III
II
I
45Ca Activity Remaining in Water (%)
Flux Period (h)

Figure 28

Calcium influx, efflux and net flux (shaded area) during the ^{45}Ca flux experiments conducted at a range of external zinc concentrations. All values represent the $\bar{x}$ ± se of 10 fish. The statistical comparisons of all possible pairs of means are also shown.

NS Not significant ** $P < 0.01$ *** $P < 0.001$

Test	$[Zn^{2+}]$ (µM)
I	0.10
II	0.66
III	4.06
IV	15.43

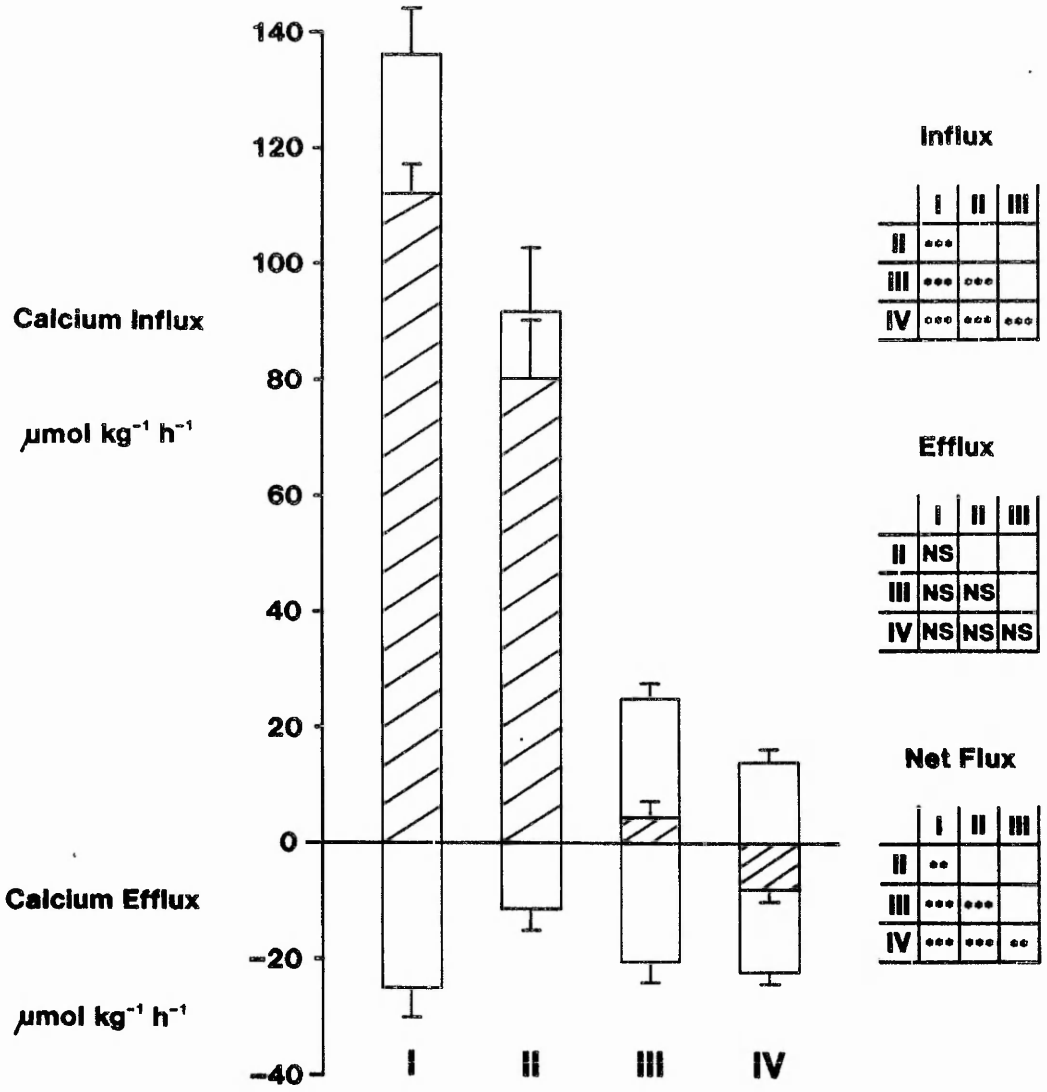

140
120
100
80
60
40
20
0
-20
-40
Calcium Influx
µmol kg⁻¹ h⁻¹
Calcium Efflux
µmol kg⁻¹ h⁻¹
I
II
III
IV
Influx
Efflux
Net Flux

Figure 29

Calcium uptake by the plasma and whole fish at the conclusion of the ^{45}Ca flux experiments conducted at a range of external zinc concentrations. All values represent the $\bar{x}$ ± se of 10 fish. The statistical comparisons of all possible pairs of means are also shown.

* $P < 0.05$ ** $P < 0.01$

Test	$[Zn^{2+}]$ (μM)
I	0.10
II	0.66
III	4.06
IV	15.43

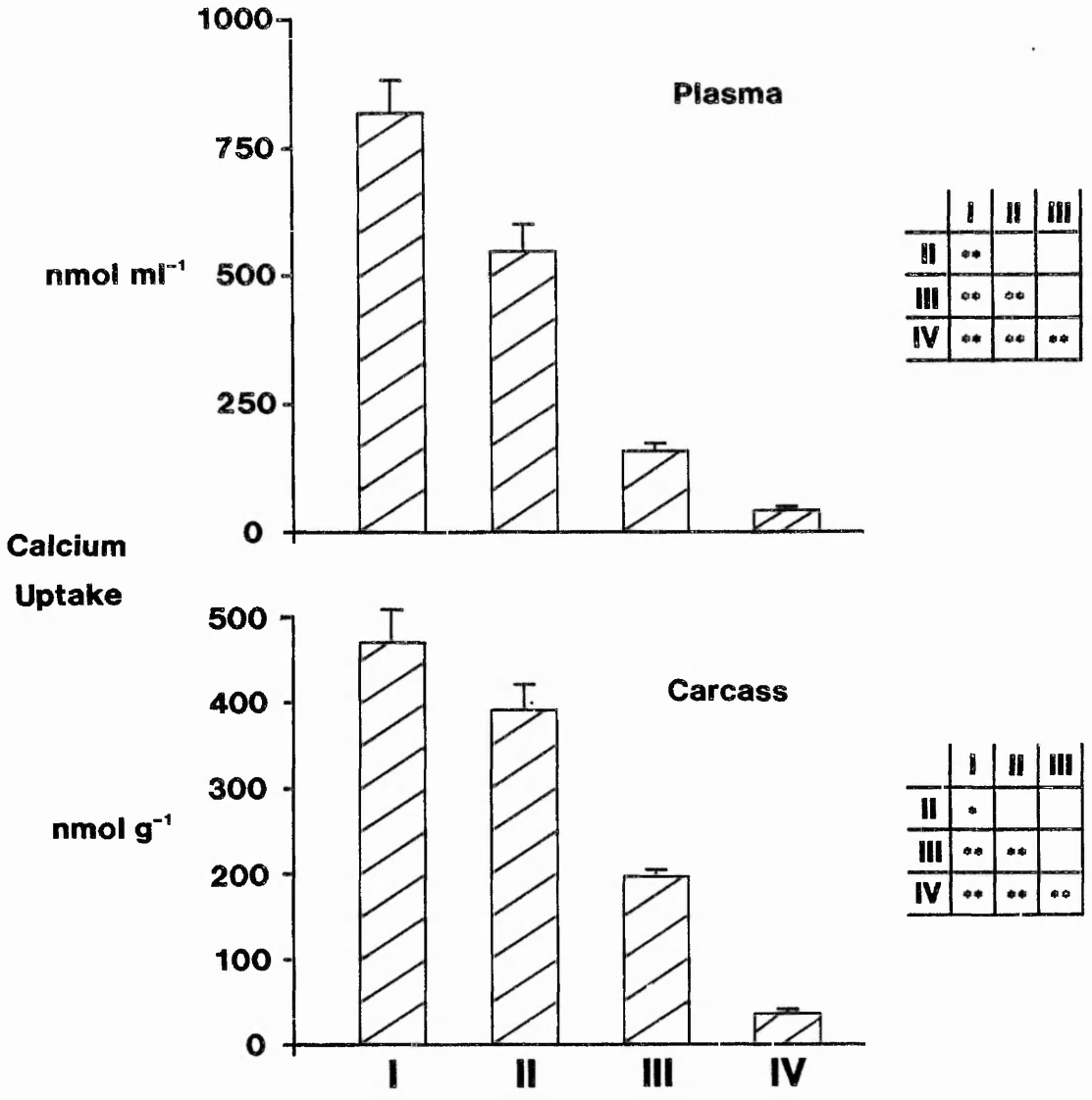
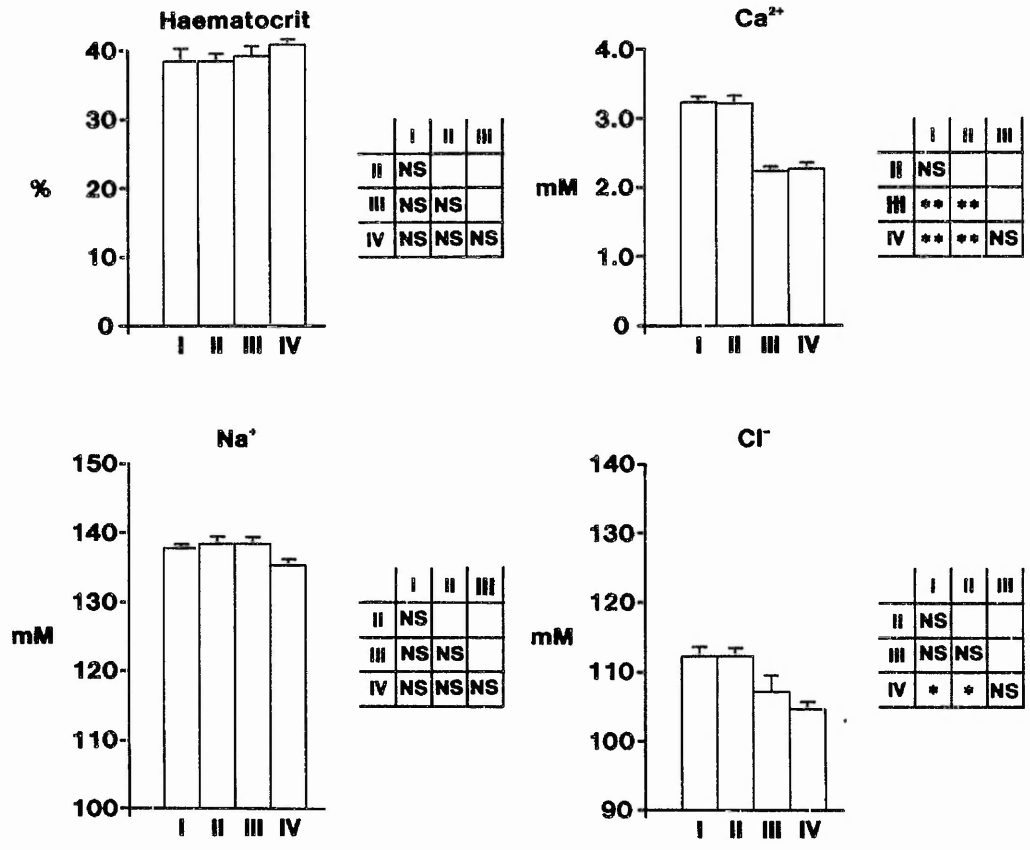

