THE EFFECTS OF A DIETARY BACTERIAL PROTEIN ON MINERAL BALANCE IN RAINBOW TROUT (S. gairdneri Rich.)

Submitted by JONATHAN DOUGLAS ANGLESEA

to The University of Aston in Birmingham

for the degree of Doctor of Philosophy

October 1982

The University of Aston in Birmingham

THE EFFECTS OF A DIETARY BACTERIAL PROTEIN ON MINERAL BALANCE IN RAINBOW TROUT (S. gairdneri Rich.)

Submitted by Jonathan Douglas Anglesea

for the degree of Doctor of Philosophy

October 1982

A bacterial protein (I.C.I.'s "Pruteen") was tested in diets for rainbow trout, to investigate the effects of the new feedstuff on the mineral balance of the fish as expressed by the carcass and tissue mineral levels.

Supplements of the major and trace elements were added to trout diets in various combinations and with Pruteen, casein or fishmeal as protein sources in order to test the effects of these dietary factors on mineral balance. Interactions between dietary factors were investigated and their effects were related to the growth of the fish, the voluntary intake of food by fish, and the mineral balance.

Growth improved in fish fed diets containing 65% Pruteen and supplemented with 4% major-mineral mix containing calcium, magnesium, phosphate, iron, sodium, potassium and chloride. Calcium, magnesium and iron are believed to have been the most active minerals in this mix.

In order to estimate the abilities of fish to tolerate water of different calcium content, identical diets were fed to groups of fish held in water of two different hardnesses (20 and 40ppm.). The growth was suppressed in fish held in water of 40 ppm. hardness, which appeared to induce a temporary deficiency of copper. This effect was not detectable after 50 days.

Pruteen was found to increase the calcium, phosphorus and magnesium contents of the fish.

The palatability of the three protein sources was determined as fishmeal > Pruteen > casein/gelatin mix.

Supplementation of fish diets with calcium was found to decrease the calcium content of the carcass, and especially the calcium and magnesium content of the hard tissues, scales and bone.

Mineral levels in the water in which fish were held were estimated by a new method which involved the use of ion exchange resin. This had several advantages when compared with conventional water sampling techniques.

To facilitate handling the large quantities of data generated by these studies, a number of computer programs were developed, which will be useful in experimental fish culture.

KEYWORDS: Minerals, Nutrition, Single-cell Protein, Trace Elements, Trout

CONTENTS

Section		Page
1.	Preface	1
2.	Introduction	4
2.1.	Mineral Metabolism: General Considerations	4
2.1.1.	Calcium	7
2.1.2.	Phosphorus	18
2.1.3.	Magnesium	23
2.1.4.	Zinc	25
2.1.5.	Iron	27
2.1.6.	Manganese	29
2.1.7.	Copper	31
2.2.	Single-cell Proteins and Mineral Metabolism	33
3.	Methods and Materials	38
3.1.	Experiments	38
3.1.1.	Experiment Construction	38
3.1.2.	Diet Formulation	41
3.1.3.	Individual Experimental Designs	50
3.1.4.	Diet Preparation	57
3.1.5.	Proximate and Mineral Composition of Diets	59
3.2.	Fish	67
3.2.1.	Species Used	67
3.2.2.	Quarantine Procedure	67
3.2.3.	Weighing	68
3.2.4.	Anaesthesia	69
3.2.5.	Freeze Branding	69
3.2.6.	Experimental Procedure	72

CONTENTS (Cont.)

Section		Page
3.3.	Fish Tanks and System Design	73
3.3.1.	General Considerations	73
3.3.2.	The Recirculating System	75
3.3.3.	The Through-flow System	80
3.3.4.	System Monitoring: Environmental Parameters	84
3.4.	Treatment and Analysis of Samples	85
3.4.1.	The Nature of Samples	85
3.4.2.	Water Sample Analysis	86
3.4.3.	The Method of Water Sampling and Treatment	92
3.4.4.	The Wet Combustion of Samples	94
3.4.5.	Mineral Analyses	98
3.5.	Statistics	100
3.5.1.	Growth Statistics	100
3.5.2.	Feed Intake and Conversion Statistics	103
3.6.	Computer Programming	106
4.	Results	108
4.1.	Experiment II	109
4.1.1.	Growth and Feeding	109
4.1.2.	Mineral Analyses	111
4.2.	Experiment III	113
4.2.1.	Growth and Feeding	113
4.2.2.	Mineral Analyses	115
4.3.	Experiment V	120
4.3.1.	Growth and Feeding	120
4.3.2.	Mineral Analyses	122

CONTENTS (Cont.)

Section		Page
4.4.	Experiment VI	125
4.4.1.	Growth and Feeding	125
4.4.2.	Mineral Analyses	128
4.5.	Experiment VII	134
4.5.1.	Growth and Feeding	134
4.5.2.	Mineral Analyses	137
4.5.2.1.	Fish Carcasses	137
4.5.2.2.	Bone	140
4.5.2.3.	Eye	143
4.5.2.4.	Kidney	146
4.5.2.5.	Muscle	149
4.5.2.6.	Liver	152
4.5.2.7.	Skin	155
4.5.2.8.	Viscera	168
4.5.2.9.	Faeces	171
4.5.2.10.	Ca:Mg Ratios in Tissues	173
4.6.	Water Analyses	175
4.6.1.	Water Quality	175
4.6.2.	Water Minerals	180
5.	Discussion	193
5.1.	Experiment II	193
5.1.1.	Growth and Feeding	194
5.1.2.	Mineral Analyses	196
5.2.	Experiment III	197
5.2.1.	Growth and Feeding	197

CONTENTS (Cont.)

Section		Page
5.2.2.	Mineral Analyses	199
5.3.	Experiment V	201
5.3.1.	Growth and Feeding	202
5.3.2.	Mineral Analyses	203
5.4.	Experiment VI	206
5.4.1.	Growth and Feeding	206
5.4.2.	Mineral Analyses	208
5.5.	Experiment VII	210
5.5.1.	Growth and Feeding	210
5.5.2.	Carcass Minerals	212
5.5.2.1.	Bone	215
5.5.2.2.	Eye	216
5.5.2.3.	Kidney	217
5.5.2.4.	Muscle	218
5.5.2.5.	Liver	218
5.5.2.6.	Skin	219
5.5.2.7.	Viscera	212
5.5.2.8.	Faeces	212
5.5.3.	Mineral Balance	222
5.6.	Water Analyses	229
6.	Conclusions	231
7.	References	234

LIST OF TABLES

Table		Page
1.1.	Abbreviations	2
2.2.1.	Minerals in Fishmeal and 'Pruteen'	36
3.1.2.1.	Formulations of the Diets: Expts. II & III	43
3.1.2.2.	Formulations of the Diets: Expt. V	44
3.1.2.3.	Formulations of the Diets: Expt. VI	44
3.1.2.4.	Formulations of the Diets: Expt. VII	45
3.1.2.5.	Formulation of the Premix	46
3.1.2.6.	Formulation of the Vitamin mix	47
3.1.2.7.	Formulation of the Mineral Mix	48
3.1.2.8.	4% Mineral Mix Supplies:	48
3.1.2.9.	Formulation of the Trace Element Mix	49
3.1.2.10.	0.02% Trace Element Mix Supplies:	49
3.1.5.1.	Methods of Proximate Analysis	59
3.1.5.2.	Proximate Compositions of the Diets: Expt. II	60
3.1.5.3.	Mineral Levels in Dry Diets: Expt. II	60
3.1.5.4.	Proximate Compositions of the Diets: Expt. III	61
3.1.5.5.	Mineral Levels in Dry Diets: Expt. III	62
3.1.5.6.	Proximate Compositions of the Diets: Expt. V	63
3.1.5.7.	Mineral Levels in Dry Diets: Expt. V	63
3.1.5.8.	Proximate Compositions of the Diets: Expt VI	64
3.1.5.9.	Mineral Levels in Dry Diets: Expt VI	64
3.1.5.10.	Proximate Compositions of the Diets: Expt. VII	65
3.1.5.11.	Mineral Levels in Dry Diets: Expt. VII	66
3.4.2.1.	Detection Limits: Perkin Elmer 303	87

LIST OF TABLES (Cont.)

Table		Page
4.1.1.1.	Factorial Effects on Growth and Feed Intake: Expt. II	109
4.1.1.2.	Growth and Feed Conversion Statistics: Expt. II	110
4.1.2.1.	Carcass Mineral Contents: Expt. II	112
4.2.1.1.	Factorial Effects on Growth and Feed Intake: Expt. III	113
4.2.1.2.	Growth and Feed Conversion Statistics: Expt. III	114
4.2.2.1.	Factorial Effects on Mineral Contents: Expt. III	116
4.2.2.2.	Carcass Mineral Contents: Expt. III	117
4.2.2.3.	Carcass Ca Levels (%) During Expt. III	118
4.2.2.4.	Carcass Mg Levels (ppm.) During Expt. III	118
4.2.2.5.	Carcass Zn LEvels (ppm.) During Expt. III	118
4.2.2.6.	Carcass Fe Levels (ppm.) During Expt. III	118
4.2.2.7.	Carcass Cu Levels (ppm.) During Expt. III	119
4.2.2.8.	Carcass Mn Levels (ppm.) During Expt. III	119
4.2.2.9.	Carcass P Levels (%) During Expt. III	119
4.3.1.1.	Factorial Effects on Growth and Feed Intake: Expt. V	120
4.3.1.2.	Growth and Feed Conversion Statistics: Expt. V	121
4.3.2.1.	Expt. V: Factorial Effects on Mineral Content After 25 Days	122

LIST OF TABLES (Cont.)

Table		Page
4.3.2.2.	Expt. V: Factorial Effects on Mineral Content After 50 Days	123
4.3.2.3.	Carcass Mineral Contents After 25 Days: Expt. V	124
4.3.2.4.	Carcass Mineral Contents After 50 Days: Expt. V	124
4.4.1.1.	Factorial Effects on Growth and Feed Intake: Expt. VI	126
4.4.1.2.	Growth and Feed Conversion Statistics: Expt. VI	127
4.4.2.1.	Expt. VI: Factorial Effects on Mineral Contents After 42 days	129
4.4.2.2.	Expt. VI: Factorial Effects on Mineral Contents After 84 Days	131
4.4.2.3.	Carcass Mineral Contents After 42 Days: Expt. VI	132
4.4.2.4.	Carcass Mineral Contents After 84 Days: Expt. VI	132
4.5.1.1.	Factorial Effects on Growth and Feed Intake: Expt. VII	135
4.5.1.2.	Growth and Feed Conversion Statistics: Expt. VII	136
4.5.2.1.1.	Expt. VII: Factorial Effects on Mineral Contents After 112 Days	138
4.5.2.1.2.	Carcass Mineral Contents After 112 Days: Expt. VII	139

LIST OF TABLES (Cont.)

Table		Page
4.5.2.2.1.	Factorial Effects on Minerals in Bone: Expt. VII	141
4.5.2.2.2.	Tissue Mineral Contents: Bone	142
4.5.2.3.1.	Factorial Effects on Minerals in Eye: Expt. VII	144
4.5.2.3.2.	Tissue Mineral Contents: Eye	145
4.5.2.4.1.	Factorial Effects on Minerals in Kidney: Expt. VII	147
4.5.2.4.2.	Tissue Mineral Contents: Kidney	148
4.5.2.5.1.	Factorial Effects on Minerals in Liver: Expt VII	150
4.5.2.5.2.	Tissue Mineral Contents: Liver	151
4.5.2.6.1.	Factorial Effects on Minerals in Muscle: Expt VII	153
4.5.2.6.2.	Tissue Mineral Contents: Muscle	154
4.5.2.7.1.	Factorial Effects on Minerals in Skin: Expt. VII	156
4.5.2.7.2.	Tissue Mineral Contents: Skin	157
4.5.2.8.1.	Factorial Effects on Minerals in Viscera: Expt. VII	169
4.5.2.8.2.	Tissue Mineral Contents: Viscera	170
4.5.2.9.1.	Faecal Mineral Levels: Expt. VII	172
4.5.2.10.1.	Factorial Effects on Ca:Mg Ratios in Tissues: Expt. VII	174
4.6.1.	Mean Levels of Minerals In Water	192

LIST OF PLATES

Plate		Page
4.5.2.7.1.	Scanning Electron Micrograph of Scales from Fish Fed Diet C	158
4.5.2.7.2.	Scanning Electron Micrograph of Scales from Fish Fed Diet CCa	159
4.5.2.7.3.	Scanning Electron Micrograph of Scales from Fish Fed Diet CFe	160
4.5.2.7.4.	Scanning Electron Micrograph of Scales from Fish Fed Diet CCaFe	161
4.5.2.7.5.	Scanning Electron Micrograph of Scales from Fish Fed Diet B	162
4.5.2.7.6.	Scanning Electron Micrograph of Scales from Fish Fed Diet BCa	163
4.5.2.7.7.	Scanning Electron Micrograph of Scales from Fish Fed Diet BFe	164
4.5.2.7.8.	Scanning Electron Micrograph of Scales from Fish Fed Diet BCaFe	165
4.5.2.7.9.	Scanning Electron Micrograph of Scales from Fish Fed Diet EM	166

LIST OF FIGURES

Figure		Page
3.2.5.1.	Freeze Branding Iron Design and Use	70
3.3.1.1.	Diagram of the Recirculating System (Before Conversion)	74
3.3.2.1.	The Modified Recirculating System	77
3.3.3.1.	Diagram of the Through-flow System	81
4.6.1.1.	Graph of Water Environmental Parameters (Expt. III)	176
4.6.1.2.	Graph of Water Environmental Parameters (Expt. V)	177
4.6.1.3.	Graph of Water Environmental Parameters (Expt. VI)	178
4.6.1.4.	Graph of Water Environmental Parameters (Expt. VII)	179
4.6.2.1.	Graph of Water Na Levels v. time.	181
4.6.2.2.	Graph of Water Na Levels v. time. (By digestion of resin.)	181
4.6.2.3.	Graph of Water K Levels v. time.	182
4.6.2.4.	Graph of Water K Levels v. time. (By digestion of resin.)	182
4.6.2.5.	Graph of Water Ca Levels v. time.	183
4.6.2.6.	Graph of Water Ca Levels v. time. (By digestion of resin.)	183
4.6.2.7.	Graph of Water Mg Levels v. time.	184

LIST OF FIGURES (Cont.)

Figure		Page
4.6.2.8.	Graph of Water Mg Levels v. time. (By digestion of resin.)	184
4.6.2.9.	Graph of Water Zn Levels v. time.	185
4.6.2.10.	Graph of Water Zn Levels v. time. (By digestion of resin.)	185
4.6.2.11.	Graph of Water Fe Levels v. time.	186
4.6.2.12.	Graph of Water Fe Levels v. time. (By digestion of resin.)	186
4.6.2.13.	Graph of Water Cu Levels v. time.	187
4.6.2.14.	Graph of Water Cu Levels v. time. (By digestion of resin.)	187
4.6.2.15.	Graph of Water Mn Levels v. time.	188
4.6.2.16.	Graph of Water Mn Levels v. time. (By digestion of resin.)	188
4.6.2.17.	Graph of Water Pb Levels v. time. (By digestion of resin.)	189
4.6.2.18.	Graph of Water Cd Levels v. time. (By digestion of resin.)	189
4.6.2.19.	Graph of Water Co Levels v. time. (By digestion of resin.)	190
4.6.2.20.	Graph of Water Ni Levels v. time. (By digestion of resin.)	190

1. PREFACE

This thesis has been constructed to prevent repetition where possible. The experiments all had some common features, and methodology was standardised within the constraints imposed by individual experimental designs. Thus the methodology, results, discussion and conclusions from each experiment are combined in appropriate chapters. This was especially important in the chapters where it was necessary to refer to results of previous or subsequent experiments. For brevity, some abbreviations have been adopted in this document, and are described in Table 1.1.

No results are given for experiments I and IV. These experiments were abandoned because many fish died, but the original experiment numbers (II-VII) were retained for simplicity.