Calcium Uptake
1000
750
500
250
0
nmol ml⁻¹
Plasma
I II III
II **
III ** **
IV ** ** **
500
400
300
200
100
0
nmol g⁻¹
Carcass
I II III
II *
III ** **
IV ** ** **
I
II
III
IV

Figure 30

Haematocrit and plasma ion concentrations measured in blood samples taken from fish at the end of the ^{45}Ca flux experiments conducted at a range of external zinc concentrations. All values represent the $\bar{x} \pm se$ of 10 fish. The statistical comparisons of all possible pairs of means are also shown.

NS Not significant * $P < 0.05$ ** $P < 0.01$

Test	$[Zn^{2+}]$ (μM)
I	0.10
II	0.66
III	4.06
IV	15.43


Haematocrit
%
40
30
20
10
0
I II III IV
I II III
II NS
III NS NS
IV NS NS NS
Ca2+
mM
4.0
3.0
2.0
1.0
0
I II III IV
I II III
II NS
III ** **
IV ** ** NS
Na+
mM
150
140
130
120
110
100
I II III IV
I II III
II NS
III NS NS
IV NS NS NS
Cl-
mM
140
130
120
110
100
90
I II III IV
I II III
II NS
III NS NS
IV * * NS

significantly depressed at the two highest [Zn^{2+}].

The results of the experiment investigating the reversibility of these effects of zinc are shown in Figures 31 and 32. Zinc was again demonstrated to inhibit the influx of calcium (period I), and on this occasion influx was shown to remain inhibited immediately following the removal of zinc (period II). After a further 48 h in "clean" water however (period III), influx was significantly higher and approached levels obtained from fish of groups (III) and (V) of the first series of experiments. Normal calcium balance was therefore, more or less completely restored 48 h after the cessation of zinc exposure. Again, little effect of zinc on the efflux of calcium was seen. The calcium uptake data of Figure 32 illustrates that even though the five fish comprising groups (I) and (II) received a total of 6 h exposure to ^{45}Ca they did not accumulate as much isotope as those five fish maintained in water containing ^{45}Ca for 3 h, 48 h after being exposed to zinc.

4.3.3 The effect of lanthanum on the uptake of zinc and calcium

Addition of 10 μM lanthanum resulted in a marked change in the rate of ^{45}Ca disappearance from the media. The result from the single fish exposed to this concentration in the pilot experiment is shown in Figure

Figure 31

The influx, efflux and net flux (shaded area) of calcium during the ^{45}Ca flux experiments investigating whether the zinc-induced inhibition of calcium influx was reversible. All values represent the $\bar{x} \pm$ se of five fish.

Period I: Fluxes during exposure to 3.49 µM $[Zn^{2+}]$.

Period II: Fluxes immediately following the removal of zinc.

Period III: Fluxes 48 h after the removal of zinc.

Means of I vs II and III vs II compared by Student's t-test.

NS Not significant ** $P < 0.01$

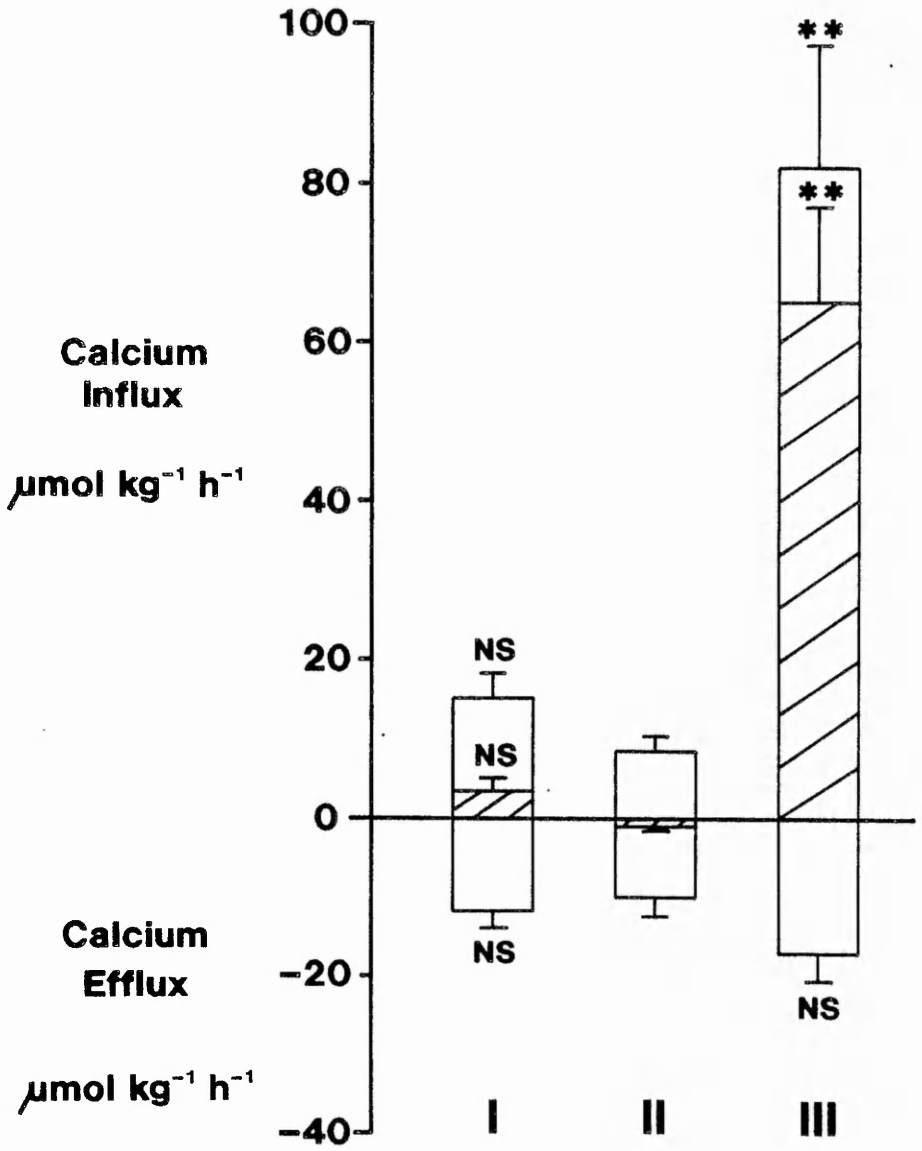
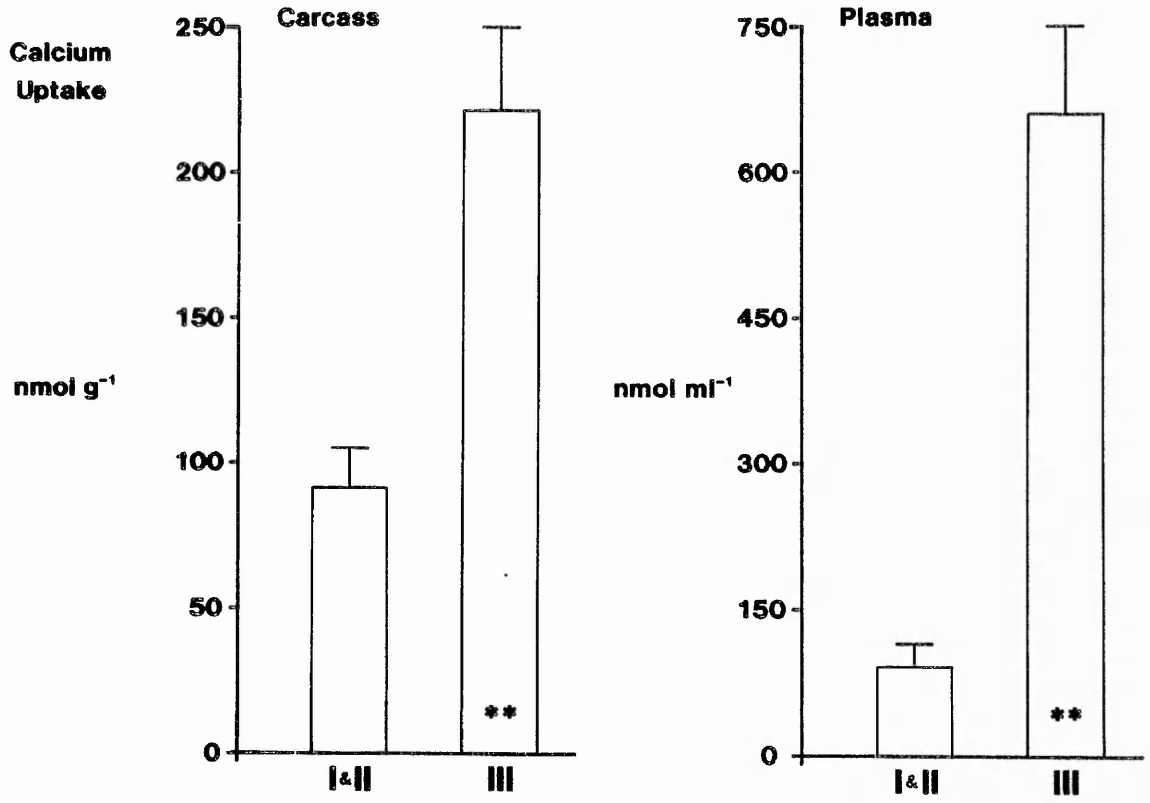