Table 1.1. Abbreviations

Abbreviation	Meaning
F	Fishmeal diet
FM	Fishmeal diet with minerals
FT	Fishmeal diet with trace elements
FMT	Fishmeal diet with minerals and trace elements
B	Pruteen diet (Bacterial protein)
BM	Pruteen diet with minerals
BT	Pruteen diet with trace elements
BMT	Pruteen diet with minerals and trace elements
E	Dry commercial trout food
B0-	Pruteen diet fed in water filtered through gravel
B4-	Pruteen diet with minerals fed in water filtered through gravel
B0+	Pruteen diet fed in water filtered through oyster shell
B4+	Pruteen diet with minerals fed in water filtered through oyster shell
BA	Pruteen diet with added sodium phytate
BAM	Pruteen diet with sodium phytate and minerals
BAT	Pruteen diet with sodium phytate and trace elements
BAMT	Pruteen diet with sodium phytate, minerals and trace elements
C	Casein/gelatin diet
CCa	Casein/gelatin diet with calcium
CFe	Casein/gelatin diet with iron
CCaFe	Casein/gelatin diet with calcium and iron
BCa	Pruteen diet with calcium
BFe	Pruteen diet with iron
BCaFe	Pruteen diet with calcium and iron
EM	Diet of moistened commercial trout food
AA	Atomic Absorption
AAS	Atomic absorption spectrophotometry
AMP	Adenosine mono-phosphate
A.O.A.C.	Association of Analytical Chemists
A.P.H.A.	Association of Public Health Analysts
D. Lt.	Detection Limit (as of a spectrophotometer)
EDTA	Ethylene-diamino tetra-acetic acid
Eth. Ext.	Ether extractables
FCR	Feed conversion ratio
I.C.I.	Imperial Chemical Industries
M.A.F.F.	Ministry of Agriculture, Fisheries and Food
MPS	Muco-polysaccharide

Table 1.1. (Cont.) Abbreviations

Abbreviation	Meaning
N.F.E.	Nitrogen-free extract
NPN	Non-protein nitrogen
ppm.	parts per million
ppb.	parts per billion (10^9)
SCP	Single-cell protein
S.E.M.	Standard error about any mean
SGR	Specific growth rate
UMB	Universal media bottle
UV	Ultra-violet
WRC	Water Research Centre

2. INTRODUCTION

2.1. Mineral Metabolism: General Considerations

Nutrient metabolism may be defined as the 'changes undergone by nutritive substances in the body' (Simkiss, 1972). Strictly speaking, therefore, minerals are not metabolised. They are absorbed as elemental ions, and are excreted as elemental ions and remain unaltered in the meantime. Mineral metabolism thus differs from the metabolism of protein, lipid or carbohydrate. In this thesis, mineral metabolism will be taken to mean the uptake, transport, storage, utilisation and excretion of minerals, and the mechanisms and factors which affect and control these processes.

Various techniques have been used to investigate aspects of the mineral metabolisms of fish, the which techniques may be categorised as follows:

i) Studies of fish growth to determine the dietary mineral requirement for growth.

ii) Studies using radioactive isotope tracers to investigate the movement and accumulation of minerals into, out of and within fish.

iii) Studies of the effects of environmental factors on the mineral content of fish and fish tissues.

iv) Studies of the effects of hormone administration on mineral levels in fish and fish tissues.

v) Studies on the osmoregulatory abilities of fish.

vi) Studies on the toxicity of minerals in water to fish.

Interest in the mineral metabolisms of fishes has greatly increased in recent years, stimulated by the demand from the aquaculture industry for complete, dry fish diets. The formulation of an 'artificial' fish diet requires precise knowledge of the levels of nutrients which will produce optimal growth with an economic cost of production. The perfect diet is an ideal, but will be approached more closely with greater knowledge of fish nutrient requirements.

Minerals for which possible nutritional requirements have been suggested include calcium, phosphorus, magnesium, zinc, iron, manganese, copper, iodine and selenium. The levels required have not, however, been determined for any one species, and studies have shown interspecific differences in the requirement for given minerals. It may be impossible to define a mineral requirement for even a single species, since many different factors may influence it.

The list of minerals given above is by no means inclusive of all minerals utilised by fishes, merely those for which optimal dietary levels have been proposed. There are many other essential elements for which a dietary optimum may yet be found, and others which, although essential, are present in natural waters at levels sufficient for fish to absorb them directly.

In this introduction, the current data available on the metabolism of calcium, phosphorus, magnesium, zinc, iron, copper and manganese are reviewed, as are the data published on the possible effects of single-cell proteins (SCP's) on these. The

elements reviewed are those which were investigated in the research described herein. These were limited by the time available and the quantity of material analysed. The notable omissions are sodium, potassium and chlorine. Analysis of the fish for chlorine was impossible as the digestion method by which the samples were dissolved used perchloric acid. Sodium and potassium levels were not determined, since no dietary requirement has been demonstrated for these elements in fish, any deficiency or excess being rapidly adjusted by movement across the gills and body surface. Macleod (1978) found no effect on growth or FCR in rainbow trout fed NaCl at levels up to 8.5% of the diet. Shaw et al. (1975) reported similar results in Atlantic salmon. Some workers (e.g. Knox et al., 1981, Mudge and Neff, 1971) have shown disturbances of the sodium and potassium levels in fish caused apparently by other factors, but the meaning of this variation is unclear. These reasons are believed to justify their omission, although the decision to omit analysis of samples for these elements was primarily taken to allow time for analysis of the other, potentially more important minerals.

2.1.1. Calcium

Calcium and phosphorus metabolism are often researched simultaneously, as these minerals are compounded in the bone and scales. They are the most abundant minerals found in fish, because of this accumulation in the hard tissues. Besides a structural role, however, both calcium and phosphorus have physiological functions independent from the other. For this reason, and as the pathways of uptake of the two elements are different, calcium and phosphorus metabolism are here reviewed separately.

Calcium is required by fish and other animals for a number of reasons. Calcium is involved in the maintenance of membrane potentials and is often found associated with proteins in the plasma. Calcium ions are necessary for the coagulation of blood and are involved in the regulation of cell metabolism by cyclic AMP.

In the majority of vertebrates, the skeleton serves two roles, as a rigid supporting structure upon which the locomotory muscles act, and as a reserve of calcium and phosphorus. The two minerals are constantly recycled into and out of the bones by specialised cells, the osteocytes. Most fishes have no osteocytes, which appear only in primitive groups of the osteichthyes. As a consequence, the mobility of calcium and phosphorus in the bones of the higher teleosts is almost nil (Simkiss 1972).

To maintain a constant level of plasma calcium, which appears to be closely regulated, fish seem to modify the rates of uptake

from and/or excretion into the external media. This appears to be achieved by alteration of the permeability of the epidermis, and its active transport mechanisms, but the precise method of control is as yet not fully understood.

Much work has been done to investigate the uptake of calcium from the external environment, generally by the use of radioactive isotopes. The most often used is ^{45}Ca although ^{90}Sr has been employed as a substitute. The use of the strontium isotope is justified by the observation that the element behaves similarly to calcium in biological systems, and is taken up by the gills at a rate almost equal to that at which calcium is absorbed. (Rosenthal 1960). This behaviour has been shown to change if the sodium ion concentration falls below a certain limit. (Rosenthal 1963). Berg (1968) used ^{90}Sr to determine the Sr:Ca discrimination of the intestine in goldfish (Carassius auratus). This was found to be far greater than the discrimination at the gills. Several other workers have found significant discrimination factors of Sr:Ca. Ichikawa, Oguri and Takada (1962) and Ichikawa and Oguri (1960) have demonstrated a discrimination factor in favour of calcium of 0.7 in the eel (Anguilla japonica). This discrimination diminishes the justification for the use of strontium isotopes instead of calcium isotopes. In the natural environment Ichikawa (1960) found that the ratio of Sr:Ca in natural water to Sr:Ca in fish tissue was 0.2. This indicates that in the natural state, fish discriminate against strontium and preferentially absorb Ca. Berg (1968) also found the proportion of the calcium requirement of the fish supplied by the food via the intestine. This was found

to be less than 50% in water of calcium content 4.0 ppm (low compared to most natural fresh waters). The cells of the intestinal wall have been shown to excrete calcium ions from the fish's blood into the lumen. Mashiko and Jozuka (1964) injected ^{45}Ca into the dorsal musculature of crucian carp (Carassius carassius) and observed the appearance of the isotope in the intestinal fluid during the following 2-6 hours. The intestine may perform an important elimination of calcium in salt water fishes, where the water swallowed has a high concentration of Ca, above that of the fish tissues. Smith (1930) first demonstrated that marine fish continually swallow water. He used a dye (carmine red) in the water which was seen to appear and be concentrated in the intestine. This indicated that the fish were swallowing water and then absorbing it through the intestinal wall. The water absorbed replaces the water lost by osmosis across the gill and body surface. Umehara and Oguri (1978) showed that plasma calcium increased in goldfish transferred from fresh water to 1/3 strength sea water. When transferred from fresh water to water with supplemental calcium only, of the same concentration as that of 1/3 strength sea water without other salts, no increase in plasma calcium was observed. This indicated that the calcium was absorbed not simply by diffusion across a concentration gradient, but from water which was ingested in the 1/3 strength sea water to replace water lost by osmotic dehydration. Thus the gills were shown to be able not only to absorb calcium, but also to exclude or excrete it. In sea water adapted rainbow trout (Salmo gairdneri), the mucus tube which envelopes the faeces has been shown to contain large amounts of

calcium carbonate, apparently formed from the calcium ingested with the sea water, and subsequently secreted by the intestine. In starved specimens, the mucus contained the equivalent of 64% of the ingested Ca. (Simkiss 1972 after Shehadeh and Gordon 1969). Hickman (1968) showed that in the Southern flounder (Paralichthys lethostigma) the kidneys were responsible for the excretion of only 11.4% of the ingested calcium. Hickman and Trump (1969) summarised data from a number of sources which showed that the renal excretion of calcium was low in most fish species studied. The evidence seems to suggest that the kidney is not of great importance in the regulation of calcium metabolism in fish. This is in contrast to its role in most higher vertebrates.

Mashiko and Jozuka (1964) continued their earlier work demonstrating the uptake of radiocalcium from water by marine and freshwater fish (Mashiko and Jozuka 1961, 1962 and Jozuka 1963). In the 1964 paper these workers investigated the routes followed by calcium absorbed from the water. They showed that the fins of a marine fish (D.flagellifera) performed a greater exchange of calcium than the gills, whilst in the freshwater crucian carp, the situation was reversed. Calcium was shown to be both absorbed and excreted by the caudal fin of the carp, and when experiments were performed isolating the fins, the calcium thus absorbed was seen to be rapidly transferred to the gills. This effect indicates that the minerals seen in the gills are not necessarily those which have crossed the gill epithelium. From the data available, it is evident that the uptake and excretion of calcium by

freshwater and sea fishes can take place against concentration gradients. There is, presumably an active transport system, possibly operating by means of a carrier protein. So far, no data have been published defining the exact sites within tissues at which ion movement takes place, nor on the sub-cellular processes by which calcium transport is achieved. In the higher vertebrates, calcium homeostasis is achieved by the antagonism of two hormones, parathyroid hormone (parathormone) and calcitonin against vitamin D.

Parathyroid hormone lowers the overall loss of Ca from the organism by increasing renal phosphate excretion, and mobilisation of bone calcium. Parathormone is released by the parathyroid glands in response to falling levels of plasma calcium. Calcitonin depresses the mobilisation of calcium from bone and may enhance bone deposition. Calcitonin is secreted by the ultimobranchial body in response to rising levels of plasma calcium. Vitamin D acts primarily on the intestine and regulates the uptake of calcium from food. It is synthesised in the skin and production is favoured by incident UV light.

As in other aspects of calcium metabolism, the calcium homeostasis of fish is somewhat different from that of the higher vertebrates.

The most fundamental difference between fishes and higher vertebrates is the structure of the bone (see above). Cellular bone, seen in all higher vertebrates, is found only in primitive fish groups, and is absent in the majority of teleosts. (Moss 1961a, b; Fleming, 1967; Simmonds, 1971). Most fish have evolved

a so-called 'acellular' bone, which is formed by osteoblasts which move away from the site of mineralisation as bone deposition occurs. The cartilaginous fibres (Sharpey's fibres) from which all bone develops, are progressively mineralised by the deposition of crystalline compounds of calcium and phosphorus (apatites). In acellular bone, the Sharpey's fibres are completely embedded in the inorganic matrix of the bone, which is dense and impermeable. In cellular bone, the matrix is permeated with fine canaliculi which allow the access of the osteblasts and osteoclasts (bone degrading cells). These are thus able to deposit or remove calcium from all areas of the bone. The disappearance of the osteoclasts and the permeating canals from fish bone may indicate the importance of bone mineralisation in fish. The most primitive fish groups (Agnathi) have cartilaginous bone. The primitive osteichthyes may have cellular bone or a mixture of cellular and acellular bone, whilst the higher groups have totally acellular bone. It is possible that as the ability of the fishes to regulate the transport of ions improved, the ability to use the bone as a reservoir of calcium and phosphorus became less important and was lost. This argument is not invalidated by the cartilaginous bone of the elasmobranchs, since these fish osmoregulate by adaptation of the osmotic pressure of the blood, rather than by ionic manipulation. In the higher vertebrates, which evolved in semi-aquatic habitats, the skeleton became not merely a rigid framework for the attachment of muscles, but a support for the whole weight of the body. This required a stronger bone than that of fish, which are supported

by the water in which they swim. Fish bone has only to resist the stresses imposed by the different muscles which act upon it, and may thus be mineralised to a lesser extent than that of terrestrial vertebrates. There is evidence to suggest that the structural role of calcium in fish bone is of secondary importance to its other physiological functions. In an experiment to investigate bone repair, Moss (1962) found that under conditions of low calcium availability, bone callus material was principally cartilaginous. The callus material remained unmineralsised until an adequate calcium supply was provided. The growth of the fish was not unduly affected even when the calcium was insufficient for bone repair. This contrasts with the human condition of ricketts, where low availability of calcium (due to vitamin D deficiency) results in bone deformation. This deformation is most pronounced in the weight bearing bones of the legs. In fish, which are effectively weightless, no condition comparable to ricketts has been seen in conditions of Ca deficiency. Moss also compared the ability of fish with acellular and cellular bone to perform bone repair, and concluded that cellular bone had a greater ability to maintain calcium homeostasis as measured in terms of the ability to form bone callus material. Moss decided that acellular bone was not available for resorption. This result was contradicted by those of Norris, Chavin and Lombard (1963) who showed that the bone of the Nassau grouper was resorbed, even though no osteocytes or osteclasts were identified in this fish. Simmonds, Simmonds and Marshall (1970) used radioactive material to investigate bone

healing in the toadfish (Opsanus tao). Calcium was shown to concentrate at sites of bone growth, and once incorporated, was unavailable for resorption into the blood plasma. The isotope, once incorporated into the bone did not diffuse deeper into the skeleton over a period of time, as it would in cellular bone. Hevesy (1945) used an isotope of phosphorus to investigate the bone metabolism of the three-spined stickleback (Gasterosteus aculeatus) and reached the conclusion that the rate of replacement of the skeleton, allowing for growth, was almost nil.

Given the metabolic inactivity of fish bone, several workers have attempted to identify the means by which homeostasis is achieved. The scales may have a significant function as a renewable source of calcium in fish. Unlike the acellular bone, scales have been shown to be capable of resorption. This process was first shown by Crichton (1935), and subsequently investigated by Van Someren (1937), Yamada (1956), Ichikawa (1953) and Wallin (1957). These studies have shown that the scales are resorbed under conditions of calcium shortage (e.g. the starvation which occurs during the spawning runs of anadromous salmonids, the enforced starvation of cyprinids.). The skin of fish appears to be an important organ of calcium storage. Simkiss and Yarker (Unpublished data cited in Simkiss, 1972) found that the skin of trout contained 40% of the total carcass calcium. Garrod and Newell (1958) showed that the calcium content of the scales decreased in association with development of the ovary, an observation which accords with the rise in plasma calcium noted

in the plains killifish (Fundulus kansae) during the period prior to spawning. (Fleming, Stanley and Meir, 1964). This rise was seen only in female fish, and the calcium was shown to be bound to lecithal proteins synthesised by the liver, which were being transported via the blood to the ovaries. The calcium ions attached to these vitellins and lecithins may provide protection against degradation during transport. The deposition of calcium in scales has been shown to be correlated with temperature (metabolic rate) rather than the food supply (Bhatia 1932).