100
80
60
40
20
0
-20
-40
Calcium
Influx
µmol kg⁻¹ h⁻¹
Calcium
Efflux
µmol kg⁻¹ h⁻¹
**
**
NS
NS
NS
NS
I
II
III

Figure 32

Calcium uptake by the plasma and whole fish at the conclusion of the ^{45}Ca flux experiments investigating whether the zinc-induced inhibition of calcium influx was reversible. Groups I and II represent the same group of five fish tested initially in the presence of zinc and subsequently immediately after the removal of zinc. These fish therefore received a total of 6 h exposure to ^{45}Ca compared to the 3 h received by the fish of Group III. All values represent the $\bar{x} \pm$ se of five fish. Means were compared by Student's t-test.

** $P < 0.01$


Calcium
Uptake
nmol g⁻¹
250
200
150
100
50
0
Carcass
**
I&II
III
nmol ml⁻¹
750
600
450
300
150
0
Plasma
**
I&II
III

33. From this it is fairly clear that lanthanum caused an inhibition of calcium uptake, a change that was evident very soon after its introduction and was therefore, likely to have been more or less instantaneous in its inhibitory action. The effect of this same [La^{3+}] on the influx and subsequent tissue content of calcium and zinc are shown in Figures 34 and 35. The results for influx showed that lanthanum inhibited the uptake of both ions but with its effect on calcium being approximately twice as great as its effect on zinc. As was the case for calcium, the inhibition of zinc influx was also clear very soon after the addition of lanthanum suggesting that the inhibition was virtually instantaneous. The results of the tissue analyses reflected these trends indicating that lanthanum prevented the entry of both calcium and zinc.

4.3.4 Radiocalcium space

The results of the two experiments designed to determine the size of the radiocalcium space of brown trout are shown in Table 14. Comparing the results obtained at 10 h in these two experiments it is evident that the low external calcium acclimation procedure caused a drop in plasma [Ca^{2+}] but little or no change in the size of the calcium space. As calcium space determined at 24 h was considerably greater than that measured at 10 h it is likely that equilibrium had not

Figure 33

Disappearance of ^{45}Ca from the medium before and after the addition of 10 μM lanthanum. The result shown is that from the single fish exposed to this particular $[La^{3+}]$ during the pilot experiment undertaken to establish the $[La^{3+}]$ to be used in subsequent experiments.

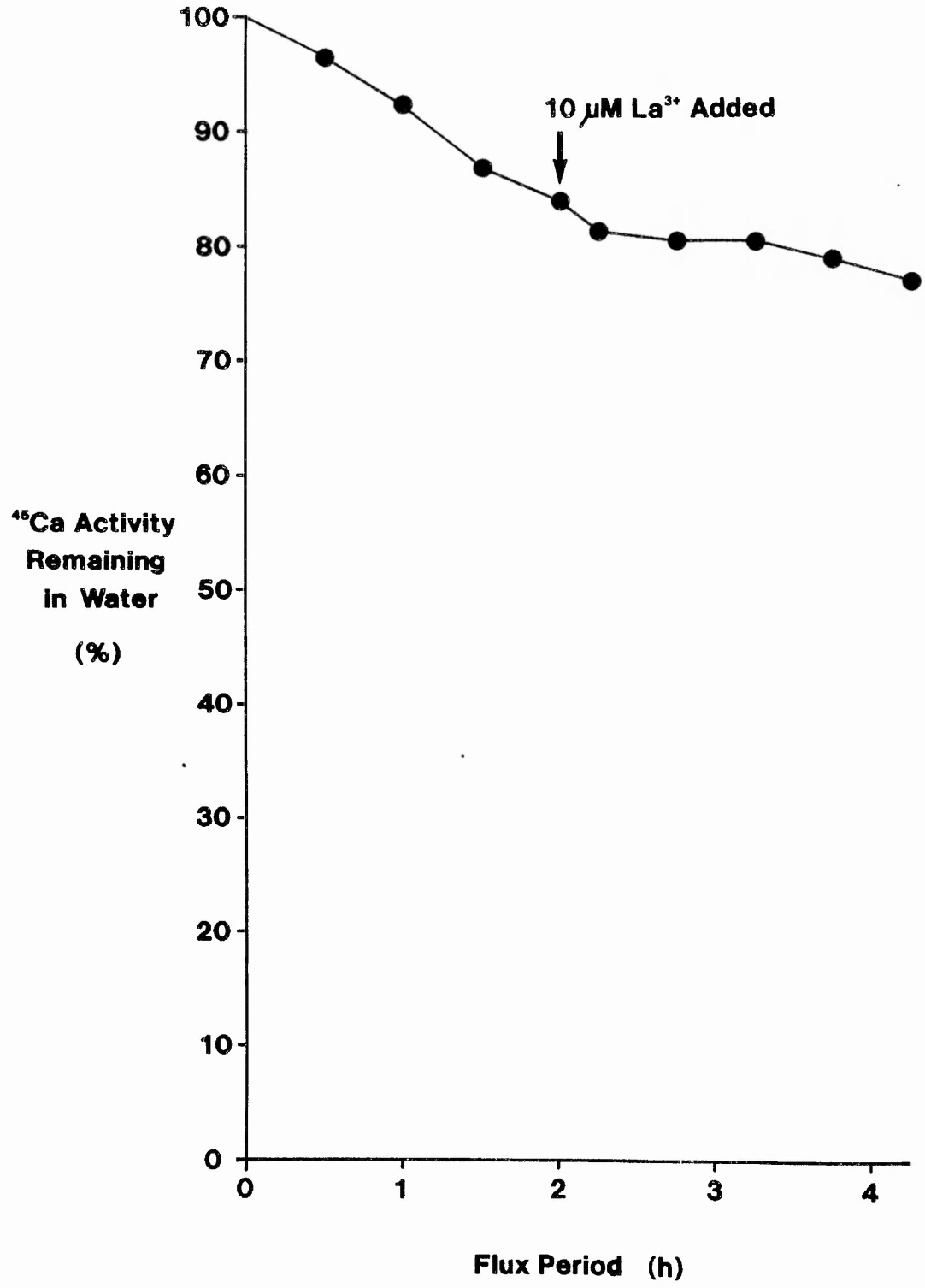
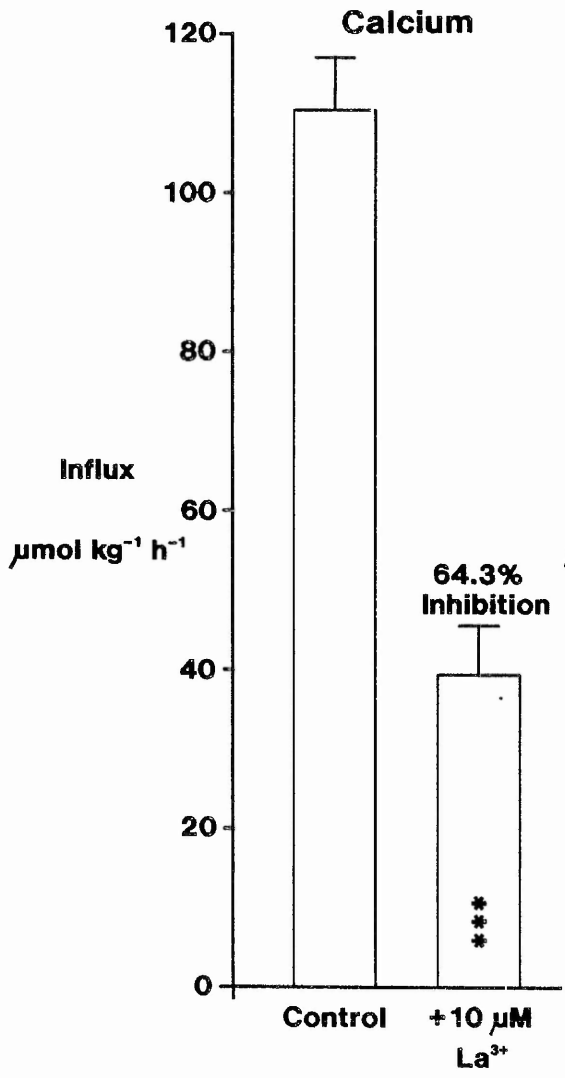

100
90
80
70
60
50
40
30
20
10
0
10 μM La3+ Added
45Ca Activity Remaining in Water (%)
0
1
2
3
4
Flux Period (h)

Figure 34

The effect of 10 μM lanthanum on the influx of calcium and zinc. All values represent the $\bar{x}$ ± se of five fish. Sample means compared by Student's t-test.