Scales may serve some of the functions in fish performed by cellular bone in higher vertebrates, but the system of calcium homeostasis in fish is still not comparable. Fish do not possess a parathyroid gland or the equivalent. Experiments involving the injection of fish with extracts of mammalian glands have, not surprisingly therefore, shown a near complete lack of response, plasma calcium levels being unaffected. (e.g. Fleming, Stanley and Meir, 1964). As opposed to parathormone, calcitonin has been shown in fish, and is produced by the ultimobranchial body (Copp et al., 1970).

Calcitonin has been detected in fish by bioassay, and levels appear to be higher than in other vertebrates. The evidence for the response of fish to calcitonin is however, contradictory. Chan, Chester-Jones and Smith (1968) claimed to have induced a lowering of blood calcium in the eel by injections of mammalian hormone. No response was observed in the plains killifish (F. kansae) when a similar experiment was performed by Pang and Pickford (1967). Hypocalcaemia and hypophosphaturia were seen in

the catfish (Ictalurus melas) in response to injection of the hormone. (Louw, Sutton and Kenny, 1967).

Vitamin D has been shown to be synthesised by the cod (Gadus morhua), (Blondin, Kulkarni and Kes, 1967) and is found in abundance in the livers of teleosts. Little response is shown to administration (Moss, 1963), although Halver (1972) reported symptoms of hypervitaminosis in fish fed excessive amounts. Launer, Tiemieer and Deyoe (1978) could not show vitamin D deficiency in channel catfish (Ictalurus punctatus) fed diets lacking it. No effect on the calcium or phosphorus retention of these fish was produced by variation of the vitamin D3 levels in the diets.

The ease with which calcium homeostasis is maintained by fish in natural waters has prevented the production of a pathological deficiency syndrome. The only documented effect of a dietary deficiency of calcium is reduced growth. Andrews et al. (1973) found that 1.5% calcium in the diet of catfish gave optimal growth and feed conversion, but that bone mineralisation was maximal at higher levels of inclusion. Arai et al. (1975) observed that calcium supplements to a diet in which the protein source was a yeast, low in Ca, improved the growth of rainbow trout. The optimal level of calcium in the diets was given as 0.6%. Ogino and Takeda (1978) showed no effect on the growth of rainbow trout by varying the calcium content of the diet, although the levels used (0.03-0.34%) fell below the range used by Arai. Ogino and Takeda (1978) tested the effect of variation in the calcium content of diets for carp, and found no difference

in the growth of fish given 0.028-0.97% calcium in the food. Sakamoto and Yone (1973) found that the optimal ratio of Ca:P for growth of red sea bream was 1:2 with 0.34% calcium in the diet. This result may be significant, as the red sea bream is a marine species, and would therefore be under a net calcium influx as a result of its osmotic regulation. A dietary calcium requirement is unexpected, and may indicate that calcium can have an effect on growth by chemical interaction with the diet whilst digestion occurs, rather than physiological effects after absorption. The existence or otherwise of optimal Ca:P ratios in the diets of fish are fully discussed in section 2.1.2. (Phosphorus).

2.1.2. Phosphorus

Phosphorus has a major role in lipid metabolism, as phospholipids are primary constituents of all cell membranes. Phosphorus is also required for the efficient metabolism of energy sources. The phosphate ion is a component of buffer systems. Phosphorus is combined with calcium in the apatites, the inorganic compounds which form the inorganic matrix of bone.

In contrast to calcium, fish do not appear to be able to obtain significant amounts of phosphorus from water by direct uptake. This may be due to the low concentration of phosphate ions present in most natural waters, or because the fish lack the necessary transport mechanisms. The former seems the most likely explanation. Coffin (1949) showed that radioactive phosphorus added to a Canadian natural lake took 2 weeks to appear in the fish tissues, whereas the isotope was seen in the zooplankton almost immediately. The radiophosphorus appeared first in the gut of the fish and then dispersed into the skeleton and other tissues. This was believed to indicate that uptake of phosphorus was via the zooplankton eaten, rather than directly from the water. The ability of the organisms in the lake to concentrate phosphorus was determined and concentration factors of 40,000 were seen. Other workers (Tomiyama et al. 1956,b, Shekhanova 1956, Srivastava 1960) published data which indicate a direct uptake from the water of inorganic phosphate. Whether this actually takes place or not, the food is the main source of phosphorus for fish. As noted above, calcium deficiency symptoms

have not been seen in fish, but symptoms of phosphorus deficiency are well documented. Andrews, Murai and Campbell (1973) recorded the effects of dietary deficiency of phosphorus in fingerling channel catfish (Ictalurus punctatus). These were poor growth and feed conversion, low bone ash and low haematocrit. Murakami (1970) reported that the addition of 5% sodium phosphate to the diet of carp (which mainly consisted of white fish meal), prevented deformity of the bones of the head, and enhanced growth. Cranial deformity appears to be characteristic of phosphorus deficiency in carp, recorded not only by Murakami but also by Ogino and Takeda (1976). The latter described other deficiency symptoms which included deformity of the vertebrae and low calcium and phosphorus in the tissues, especially the skeleton. These workers also noted that the level of phosphorus which produced maximal bone mineralisation was higher than that which gave maximal growth. (Vide, Andrews et al., sect 2.1.1.) Ogino and Takeda (1976) investigated the calcium and phosphorus requirements of rainbow trout (S. gairdneri). The phosphate requirement was shown to be around 0.8% of the diet for optimal growth, but was higher if the dietary calcium level was proportionately higher. These results support the hypothesis that the ratio of Ca:P in the diet has an effect on the requirement for each. The deleterious effects of high calcium levels in the diet noted by Andrews et al (1973) were not found to be alleviated by increasing the phosphorus level of the feed. This caused some authors to suggest that there is for each species an optimal ratio of Ca:P in the diet. Given that there is for each

species an optimal Ca and an optimal P concentration in the diet, there will be an optimal Ca:P ratio. However, it is now generally accepted that if there be an insufficiency or excess of either mineral, mere alteration of the other to give the apparent optimal Ca:P ratio will be of little value. Moreover, of the two minerals, phosphorus will be the more important, as the fish do not have the option of balancing the intake by uptake from the water. This was shown in an experiment by McCay et al. (1936) who raised brook trout (Salvelinus fontinalis) fingerlings on a diet of beef liver, which is high in phosphorus but low in calcium. They reported no difference in the calcium and phosphorus contents of the carcasses of fish (whether given calcium supplements or not,) although the Ca:P ratios of the diets were different. The water was, however, of relatively high calcium content (43 ppm), whilst the water in which Ogino and Takeda (1976) carried out their experiments contained 20 ppm. Phillips (1962) investigated the effect of water temperature and diet on the phosphorus content of brook trout. The availability of phosphorus from meat meal was found to be lower than from fresh meat. High dietary levels of meat meal phosphorus failed to give blood phosphorus levels as high as those in fish fed low phosphorus fresh meat diets. Blood phosphorus levels were seen to decrease as the water temperature rose, and this was attributed to the increase in the metabolic rate. Whole blood phosphorus was far higher than serum phosphorus, due to the presence of the erythrocytes in the former. Cell membranes contain large amounts of phospho-lipids. Different feedstuffs may contain phosphorus in

several forms, which may in turn be more available to one species of fish than another. Murakami (1970) found that although the phosphorus content of white fishmeal was c. 2%, carp fed this material became phosphorus deficient. This was believed to be due to the form of the phosphorus in fishmeal. Most of the phosphorus in this material is present in the form of apatite (see above) from the hard tissues of the powdered fish. This compound is insoluble in neutral or alkaline media and is therefore poorly digested in the carps, which lack an acid stomach. Black sea bream, which, like the salmonids, possess a stomach with acid digestive secretions, were found to digest and absorb fishmeal phosphorus well (Yone and Toshima 1979). Phytin phosphorus has been investigated as a possible source of dietary phosphorus because of the increased interest being shown towards plant proteins for fish feedstuffs (e.g. Cowey et al. 1971, 1974). Up to 60% of the phosphorus in soybean meal may be in the form of phytate, and this phosphorus has been shown to be readily available to rats (Likuski and Forbes 1965). Andrews et al. (1973) found phytin phosphorus to be less available than inorganic phosphate to channel catfish, however the phosphate from phytin appeared to alleviate haematocrit reduction in these fish. This contradictory result, in which the phosphorus appeared able to satisfy one physiological requirement (increase of haematocrit) but not another (growth), was related to the observation made by Waldroup et al. (1963) in chickens. Here phytin phosphorus allowed adequate bone mineralisation but was unavailable for growth.

Besides plant and animal proteins, a new class of proteins have lately become available for incorporation into fish diets: the single cell proteins or SCP's. These appear to be very good sources of phosphorus, which occurs as a component of nucleic acids. Feed proteins contain some nucleic acids, but in these compounds, which are produced from simple organisms (prokaryotes) lacking a nuclear membrane , up to 5% of the nitrogenous material may be non-protein nitrogen (NPN) - mostly nucleic acids. These compounds contain organic phosphorus, which is available to all types of fish. The role of these proteins as sources of phosphorus (and other minerals is described in section 2.2.)

2.1.3. Magnesium

Magnesium is an essential dietary mineral for all freshwater fishes. It is involved in many enzyme systems as a cofactor and may occur as a component of metalloenzyme molecules.

The primary source of magnesium for freshwater fish is the food, and deficiency symptoms have been described by several authors. Characteristic symptoms include extreme sensitivity to external stimuli, such as light, vibration or movement, and metastatic calcification of the soft tissues, especially the kidney. This is accompanied by an increase in the total calcium content of the carcass.

Ogino and Chou (1976) determined the dietary requirement for carp to be a minimum of 400-500 ppm of the diet. Deficiency induced high mortality, poor growth, sluggishness and convulsions. Cowey et al. (1977) have described a renal calcinosis in rainbow trout produced by a dietary imbalance of Ca:Mg. O'Dell (1960) showed that a high intake of dietary calcium and phosphorus aggravated a magnesium deficiency in terrestrial animals, and increased the magnesium requirement. It is not clear if this situation would occur in fish, whose physiologies are considerably more 'open' than those of non-aquatic animals. Arai et al. (1974) suggested a magnesium requirement for the eel as 400 ppm in the diet, a value similar to that given above for carp. Ogino (1975) found the requirement of rainbow trout to be 600-700 ppm. of the diet.

In salt water, magnesium deficiency is unlikely to occur, as

the Mg ion is present at high concentration. As fish drink continually in the sea, a constant intake of magnesium is assured. The major part of the magnesium thus ingested remains in the lumen of the intestine, despite the presence of a concentration gradient favouring its removal (Hickman, 1968; Shehadeh and Gordon 1969). The walls of the intestine seem to partly exclude both magnesium and sulphate ions. An average of 15.5% of the magnesium and 11.3% of the sulphate ions are seen to cross the intestinal wall into the bloodstream and are subsequently excreted by the kidney. The exclusion can therefore be seen to be selective, the more so when the diffusion rate of magnesium has been shown to be 37% faster than that of the sulphate ions, despite the diameter of the hydrated magnesium ion being larger than that of the sulphate ion (Potts and Parry 1964). Since more magnesium than sulphate enters the blood, proportionately more magnesium is excreted by the kidney. There is therefore a greater ratio of magnesium to sulphate in fish urine than in sea water.

The above explains the failure to define a magnesium requirement for sea fish such as red sea bream (Sakamoto and Yone 1979), and Atlantic salmon (Lall and Bishop 1977).

There is little storage of magnesium in fish tissue, relative to calcium, and the regulation of magnesium in the carcass is uncertain. In man, the renal excretion of magnesium has been shown to fall in response to low levels of dietary magnesium, but no data for fish in similar circumstances are available.

2.1.4. Zinc

The high toxicity of dissolved zinc to fish has been extensively studied, and there exists a considerable literature on this subject. Only in the past few years, however, has zinc been identified as an essential trace element in the diets of fish. The biochemical role of zinc is primarily as an enzyme cofactor, for digestive and other enzymes, notably the carboxypeptidases in the acrosome of spermatozoa. Radioactive tracer studies have shown that zinc is concentrated in the acrosome. Deficiency of zinc has been shown to produce defective spermatogenesis, and reduced fertility. Ketola (1978) demonstrated deficiency symptoms in trout to be poor growth and bilateral cataract. These fish were fed diets containing white fish meal, which had a zinc content of 60 ppm., but the symptoms were alleviated by a further 150 ppm. hydrated zinc sulphate. This level was high compared to that suggested by Ogino and Yang (1978) for rainbow trout. These authors found that growth was optimal in fish given 15-30 ppm. zinc in a test diet. This does not however invalidate the results of Ketola, as the diets used by the latter contained fishmeal, which is known to contain large amounts of minerals such as calcium and phosphorus. It is possible that the presence of these minerals may affect the zinc availability. Ogino and Yang (1978) studied the symptoms of zinc deficiency and performed an experiment to investigate recovery from it. Deficiency was seen to be characterised by an initial lack of symptoms, the first response being a decrease in appetite

after six weeks on the deficient diet. After eight weeks the fins were seen to degenerate by erosion and eye cataracts developed. Mortalities occurred, and when the fish were returned to a diet containing adequate zinc a further 20% mortality occurred during recovery. The appetite improved after only a short period of feeding with the complete diet. The cataract was however irreversible. The eyes of the fish were analysed for zinc and were found to be low in zinc in deficient fish. As the levels of zinc in the diet were decreased, so also did the zinc content of the whole carcass, and especially the intestine and the eyeball. Protein digestibility was decreased by 30% as a result of zinc deficiency, and the digestion of carbohydrates was also impaired. 5 ppm dietary zinc was shown to prevent the onset of deficiency symptoms, but the growth of these fish was less than that of fish fed 15-30 ppm. Zinc deficiency induced high ash levels in the carcasses, and iron and copper levels inversely proportional to the zinc level.

Lunde (1973) showed that the zinc content of the meal produced from sea fish had in it a higher ratio of zinc to cadmium than sea water, indicating a preferential retention or uptake of zinc over cadmium in these fish.

2.1.5. Iron

Iron is essential for the formation of the respiratory pigment haemoglobin, and is an important enzyme cofactor. A deficiency syndrome has been demonstrated in both marine and freshwater species, and is characterised by an hypochromic microcytic anaemia. This has been seen in brook trout (Kawatsu 1972), carp (Sakamoto and Yone 1978a), red sea bream (Ikeda et al. 1973) and eels (Arai et al. 1975). Fish appear able to absorb some iron from the water, for Roeder and Roeder (1966) showed that the growth of the swordtail (Xiphophorus helleri) and platyfish (Xiphophorus maculatus) improved when ferrous sulphate was added to the aquarium water. Ferric nitrate had no effect, and the effect of ferrous sulphate was shown to be temporary, continual supplementation being necessary for a continued effect. The ferrous salt was observed to be rapidly converted to a precipitate of the inactive ferric hydroxide. The effect of iron in the water was not seen after the fish were sexually mature, and maturity occurred earlier in the fish in supplemented water.

Hevesy et al (1964) showed that plasma iron was absorbed by fish erythrocytes at a rate dependent upon temperature. Sakamoto and Yone (1979B) tested three different iron compounds in the diet of yellowtail and found that the ferrous salts were absorbed more than ferric salts. Ferric chloride was found to be better absorbed than ferric citrate. Hydrochloric acid secreted by the stomach was shown to increase the absorption of ferric salts to levels as high or higher than levels of absorption of ferrous

salts. Ascorbic acid is known to improve iron absorption, possibly by conversion of ferric to ferrous forms, or by forming a readily absorbed complex with the mineral (M.A.F.F. 1976).