* P < 0.05 *** P < 0.01


Calcium
120
100
80
60
40
20
0
Influx
μmol kg⁻¹ h⁻¹
64.3%
Inhibition
***
Control
+10 μM
La3+

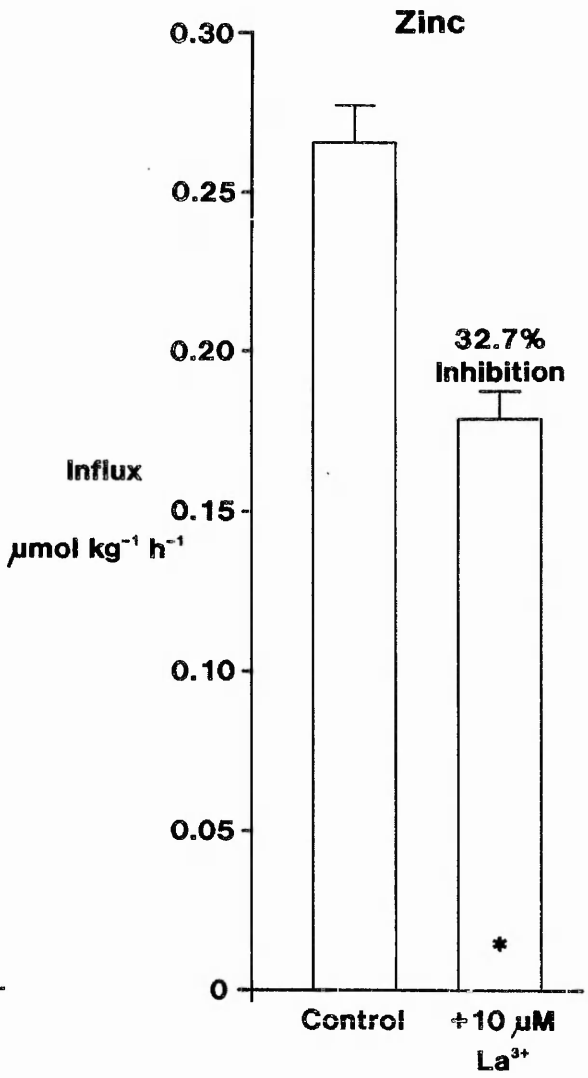

Zinc
0.30
0.25
0.20
0.15
0.10
0.05
0
Influx
μmol kg⁻¹ h⁻¹
32.7%
Inhibition
*
Control
+10 μM
La3+

Figure 35

The effect of 10 μM lanthanum on the uptake of calcium and zinc by plasma and the whole fish. All values represent the $\bar{x}$ ± se of five fish. Sample means compared by Student's t-test.

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

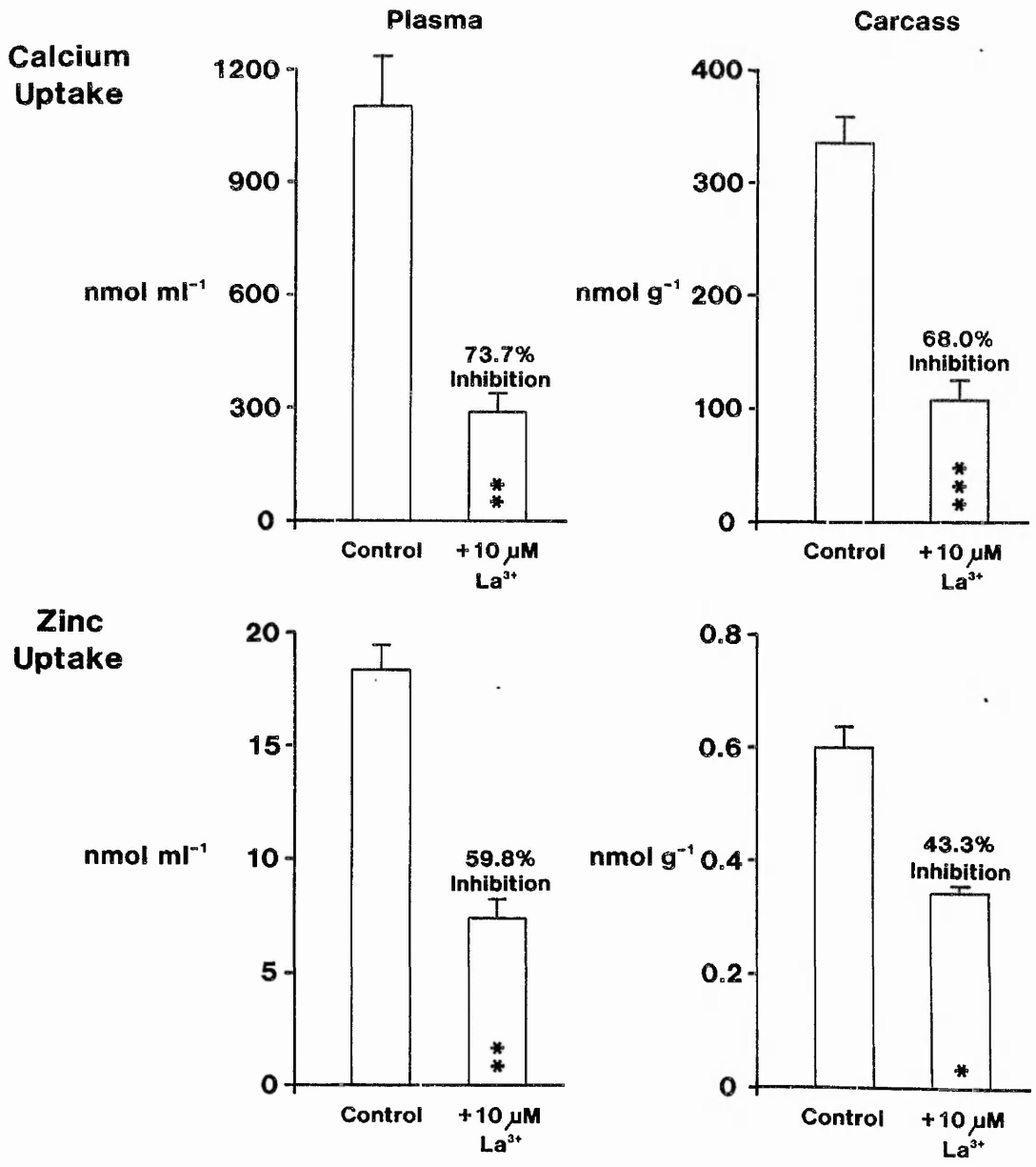

Plasma
Carcass
Calcium Uptake
nmol ml⁻¹
nmol g⁻¹
1200
900
600
300
0
400
300
200
100
0
73.7% Inhibition
68.0% Inhibition
Control
+10 μM La³⁺
Zinc Uptake
20
15
10
5
0
0.8
0.6
0.4
0.2
59.8% Inhibition
43.3% Inhibition

Table 14

Plasma $[Ca^{2+}]$ and calcium spaces determined at 10 and 24 h (first experiment) on fish long-term acclimated to ambient water conditions, and 10 h only (second experiment) on fish acclimated and tested in water of low calcium content.

All values are $\bar{x} \pm$ se

	Plasma $[Ca^{2+}]$ (mM)	Calcium space (ml kg^{-1})
1st Expt.(10 h)(n=4)	---	2418 ± 114
1st Expt.(24 h)(n=4)	2.58 ± 0.06	3957 ± 337
2nd Expt.(10 h)(n=5)	1.88 ± 0.07	2248 ± 62

been fully reached at 10 h, the process of ^{45}Ca redistribution throughout the calcium space from the site of injection still being in progress. The lack of any measurements beyond 24 h means that it cannot be stated with any certainty whether or not equilibrium had been reached at 24 h. For purely comparative purposes an attempt was made to calculate the whole body exchangeable calcium content from the data in Table 14. Using the calcium spaces determined at 10 h and plasma $[Ca^{2+}]$ at 24 h (first experiment) and 10 h (second experiment) approximate values of 6240 and 4220 mmol kg^{-1} were obtained, suggesting that associated with the acclimation to low external $[Ca^{2+}]$ was a considerable reduction in the exchangeable calcium pool of the fish.

4.4 DISCUSSION

4.4.1 Calculation of calcium fluxes

The use of the same equation for the calculation of calcium influx as that used in the calculation of zinc influx meant that the same assumptions already discussed for the latter were applicable in the calculation of the former. Thus, it was assumed that ^{45}Ca disappearance from the media was fully accounted for by that appearing in the fish, and, that the backflux of isotope from the fish to the media remained negligible throughout the duration of the experiment. Regarding the latter point, it was stated in chapter 3 that in order to measure backflux an idea of the size of the exchangeable pool of the ion in question is required. One of the parameters necessary in order to measure this is the so-called equilibrium radiospace, the apparent volume occupied by the exchangeable pool of the ion in question when uniformly distributed at the same concentration as that of plasma (Mayer and Nibelle, 1969). Thus, from a knowledge of plasma $[Ca^{2+}]$ and equilibrium calcium space it is possible to gain an estimate of the size of the exchangeable calcium pool. Reader (1986) however, has argued that measurements of calcium space are unsuitable for use in the calculation of whole body exchangeable calcium content, basing his argument on the results of a

number of studies that have shown that the uptake of calcium is not confined to the gills but additionally occurs in significant quantities over the body surface in general, particularly the fins and scales (Mashiko and Jozuka, 1964; Rodgers, 1984; reviews: Simmons, 1971; Dacke, 1979). In particular, the results of a study on rainbow trout (Perry and Wood, 1985) suggested that as much as 50% of calcium uptake normally occurs through the skin. A net uptake of calcium was also demonstrated in air-breathing mudskippers (Periophthalmodon schlosseri) and marble gobies (Oxyeleotris marmorata) where the possibility of branchial uptake had been abolished by holding them on damp filter paper (Fenwick and Lam, 1988). In support of this extra-branchial uptake of calcium Flik et al. (1984a) have reported the presence of the calcium binding protein calmodulin in the mucus of the body surface of tilapia and have demonstrated that the concentration of calmodulin is inversely related to external $[Ca^{2+}]$. Reader argued that, at least during short-term experiments, an extra-branchial influx of calcium was unlikely to exert a significant effect on the calcium dynamics of the blood and, as a result, injected radioisotope is likely to considerably under-estimate the size of the exchangeable calcium pool. It was not a primary aim of the present work to determine accurately the size of this pool however. Instead, an approximate measure was required to allow an estimate of backflux to

be made. Bearing in mind Reader's argument that exchangeable calcium determined from measurements of calcium space is likely to be significantly under-estimated, if it could be demonstrated that backflux calculated using this figure was insignificant then, if exchangeable calcium was under-estimated, backflux would only be further reduced if the true figure had been used.

Hobe et al. (1984a) measured calcium spaces in rainbow trout fitted with dorsal aortic cannulae through which ^{45}Ca was introduced directly into the bloodstream. Using this technique it was found that the calcium space was apparently filled to equilibrium by 10 h post-injection as no further change was evident at 24 h. A figure of approximately 1850 ml kg^{-1} was obtained, with calcium space not being affected by acclimation of fish to a wide range of external $[Ca^{2+}]$. In the experiments of this study calcium spaces of 2350 ml kg^{-1} were obtained after 10 h. Again these were relatively unaffected by the acclimation history of the fish. Thus, the results from these two studies were reasonably similar. Unlike the results of Hobe and co-workers however, equilibrium had apparently not been reached by 10 h in this study. It is to be expected that an intra-peritoneal injection of isotope would take longer to distribute to equilibrium than isotope infused directly into the bloodstream. The considerably greater calcium space measured after 24 h in

this study compared to that at 24 h measured by Hobe and co-workers is difficult to explain however. Inter-specific differences are unlikely to account for all of the observed variation. Further work is obviously required examining the effect of varying the route of isotope introduction on the subsequent measurement of calcium space.

For the purposes of the backflux calculation it was decided to err on the side of caution and use the calcium space determined at 10 h as this produced the lower estimate of exchangeable calcium. Backflux was estimated according to Maetz (1956) whereby internal and external specific activities at the end of the experiment were compared. In this way it was calculated that internal specific activity was never greater than 5% of external specific activity, an indication that backflux was likely to be insignificant.

The second assumption necessary when using the influx equation of Kirschner (1970) is that isotope disappearance from the media fully represents that entering the fish. Adsorption to the apparatus itself was shown to be negligible, a similar result to that found by Reader (1986) but one in marked contrast to that of Hobe et al. (1984a) who, using "Perspex" as the flux apparatus construction material, noted the adsorption of considerable quantities of ^{45}Ca to the apparatus. Flik et al. (1985a) however, also used "Perspex" as the

construction material and reported no self-adsorption, a result highlighting the need to perform adequate control experiments in studies of this type, a factor often overlooked in earlier work using radioisotopes.

Regarding the possible adsorption of isotope onto the surface of the fish unaccompanied by subsequent entry into the fish, Hobe et al. (1984a) obtained figures for influx approximately 2.5 times higher when measured from the disappearance of isotope from the water than when measured indirectly from efflux and net flux calculated following the injection of isotope into the blood. They proposed that a considerable quantity of ^{45}Ca was simply adsorbed to the surface of the fish and did not subsequently enter the animal, so resulting in the considerable over-estimation of influx when calculated from the disappearance of ^{45}Ca from the water. As discussed earlier however, a number of studies have demonstrated the extra-branchial uptake of calcium. It was considered therefore, that the measurement of calcium influx in the present study through the monitoring of isotope disappearance from the water was a valid technique, although the possibility existed that influx calculated in this way would be somewhat greater than true values. Through the measurement of plasma ^{45}Ca levels it was considered that the true trends of calcium uptake under a variety of external conditions could be determined. In the event it was obvious that these trends

confirmed those reported for influx, thereby providing further evidence that the latter were real rather than an artifact of the experimental design.

4.4.2 Normal calcium balance

Calcium flux rates, particularly in relation to external [Ca^{2+}], have been the subject of considerable attention in recent years. Published data of flux rates cover a wide range of values with those obtained in this work tending towards the upper end of this range. All rates reported below are in μmol kg^{-1} h^{-1}. Reader (1986) worked on brown trout alevins and typically obtained values for influx of less than 40. Similarly, most other published data for calcium influx, both for salmonids (Hobe et al., 1984a; Perry and Wood, 1985; Wagner and Copp, 1985) and other freshwater species (Berg, 1968; Fleming 1973; Flik et al., 1985a) lie below 40.

Rates of influx obtained in the absence of zinc in this study ranged from 27 to 122, the former obtained from fish acclimated and tested in water of low external [Ca^{2+}] (0.02 mM), and the latter from fish acclimated to this low [Ca^{2+}] but tested in one considerably higher (0.25 mM). Despite the majority of published data being lower than these figures comparable data are available. Ichii and Mugiya (1983) cite a rate of 149 from unpublished data on C. auratus. Mayer-Gostan et al.

(1983) measured rates as high as 130 in F. heteroclitus, whilst in this same species a rate of 190 was reported by Pang et al. (1980). Flik et al. (1986a) obtained a maximum rate of 116 by linear extrapolation of their data for 10-30 g tilapia to a fish of weight 1 kg. Flik and co-workers state however that the linear extrapolation of data, particularly for fish of small size, is likely to result in an over-estimate of true flux rates as they found that calcium flux rates were not necessarily directly related to body weight. It is possible therefore, that the rates measured in the work presented here are also somewhat over-estimated though probably to a lesser extent than those reported by Flik and co-workers as the fish used in their work were substantially smaller than those used in this study.

In contrast to the relatively high rates of influx measured in this work, rates of efflux were generally low, irrespective of treatment. Extremes of 13 and 34 were measured in the absence of zinc, rates that are comparable with much of the published data; 10-26 in brown trout alevins (Reader, 1986), 3-11 in rainbow trout (Hobe et al., 1984a), and 8-16 in tilapia (Flik et al., 1985a, 1986a). These low rates of efflux relative to influx, meant that all fish were in a state of net positive calcium balance, a situation typical of that found in fish undergoing rapid growth, where dietary calcium deficiency can be compensated by increased uptake

from the water (Ichii and Mugiya, 1983).

Irrespective of the possible errors discussed earlier regarding the calculation of influx, it was considered that the most important conclusions to be drawn from these ^{45}Ca flux experiments would be gained from the overall trends observed rather than the actual flux values themselves.

Statistical analysis of the flux data suggested that the one week acclimation period in low calcium stimulated the calcium uptake mechanism of the fish resulting in a greater influx of calcium relative to test $[Ca^{2+}]$ acclimated fish when the low $[Ca^{2+}]$ acclimated fish were subsequently placed in media of elevated $[Ca^{2+}]$. In those experiments where fish were acclimated to water of the $[Ca^{2+}]$ used in the subsequent test influx rates were similar at the two higher concentrations (0.25 and 0.50 mM) but were significantly lower at the lowest concentration (0.02 mM), although even at this low concentration net calcium uptake persisted.