2.1.6. Manganese

Manganese, like magnesium, zinc and iron, is an important enzyme cofactor, and has been shown to be essential for adequate growth in fish, both freshwater and marine. Lall and Bishop (1977) demonstrated a requirement for dietary manganese in the Atlantic salmon, (*Salmo salar*), grown in sea water. Ogino and Yang (1980) suggested that the optimal level of dietary Mn for growth of rainbow trout was between 4-13 ppm. The lower level was observed to induce poor growth and bone deformities, notably a shortening of the carcass and abnormal tail formation. The deficiency of dietary Mn was found to have a marked effect on the vertebral manganese content, which fell by a factor of 3 in the deficient fish. Knox et al. (1981b) used larger fish than Ogino and Yang to investigate the effects of low manganese intake on rainbow trout. They showed no effect on growth of feeding diets containing 1.3 ppm. Mn. However, this level of intake reduced the Mn concentration in the liver and the vertebrae, and also reduced activity of a manganese-containing enzyme (Mn-Superoxide dismutase) in the heart and liver. The function of the superoxide dismutases is to remove superoxides (strong oxidising agents, the occurrence of which may be increased by the ingestion of rancid fats with a high peroxide content) from the cytoplasm, thus preventing free radical damage to the cell. The vertebral Ca and P levels were shown by Knox et al. to be reduced in Mn deficient fish, and these authors related this lowering of bone mineralisation to a possible role of Mn in mucopolysaccharide

(MPS) production, specifically in the activity of glycosyltransferases. Ogino and Yang (1980) found that the dietary Mn optima for both carp and rainbow trout were similar: 12-13ppm., but the definition of the optimal level relied upon the observation that fish fed this level had a better growth rate and a higher tissue mineral content than fish given 4 ppm. In fact the true optima may lie anywhere above 4 ppm, given the data presented by these authors. Knox et al. (1981b) found no difference in growth of 15g fish given 1.3 and 33 ppm. Mn respectively. The two studies described therefore indicate that there is a dietary requirement for rainbow trout, and that the optimal level is above 4 ppm., but the exact level is uncertain, and may vary with the size or species of fish used.

2.1.7. Copper

The greater part of the literature concerning fish and copper details the high toxicity of this metal when dissolved in water. However, copper has been shown to be an essential mineral in the diet of carp and rainbow trout (Ogino and Yang 1980). The dietary optimum for both species was suggested as 3.0 ppm, although this was merely based on an observed growth improvement against one other diet containing 0.7 ppm. The optimum should therefore be above 0.7 ppm., but the exact level remains undefined. Knox et al. (1982) found no effect on growth of increasing the Cu content from 15 to 150 ppm in trout diets. The higher concentration raised liver copper by a factor of 2.4. This result contrasts with those of Murai et al. (1981) who found that more than 4ppm.copper in diets for channel catfish reduced growth and feed conversion. In this study fish given more than 8.0 ppm. Cu were found to grow significantly less than fish given 2.0 ppm. The growth was related to the feed conversion. From the results of Murai et al. (1981), Knox et al. (1982) concluded that the tolerance of rainbow trout to high dietary copper was greater than that of channel catfish. The latter workers also showed that in rainbow trout, the levels of plasma copper were not correlated with dietary copper, nor was the activity of the copper-containing enzyme Zn-Cu-superoxide dismutase in the liver.

Wilson et al. (1981) surveyed wild populations of rainbow and brown trout in environments with and without copper pollution from mine wastes. They observed that the level of copper in the

livers of healthy trout from unpolluted waters was 76 ppm., and as high as 270 ppm. in polluted water. The liver copper levels observed by Knox et al. (1982) ranged from 63 to 150 ppm. These results suggest that 15 ppm. (The lowest level used by Knox et al.) is slightly suboptimal compared to the wild fish. This suggestion is supported by the fact that the fish given 12-13ppm. Cu by Ogino and Yang had 34.4 ppm. Cu in the livers.

Knox et al.(1982) found that the levels of zinc in the diets of the fish, and the ratio of zinc:copper had no effect on the copper levels in the liver. This observation contrasted with the results of Bremner et al. (1976), who found that zinc in the diets of sheep may protect against copper toxicity. However, the results of Wilson et al. (1981) indicate that the levels of copper induced in the fish livers by Knox et al. were below those of fish in toxic waters. Thus it would appear that to induce liver copper levels in trout as high as those seen in polluted natural waters would require dietary levels above 150 ppm Cu.

Cu is involved in the mobilisation of iron reserves as the Cu-protein complex ceruloplasmin (Beerstecher 1979).

2.2. Single-cell Proteins and mineral metabolism

The rising price, fluctuating availability and variable composition of fishmeal has in recent years prompted much research into alternative sources of protein in animal feeds. Despite this, most animal feeds in the United Kingdom still contain fishmeal. In the case of feeds for fish, fishmeal may be the only protein source, included as up to 65% of the dry ingredients. This dominance by fishmeal as the protein source for fish diets has engendered considerable interest in fishmeal replacement specifically in these feeds. A number of protein sources have been tested as substitutes for fishmeal in fish food, notably soyabean meal, animal waste and industrial raw fish, and industrial by-products. Single-cell proteins (SCP's) have been shown to be among the most promising of the fishmeal replacers tested to date (Sherwood 1974; Kaushik and Luquet 1980; Tacon 1978).

Although SCP's are often discussed as a discrete class of proteins, they are manufacured from a wide range of organisms. SCP's may be of algal, fungal or bacterial origin, and each of these classes represents a growing number of species, each of which has its own particular composition and nutritional properties. Mahnken et al. (1980) found that complete substitution of fishmeal with alkane yeast in trout diets did not affect intake, growth or conversion. In salmon diets, more than 25% substitution of fishmeal suppressed growth by more than 4%. Beck et al. (1978) reported that fishmeal and other protein

sources in trout diets could be almost completely replaced by yeast or methanol bacteria, provided supplemental amino acids were given. Atack et al. (1979) found that the growth of carp fed bacterial protein was faster than that of fish fed herring meal or casein/gelatin mixture. Carp fed on yeast diets grew more slowly, and carp fed on an algal protein least of all. Nose (1974c) found that yeast diets for trout were improved by supplementation with up to 5% cysteine, but that methionine supplementation was ineffective. The growth of rainbow trout was found to be improved by cysteine and arginine supplementation (Nose 1974b). Although diverse, SCP's do possess some common properties, notably the relatively high proportion of non-amino acid or non-protein nitrogen (NPN). SCP's may contain up to 20-30% NPN (Tacon 1979) whereas conventional feedstuffs typically contain 1-2% NPN. This difference is due to the fact that the simple organisms from which SCP's are made have a greater amount of genetic material in the cell than the higher animals and plants from which other feed proteins are derived. The greater part of the NPN is nucleic acids. The metabolic end product of nucleic acids is uric acid, which is of low solubility and thus cannot be easily excreted. In vertebrates other than man and the higher primates, uric acid is converted by the enzyme urate oxidase to soluble allantoin, which is more easily excreted. Sanchez Muniz et al. (1978) found however that the uric acid levels in trout kidney were significantly raised by a diet containing 50% yeast protein (Hansenula anomala).

In SCP's, phosphorus availability is generally good. Fishmeal also contains much phosphorus, but in this case, it is combined with calcium in fragments of hard tissues, and may not be as easily digested as phosphorus from SCP's. Ogino et al (1979) reported that the digestibilty of phosphorus in SCP was 90% in rainbow trout, but fishmeal phosphorus was only 60% digestible. The absolute levels of minerals differ in different SCP's, and between SCP'S and other protein sources. In general, SCP's have a higher sodium content than fishmeal, but lower calcium, potassium and iron. In this respect, they resemble plant material rather than animal, but their amino acid spectra are usually more similar to animal proteins than plant. The comparison of SCP's with each other and the many materials which have been used in fish diets would require more space than is available here, so the table 2.2.1. of the mineral levels of fishmeal and 'Pruteen', the SCP used throughout the research described herein, must suffice to demonstrate these differences.