A number of workers have demonstrated an enhancement of calcium uptake when low $[Ca^{2+}]$ acclimated fish were subsequently exposed to elevated external $[Ca^{2+}]$ (Pang et al., 1980; Mayer-Gostan et al., 1983; Perry and Wood, 1985). The rainbow trout of Perry and Wood's work exhibited this effect after only one day in low calcium. From kinetic studies on perfused heads the authors showed that the acute (1-day) response to low calcium involved

an increase in the maximal rate of calcium uptake (J_{max}) associated with an increase in the affinity of the calcium uptake mechanism (decreased K_m). After one week however, the K_m had returned to control levels but the J_{max} remained elevated, gradually returning towards control levels but still remaining elevated after 30 days. It was speculated that the 1-day response arose from the modification of pre-existing calcium transport sites and/or an increase in membrane pemeability, possibly mediated by hormone surges or the loss of membrane-bound calcium during low calcium acclimation, such as has been documented following acute exposure to low pH (McWilliams, 1983). In the medium-term it was thought that J_{max} remained elevated due to an increase in Ca^{2+}-ATPase activity, possibly resulting from the proliferation of lamellar chloride cells that was observed, chloride cells having been implicated in the mechanisms of calcium uptake by a number of workers (Payan et al., 1981; Flik et al., 1984b, 1985b; Ishihara and Mugiya, 1987). Chloride cell proliferation in rainbow and brown trout held in soft water was also demonstrated by Laurent et al. (1985), the response being one primarily due to a reduction in ambient [NaCl] rather than a reduction in $[Ca^{2+}]$. The similar response observed by Perry and Wood (1985) occurred in the absence of changes in [NaCl], a discrepancy in results that is worthy of further study. Irrespective of the mechanism by

which J_{max} was elevated, it was considered that ultimately it returned to control levels. The long-term response to reduced external $[Ca^{2+}]$ was thought to involve other factors such as efflux modulation.

In the light of this evidence therefore, it seems likely that the acclimatory response to low calcium seen in this study was similar to the medium-term response noted by Perry and Wood. Interestingly, influx rates measured at 0.25 and 0.50 mM calcium in this study were not significantly different from each other. A similar response was observed in F. heteroclitus (Mayer-Gostan et al., 1983), calcium uptake in low calcium acclimated fish showing a very marked increase at 0.05 mM and then levelling off, such that influx at 0.25 and 0.50 mM (and indeed at much higher concentrations) remained unchanged. This result was interpreted as indicating either a rapid saturation of active transport mechanisms induced during acclimation to low calcium (transport was believed to be passive under normal conditions) or, a rapid modification of low calcium induced membrane permeability changes. Recent work has suggested that calcium uptake in fish is an active process, the initial step being passive diffusion across the apical membrane. Low intracellular $[Ca^{2+}]$ (Godfraind-DeBecker and Godfraind, 1980) is thought to facilitate this diffusion down an electrochemical gradient (Flik et al., 1985b). Calcium is subsequently bound to proteins in the cytosol, calmodulin

being strongly implicated in this role (Flik et al., 1984b, 1985b,c; review: Moore and Dedman, 1982). The ultimate step probably involves the active transport of calcium across the basolateral membrane, high affinity Ca^{2+}-ATPases located in the basolateral membrane of the chloride cells probably providing the driving force for this process (Perry and Flik, 1988).

It is likely therefore, that the increased influx of calcium observed in this and other studies following acclimation to low external $[Ca^{2+}]$ reflects an increased ability of the fish to take up calcium, the mechanism for this probably involving the hormonal stimulation of the calcium uptake mechanism either directly, through the stimulation of a calcium pump in the gills (Flik et al., 1984c), or indirectly through chloride cell proliferation (Perry and Wood, 1985; Flik et al., 1986b). The fact that influx rates measured at 0.25 and 0.50 mM in this study were similar would tend to suggest that the calcium active transport mechanism had become saturated at these concentrations.

In comparison to the marked effect of the low calcium acclimation process on the influx of calcium, that on efflux was small, though there was some suggestion of a possible stimulation of efflux under the same conditions. Calcium efflux is thought to largely follow a paracellular route (Perry and Flik, 1988). The increased rates of efflux observed may therefore, simply

have reflected an increase in branchial permeability following the low calcium treatment such as has been observed in the many studies examining ion and water exchange in relation to external [Ca^{2+}] that were discussed in chapter 2.

Published data concerning plasma [Ca^{2+}] in relation to external [Ca^{2+}] are conflicting. After 5 days exposure to 0.2 mM calcium tilapia were found to be hypocalcaemic relative to fish held in 0.8 mM calcium (Wendelaar Bonga *et al.*, 1984), though after 10 weeks at 0.2 mM these fish were hypercalcaemic relative to the others (Wendelaar Bonga *et al.*, 1985; Flik *et al.*, 1986a). Hobe *et al.* (1984a) also noted a hypocalcaemic response to low calcium, this time after a 10-14 day acclimation period. In contrast however, neither Umehara and Oguri (1978), Pang *et al.* (1980), Parker *et al.* (1985) nor Perry and Wood (1985) noted any disturbance of plasma calcium levels following acclimation to a range of [Ca^{2+}]. In the work presented here also, plasma [Ca^{2+}] was unaffected by low calcium acclimation. When these fish were subsequently exposed to elevated external [Ca^{2+}] however, the increased rates of calcium influx measured in these fish were reflected by a marked elevation of plasma [Ca^{2+}]. It was thought that this hypercalcaemia was of a transient nature though, as it was not evident in those fish acclimated to the same test [Ca^{2+}] for the week before experimentation.

Disturbances in both plasma sodium and chloride balance were also evident after one weeks' acclimation to low external [Ca^{2+}]. Sodium in particular was depressed in these fish, the effect on chloride being less clear. As discussed in chapter 2 calcium is known to exert a considerable influence on the permeability of the gill epithelium to ions. Perry and Wood (1985) reported a similar reduction in plasma [Na^{+}] and [Cl^{-}] one day after the start of acclimation to low calcium. This response was corrected at 7 days but had reappeared after 15 days. In contrast however, net fluxes of sodium and chloride were found not to be affected following 10-12 day acclimation to a wide range of external [Ca^{2+}] (McDonald et al., 1983). It is considered likely that the disturbances in sodium and chloride balance noted here were the result of short-term membrane permeability changes. The results of McDonald and Rogano (1986) are worthy of mention at this point as they found that fish held in water of low [Ca^{2+}] but normal [NaCl] did not exhibit the depression of plasma sodium and chloride observed when both external [Ca^{2+}] and [NaCl] were reduced. Indeed, under these conditions the influx of the two monovalent ions was stimulated, a result interpreted as evidence of a controlling function of calcium on the access of sodium to its transport mechanism. As already mentioned the reduction of ambient [Ca^{2+}] without an associated reduction of [NaCl] in this work resulted in

the disturbance of sodium and chloride balance, a very different result to that obtained by McDonald and Rogano (1986). It is possible that differences in water pH between these two studies were sufficient to account for the observed variation in results. All experiments in this work were completed at a pH of 6, whereas those of McDonald and Rogano (1986) were all carried out at pH 7.5. Reduction of ambient pH, though most commonly of greater magnitude than that employed here, has frequently been demonstrated to disrupt normal sodium and chloride balance. Thus, following the transfer of brown trout from neutral media to water of pH 6 a substantial reduction in sodium uptake was apparent which, after 7 days (the same time as the acclimation period employed here), had resulted in the depression of both plasma [Na^+] and [Cl^-] by 15 and 20% respectively (McWilliams, 1980). In a second study (McWilliams, 1982) the addition of calcium to media of pH 5.5 and 6.5 substantially reduced the efflux of sodium. The results of these two papers may therefore, be sufficient to explain the observed discrepancies between this work and that of McDonald and Rogano (1986) and serve to emphasise the need to report all major water quality parameters in work of this type.

4.4.3 The effect of zinc on calcium balance

The results of those experiments investigating the

turnover of calcium in the presence of a range of external zinc concentrations revealed a strong dose-dependent inhibition of calcium influx by zinc with no effect on the efflux of calcium. The inhibition of influx was such that at an external [Zn^{2+}] of 4 μM the net uptake of calcium was almost completely abolished, and at 15 μM the fish were in a state of negative calcium balance. These disturbances of influx were reflected by disturbances in plasma [Ca^{2+}], the fish at the two highest zinc concentrations being hypocalcaemic relative to those tested at the two lowest concentrations. Furthermore, these disturbances remained apparent immediately after the removal of zinc from the media, although after a further 48 h in "clean" water, influx had recovered to near-normal levels. Thus, these results supported the proposal put forward in chapter 3 that at sublethal concentrations the toxicity of zinc is intimately associated with the disruption of normal calcium balance.

The results of those experiments completed in this study using the calcium-channel blocker lanthanum were of particular interest. Before discussing the actual results themselves however, it is perhaps worth mentioning recent work undertaken concerning the possible mode of action of lanthanum. Electron microscopy has shown that lanthanum does not enter the cells of the branchial epithelium but rather, accumulates as electron dense deposits on the

apical surface of the membrane, particularly at the chloride cells (Perry and Flik, 1988). Eddy and Bath (1979) proposed that due to its replacement of calcium, a known stabiliser of membranes, lanthanum would cause considerable structural damage to fish gills and explained in this way the large efflux of sodium and chloride which they observed following the addition of 2 mM La^{3+}. Perry and Flik (1988) however, observed no penetration of lanthanum (0.2 mM) into the tight junctions between epithelial cells. In addition, the efflux of calcium was not affected by lanthanum. On this evidence therefore, it is likely that lanthanum does not exert a significant effect on the integrity of the branchial epithelium particularly when used at relatively low concentrations such as 10 μM (this study) or 1 μM (Verbost et al., 1987).

The instantaneous inhibition of calcium influx by lanthanum observed in this and other studies therefore, is considered to indicate that La^{3+} inhibits the permeation of calcium through the apical membrane. The fact that the influx of both cadmium (Verbost et al., 1987) and zinc (this study) was also instantaneously inhibited by lanthanum is considered as evidence that these metals enter the epithelium through the same La^{3+}-sensitive calcium channels. Supporting evidence for this is unfortunately lacking with regard to zinc, though for cadmium some is available. Although calcium

transporting channels are considered to be highly specific for calcium (Hess and Tsien, 1984) it has been shown in isolated amphibian atrial cells that cadmium is a potent blocker of them (Giles et al., 1983). In the work of Verbost and co-workers cadmium was found not to influence the accumulation of calcium in the gill, only its subsequent extrusion from the epithelial cells into the bloodstream, a finding that was taken to indicate that although both cadmium and calcium entered the cell through the same calcium-channel, they were not in competition for uptake sites at the apical membrane. Similarly, Part et al. (1985) demonstrated that the entry of cadmium into the gill was not affected by external $[Ca^{2+}]$. The ^{45}Ca content of the gill tissue in relation to external $[Zn^{2+}]$ was unfortunately not investigated in the work presented here so it is not possible to state with certainty whether zinc also penetrates the apical membrane without affecting the intracellular accumulation of calcium.

Although lanthanum inhibited the influx of both calcium and zinc its effect on the former was greater than that on the latter. Indeed, the inhibition of calcium influx was almost twice as great as that of zinc. A similar phenomenon was noted by Verbost et al. (1987) with regard to the accumulation of cadmium and calcium in the perfused gill tissue of rainbow trout. They interpreted their results in terms of the results of a

previous study which had demonstrated that cadmium was bound more strongly than calcium by gill-surface mucoproteins (Part and Lock, 1983). Thus, the stronger binding of cadmium meant that less was rinsed off of the gill surface during the rinse prior to the measurement of radioactivity. Cadmium accumulation was therefore, apparently greater than calcium accumulation. The study of Part and Lock (1983) concerned only the binding of calcium, cadmium and mercury to mucoproteins. It is not possible therefore, to say for certain whether zinc is bound by mucoproteins in a similarly strong manner to that of cadmium and mercury. The comparative influxes of zinc and calcium noted in the presence of lanthanum in this study would tend to suggest that this was indeed the case however. Indeed, the data concerning the appearance of ^{65}Zn and ^{45}Ca in the plasma showed only a relatively small (59.8% and 73.7%) difference in the percentage inhibition of zinc and calcium uptake by lanthanum, an indication that surface adsorbtion phenomena were probably supplementing the true influx of zinc.

Once in the cell, calcium is considered to bind to specific proteins, calmodulin, as discussed earlier, being strongly implicated in this role. In the work of Verbost *et al*. (1987) the inhibition of calcium influx by cadmium was only apparent following a 16 h pre-exposure to a $[Cd^{2+}]$ greater than 0.01 μM. A concentration as high as 1 μM did not induce an immediate inhibition of influx

such as was observed at a [Zn^{2+}] of 0.7 μM in this study. Verbost and co-workers interpreted their results as evidence of an initial buffering of cadmium by cytosolic proteins, the inhibition of calcium uptake only occurring on the exhaustion of this buffering capacity. Binding of cadmium to proteins such as calmodulin has been demonstrated in a number of studies (Chao et al., 1985; Richardt et al., 1986; Flik et al., 1987). Chao et al. (1984) demonstrated the binding of a number of metals, including zinc, to calmodulin and attributed the relative ability of these metals to substitute for calcium to their ionic radius, such that the closer the radius was to that of calcium (0.99 Å) the greater was that metals ability to substitute. Thus, cadmium (0.97 Å) was found to be more effective than zinc (0.74 Å), manganese (0.80 Å) being of intermediate potency. Manganese was also found to be of lower potency than cadmium by Richardt et al. (1986), zinc unfortunately not being investigated in this particular study. Brewer et al. (1979) suggested that subcellular zinc toxicity may be attributed to the inhibition of calmodulin, though Chao et al. (1984) demonstrated inhibition only at high concentrations, zinc being stimulatory at lower ones.

The evidence available from the literature therefore, tends to suggest that zinc, cadmium and a number of other heavy metals are able to substitute for calcium on intracellular binding proteins such as

calmodulin. It is likely though that zinc, possibly due to its smaller ionic radius, is less able to do this than cadmium. This differing ability may explain the observed differences in the time course of calcium influx inhibition between these two metals, 16 h pre-exposure being required for cadmium to exert its effect, whereas that of zinc is very rapidly exerted following the introduction of the metal to the medium. It does seem unlikely though that differences in intracellular protein binding capability can fully explain these differences. Further work is obviously required, particularly using biochemical techniques in combination with *in vitro* physiological techniques such as the perfused head preparation.

The zinc-induced inhibition of calcium influx remained apparent for at least 3 h following the removal of zinc from the medium, though after a further 48 h it had virtually disappeared. Few comparative data are available concerning the potential of unidirectional or net calcium fluxes for recovery either during or after metal exposure. Spry and Wood (1985) obtained results from two fish suggesting a possible potential for recovery of normal calcium balance actually during exposure of rainbow trout to 12.3 μM (0.8 mg l^{-1}) zinc for a number of days. Reid and McDonald (1988) demonstrated that disturbances of calcium influx by cadmium remained apparent, though at a reduced rate, 12 h

after the removal of cadmium from the medium. From these few data therefore, it can be tentatively proposed that trace metal induced disturbances of calcium balance are relatively short-lived following the removal of the metal from the medium, and also show some potential for recovery during exposure to it. It may be that the detoxification mechanisms discussed in chapter 3 are important in these processes though more work is required though before any firm proposals can be put forward.

As briefly discussed earlier, high-affinity, calmodulin-dependent Ca^{2+}-ATPases located on the basolateral membrane are believed to provide the mechanism for the translocation of calcium from the cytosol to the blood (Flik et al., 1985b,c; Perry and Flik, 1988). Verbost et al. (1987) believed that once the cadmium buffering capacity of the cytosol was exceeded cadmium was able to inhibit the basolateral calcium pump. Little direct evidence is available to confirm or deny this however. Shephard and Simkiss (1978) demonstrated the inhibition of Ca^{2+}-ATPase extracts by a number of heavy metals. Similarly, Bansal et al. (1985) demonstrated cadmium-induced inhibition of a Ca^{2+}-ATPase isolated from the gills and heart of the freshwater teleost Saccobranchus fossilis but only at the very high concentration of 500 μM. Reid and McDonald (1988) have suggested that the particular ATPase investigated by Bansal and co-workers may have had a considerable bearing

on these results as Flik and co-workers observed both high and low affinity Ca^{2+}-ATPases in the gills of tilapia, only the former being implicated in the role of a transmembrane calcium transporter. In addition, Flik et al. (1983) have questioned the validity of many earlier reports describing teleost gill Ca^{2+}-ATPase activities. Their data obtained from the same assay protocol of these earlier studies indicated that the ATPase activities described did not represent specific high affinity Ca^{2+}-ATPases but rather, non-specific phophatase activities. On the basis of in vivo kinetic studies Reader (1986) tentatively proposed that the effect of cadmium on calcium balance involved competitive inhibition of the calcium uptake mechanism. He too stated that further in vitro studies are required to clarify the situation though.

Data concerning Ca^{2+}-ATPase activity in the presence of zinc are unfortunately not available. Until they are it is not possible to do more than speculate on whether sublethal concentrations of zinc even wholly or partly exert their toxic action through the inhibition of this probable transmembrane calcium transporter. From the results presented in this work however, it would seem to be a potentially interesting area for further research.

Turning now to the efflux of calcium, zinc was found to have no effect on this parameter, even at the relatively high concentration of 15.4 µM. This result is

in general agreement with much of the literature regarding the mode of action of cadmium (Reader, 1986; Verbost et al., 1987; Reid and McDonald, 1988), providing further evidence of a possible common mode of action of these two metals. Manganese too has been shown to influence calcium balance in a similar manner (Reader, 1986) so it may eventually prove to be possible to construct a model applicable to a number of trace metals detailing the basic mechanism of toxicity, at least at sublethal concentrations.

The results obtained for plasma sodium and chloride concentrations following zinc exposure however, highlight the difficulties inherent in the construction of such a general model. It was proposed in chapter 3 that any zinc induced disturbances in normal sodium and chloride balance were likely to be of secondary importance to the large disturbances observed in calcium balance. Similarly, the work presented in this chapter revealed no effect of zinc on plasma $[Na^+]$ and only a relatively minor disturbance of plasma $[Cl^-]$ at the highest zinc concentration. The small disturbances in sodium and chloride balance noted by Spry and Wood (1985) for zinc, Reader (1986) for cadmium and manganese, and Reid and McDonald (1988) for cadmium have largely been attributed to the modification of membrane permeability by the metal under study. Copper on the other hand, has frequently been shown to exert its toxic action primarily through a

major disturbance of sodium balance (Lauren and McDonald, 1985, 1986; Stagg and Shuttleworth, 1982). This disturbance is thought to arise from the inhibition of branchial Na^+/ K^+-ATPase (Lauren and McDonald, 1987b), the inhibition possibly being of a non-competitive nature due to structural alteration of the ATPase by copper (Lauren and McDonald, 1987a). Calcium balance however, is only slightly affected by copper (Reid and McDonald, 1988). Aluminium also exerts a considerable influence on sodium balance with little effect on calcium balance (Dalziel et al., 1986; Reader et al., 1988). The reason why these large variations in the toxic action of different metals exist is still very uncertain. It is quite likely that relatively small differences in the chemistry of these metals account for the observed differences in their toxic effects (review: McDonald et al., 1986).

5 GENERAL DISCUSSION

The acute toxicity of zinc to brown trout was shown to be much lower in hard water relative to that in an artificially softened water. This differed from the hard water mainly in its elevated sodium concentration and very low concentrations of the divalent hardness ions calcium and magnesium. The modification of toxicity was attributed to the altered calcium and magnesium concentrations rather than to the altered sodium concentration. The composition of the medium during the acclimation period that preceded exposure to zinc was demonstrated to have little influence on the subsequent toxicity of zinc, the hardness regime during zinc exposure largely determining the toxicity of the metal.

Specific investigation of calcium at the same acutely toxic zinc concentration revealed a progressive reduction of toxicity with increasing external $[Ca^{2+}]$. As with increasing water hardness, the large reduction in the toxicity of zinc with increased external $[Ca^{2+}]$ remained evident even though the fish were acclimated to water of very low divalent ion content.

Using ^{65}Zn it was shown that external calcium and magnesium exerted a profound influence on the uptake of zinc from the surrounding medium, the influx of zinc being greatly reduced by increased external divalent ion concentration. In this respect magnesium was shown to

have less influence than calcium, the latter exerting greatest effect at a concentration of 0.15 mM. Acclimation to elevated external [Ca^{2+}] before zinc exposure had relatively little influence on the uptake of the metal, ambient [Ca^{2+}] during zinc exposure being the major determinant of the rate of influx.

Two possible mechanisms may explain the modification of zinc uptake by calcium and magnesium: membrane permeability changes occuring rapidly on transfer of fish between waters of differing hardness; and competition between the divalent ions for uptake sites at the gill. In this study it was shown that calcium exerts a profound influence on the uptake of zinc by the fish. Similarly, in other studies calcium has been shown to modify membrane permeability in a manner similar to that observed here for the uptake of zinc. Furthermore, magnesium has been shown in this study to be less effective than calcium in reducing the influx of zinc, and in other studies to be less effective than calcium in reducing membrane permeability. Hence, indirect evidence suggests that surface permeability changes occurring in relation to changes in the divalent ion status of the external medium are likely to be of some importance in controlling the uptake of zinc. However, evidence has been presented in this work to suggest that competition between divalent ions for uptake sites is of considerable importance in the modification of zinc uptake by calcium

and magnesium. Using ^{45}Ca it was shown that an environmentally relevant sublethal [Zn^{2+}] caused a strong dose-dependent inhibition of calcium influx accompanied by a marked hypocalcaemia. Experiments using lanthanum, a specific calcium-channel blocker demonstrated a rapid inhibition of both calcium and zinc influx following the addition of lanthanum to the external medium, a result which strongly suggests that these metals share a common uptake pathway across the apical membrane of the branchial epithelium. Other workers have reported a similar inhibition of calcium influx by cadmium and inhibition of both calcium and cadmium influx by lanthanum. These results, together with biochemical evidence concerning the mode of action of cadmium, have been interpreted as evidence for a common uptake pathway across the apical membrane for both calcium and cadmium, the cadmium-induced inhibition of calcium transport being thought to occur at the basolateral membrane where calcium is extruded into the bloodstream. Intracellular proteins such as calmodulin are capable of binding calcium and cadmium, the inhibition of calcium influx by cadmium probably occurring when the cadmium binding capacity of these proteins is exceeded.

It is proposed that zinc exerts its toxic effects, at least at sublethal concentrations, through a similar mechanism to that proposed for cadmium. Whereas the results for cadmium were interpreted in terms of a

modification of membrane permeability by calcium and magnesium causing the changes in the uptake rate of cadmium however (Part et al., 1985), it is considered here that competition between calcium and trace metals occurs intracellularly at a Ca^{2+}-ATPase on the basolateral membrane that is thought to extrude calcium into the bloodstream.

The first series of experiments using ^{45}Ca investigated the turnover of calcium by the fish in the absence of an increased external zinc concentration. The ability of the fish to take up calcium was enhanced following a period of acclimation to water of low divalent ion content. It is considered that this enhancement reflected an increase in Ca^{2+}-ATPase activity, possibly linked to a proliferation of branchial chloride cells. Apparent saturation of the calcium uptake mechanism in this and other studies was considered evidence for the transepithelial process involving, at some stage, the active transport of calcium via transport molecules. Using ^{65}Zn it was shown that although the effect of acclimation was small in comparison to the modifying influence of the external $[Ca^{2+}]$ during exposure to zinc the acclimation regime did exert an affect on the subsequent rate of influx of the metal. Acclimation to low external $[Ca^{2+}]$ before exposure to zinc in a higher $[Ca^{2+}]$ for example, resulted in a higher rate of zinc influx than that observed in fish acclimated

and tested in the same higher external $[Ca^{2+}]$. The enhancement of zinc influx following acclimation to low calcium may thus have reflected the enhanced calcium uptake capability, the somewhat lesser stimulation of zinc influx relative to calcium influx probably refecting a lower affinity of the calcium uptake mechanism for zinc. It is considered that the mechanism by which increased external $[Ca^{2+}]$ reduces the influx of zinc is likely to operate at the basolateral calcium transporter. Given that in those studies on cadmium external calcium affected only the translocation of cadmium from the epithelium to the bloodstream and not the initial accumulation of cadmium by the epithelium it is difficult to envisage membrane permeability phenomena influencing the intracellular extrusion of the trace metal. It may be that magnesium also affects the uptake of zinc through competition at the basolateral membrane. The lower ability of magnesium to do this in comparison to calcium may refect a lesser affinity of the calcium transporter for magnesium relative to its affinity for calcium and possibly zinc too. Further investigation of this hypothesis is obviously required. Kinetic studies would help to resolve possible divalent ion competition. *In vitro* studies similar to the perfused head technique used by Perry and Wood (1985) would avoid problems arising from the adsorption of radioisotope onto the surface of the fish.

The zinc-induced disturbance of normal calcium balance was reflected in the ^{65}Zn experiments by hypocalcaemia in fish exposed to zinc at the lowest external $[Ca^{2+}]$ and in the ^{45}Ca experiments by hypocalcaemia in fish exposed to the two highest zinc concentrations. The comparatively small changes observed in plasma sodium and chloride concentrations were attributed to a modification of membrane permeability and were considered to be of secondary importance relative to the hypocalcaemia.

Most zinc was accumulated by the gills, plasma and carcass, although zinc content per unit weight of tissue was greatest in the gills and plasma with lesser but significant quantities in the kidney, liver, spleen and alimentary canal. The gills were concluded to be the major site of zinc uptake, their high zinc content being considered an artifact of the relatively short duration of the experiments.

In conclusion therefore, the results of the work presented in this thesis have clearly demonstrated that the toxicity of zinc to the brown trout is greatly reduced on increasing the $[Ca^{2+}]$ of the external medium. Acclimation to elevated external $[Ca^{2+}]$ modifies toxicity very little in comparison to the actual $[Ca^{2+}]$ of the medium during exposure to the metal. Calcium appeared to exert its greatest influence on the influx of zinc at a concentration of 0.15 mM, a concentration environmentally

relevant as regards natural waters subject to acidification (NRCC, 1981). Thus, at such low calcium concentrations relatively small changes of external $[Ca^{2+}]$ will profoundly modify the uptake and subsequent toxicity of zinc. Such a phenomenon is likely to have a significant bearing on the outcome of conservation programmes such as the liming of acidified waters, the process whereby the calcium concentration of a body of water is artificially raised through the addition of calcium bearing rock, usually limestone.

As regards the mechanism(s) of toxic action of zinc, it is proposed that zinc disturbs the calcium balance of teleosts through competition with calcium at the basolateral membrane Ca^{2+}-ATPase, a similar mechanism to that suggested for cadmium. In addition, it is also considered likely that other environmentally relevant heavy metals such as copper, chromium, manganese, nickel, lead and iron may also exert similar effects at sublethal concentrations, although for some, such as copper, other mechanisms of toxicity are likely to be of greater importance. Further work investigating calcium fluxes in the presence of these heavy metals is required to confirm this view however. Experiments of similar design to those employed in this study would be suitable in an investigation of the comparative effects of these heavy metals.

If the evidence of calcium influx inhibition

obtained in this study could be supported by biochemical evidence of trace metal-induced Ca^{2+}-ATPase inhibition then the proposal that the disruption of normal calcium balance is a fundamental mechanism of sublethal heavy metal toxicity would be well-founded. Furthermore, the acidification of natural waters is frequently accompanied by the elevation in concentration of a number of trace metals, not simply one in isolation from the remainder. If it could be demonstrated for a number of these metals that a similar mechanism of toxicity exists then there is a strong liklihood that they are to some extent additive in their effects on fish populations. Indeed, the additive toxicity of trace metal mixtures has been demonstrated on a number of occasions (Lewis, 1978; Finlayson and Verrue, 1982; Roch and McCarter, 1984; Hutchinson and Sprague, 1986; review: EIFAC, 1980). Such an additive toxic action would, in itself, probably be sufficient to explain the loss of fish populations in many acidified waters, the disruption of normal calcium balance resulting in an increased metabolic cost and hence decreased fitness for survival in the modified environment.

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