Table 2.2.1. Minerals in Fishmeal and 'Pruteen'

Mineral	Level in Pruteen	Level in Fishmeal
Sodium	1.5 (%)	0.7 (%)
Potassium	0.15 ''	0.7 ''
Calcium	0.07 ''	4.34 ''
Phosphorus	2.3 ''	2.14 ''
Magnesium	0.221 ''	0.18 ''
Zinc	21.4 (ppm.)	48.7 (ppm.)
Iron	143 ''	245 ''
Copper	6.8 ''	8.3 ''
Manganese	15.4 ''	8.95 ''

The visible differences (Table 2.2.1.) provided a base on which to construct an experimental programme to investigate the incorporation of Pruteen into trout diets. An hypothesis was proposed to account for the results of previous nutritional trials using Pruteen. When Pruteen was gradually substituted for fishmeal in trout diets by workers at I.C.I., growth and feed conversion improved up to 25-30% of the dry diet. More than this suppressed growth and feed conversion. The hypothesis proposed that this effect was due to Pruteen being a better protein source than fishmeal, but an inferior mineral source. In combination, protein and mineral levels would be optimal in fishmeal diets with 25-30% of the dry matter as Pruteen. Below or above this, an imbalance of either protein or minerals suppressed growth. The

hypothesis therefore implied that if a suitable mineral supplement could be developed, the optimal level of inclusion could be raised considerably from 25-30%, perhaps even to 100%.

This thesis describes how the hypothesis was tested, the results obtained, and the conclusions drawn from these.

3. METHODS & MATERIALS

3.1. Experiments

3.1.1. Experiment Construction:

All the experiments in this research project were designed according to the factorial principle. This type of experimental design is more efficient than the single-factor or one-way design, as each observation provides information about all the treatments. This removes the need for a control, as each treatment is in effect, both an experimental and a control treatment.

The application of the factorial principle is fully described in Snedecor & Cochran (1974) and Bailey (1959).

There are many variations of the factorial design, but for simplicity of construction and analysis, the randomised block design described by Bailey was used. In horticultural trials, 'randomised block' analysis is self explanatory, experimental treatments being replicated into randomly assorted blocks of land, which allows variations of soil fertility to be discounted from the interpretation of the results by analysis of variance. In the present experiments, the concept of random blocks was transferred to individually labelled fish as replicates of a treatment, allowing the variation in response between fish to be eliminated when the variation between treatments was examined. The effects of the variation in growth between different fish were thus removed in the analysis of the results (see section 4).

Labelling fish also enabled the growth of each fish to be recorded, thus greatly increasing the number of observations which could be made during the experiment, and increasing the sensitivity of the experiments.

By the use of the factorial method, each experiment tested several null hypotheses. The five experiments each studied a number of dietary factors, but whilst conventional designs of experiment might test the effects of one factor on fish growth, the factorial analysis allowed the simultaneous testing of the effects of more than one factor, and any interactions which occurred between them.

The factorial analysis could have given spurious results in certain circumstances. Because only two levels of the factors were used, there was a risk that if a factor had an optimal value, it would be shown to have no effect or an incorrect effect, if neither value used were near-optimal. For example, Arai et al. (1975) showed a dose response curve for calcium in trout diets. If a factorial experiment set up to determine the effect of Ca in the diet had used very low and very high levels of Ca the optimal level would be missed, and the result would probably show no effect of calcium. Therefore, careful consideration of levels of dietary factors likely to be optimal was necessary before diet formulation.

The two values of each factor used were absence (zero) or presence at the concentration customarily found in trout diets. The factors were all dietary ingredients, with the exception of

Experiment V where the concentration of calcium in the water was altered and thus became a factor in the experimental design. Dietary factors were used because the research program was based on the testing of "Pruteen" which is a feed ingredient; however fish size, water flow rates, temperature, salinity or any other experimental variable could be incorporated as a factor in a factorial experiment. The choice of factors in a factorial experiment depends on the information sought.

The availability of tanks prevented more than three factors being tested simultaneously. In the 2^n design, n factors are used at 2 levels, one of which may be an absence of the factor. It will be appreciated that with three factors, 2^3, (8) treatments are needed to accomodate all the possible combinations of the factors. To test four factors, 2^4 (16) treatments would be needed, which could not be accomodated in the 8 or 9 tanks that were available. So, every factor added to the 2^n experiment doubles the number of treatments needed. This limits the number of factors that can be studied in one trial. The nine tanks could have been used in a 3^2 factorial experiment, i.e. 9 treatments needed, but in this case 3 levels of 2 factors would be tested, requiring a more complex analysis and testing fewer factors than the 2^3 design.

When 9 tanks were available, the 2^3 factorial design was used, with a ninth treatment: a commercial diet given as a comparator control. When this was compared with the other treatments, a simple one-way analysis of variance was used to check the difference between the responses to experimental treatments and the conventional diet. Thus the experimental treatments could be compared with a treatment which approximated to the 'real world'.

3.1.2. Diet Formulations

The formulations of the diets used in the experiments are shown in Tables 3.1.2.1.to 3.1.2.4. With the exception of Experiment V, differences in the diets given to different groups of fish were the only experimental variables.

As each factor was either included or excluded, it was necessary to provide a substitute when it was omitted. Pruteen, the test protein/mineral source, (B), was substituted for fishmeal, (F), the conventional protein source, giving diets with either 65% Pruteen or 65% fishmeal in Experiments II and III. In Experiment VII Pruteen (B) was substituted for a casein/gelatin mixture (C).

The mineral mix (M) (Table 3.1.2.7.) was included at 4%, and when excluded, was replaced with 4% polypropylene granules, (I.C.I. Ltd. Billingham, U.K.) . Polypropylene granules were chosen in preference to the more conventional alpha-cellulose or polythene flour as a bulking agent, because the polypropylene is in the form of smooth surfaced granules. These are c. 0.1mm in diameter, and present a low surface area for a given inclusion level when compared with other fillers. Thus the potential area on which nutrients could be adsorbed was minimised, and the possibility of interference with digestion reduced. Polypropylene was also used as an inert substitute for the trace elements (T) and sodium phytate (A), where these ingredients were tested.

All the diets in all the experiments were made from a basic premix to which other ingredients were added. This basic premix

is taken from the U.S. National Research Council (1973), the modification of the Oregon Test Diet (Oregon Fish Comm. 1972), (Table 3.1.2.5.) omitting the mineral mix and casein/gelatin mix, and substituting 7% maize oil ("Mazola") and 3% cod liver oil (The Boots Co. Ltd. Nottingham) for the 10% salmon oil specified in the Oregon formulation.

The Vitamin mix incorporated into the premix was supplied by I.C.I. Ltd., to the specifications given in Table 3.1.2.6. (U.S. National Research Council Vitamin Premix No 3. 1973).

In Experiments II, III and VI trace elements were supplied at a level of 0.02% of the diet. The formulation of the trace element mix is given in Table 3.1.2.9..

Table 3.1.2.1. Formulations of the diets: Expt.s II and III

% in Diet	F	FM	FT	FMT	B	BM	BT	BMT
Fishmeal	65.0	65.0	65.0	65.0	0.00	0.00	0.00	0.00
Pruteen	0.00	0.00	0.00	0.00	65.0	65.0	65.0	65.0
Mineral mix	0.00	4.00	0.00	4.00	0.00	4.00	0.00	4.00
Trace element mix	0.00	0.00	0.02	0.02	0.00	0.00	0.02	0.02
Polypropylene	5.00	1.00	4.98	0.98	5.00	1.00	4.98	0.98
Premix	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 3.1.2.2. Formulations of the diets: Expt. V

% in Diet	B0	B4
Pruteen	65.0	65.0
Mineral mix	0.0	4.0
Polypropylene	4.0	0.0
Premix	31.0	31.0
Total:	100.0	100.0

Table 3.1.2.3. Formulations of the diets: Expt. VI

% in Diet	B	BA	BM	BAM	BT	BAT	BMT	BAMT
Pruteen	65.0	65.0	65.0	65.0	65.0	65.0	65.0	65.0
Mineral mix	0.00	0.00	4.00	4.00	0.00	0.00	4.00	4.00
Trace element mix	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.02
Phytic acid	0.00	0.6	0.00	0.6	0.00	0.6	0.00	0.6
Polypropylene	5.00	4.4	1.00	0.4	4.98	4.38	0.98	0.38
Premix	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 3.1.2.4. Formulations of the diets: Expt. VII

% in Diet	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe
Casein	55.28	55.28	55.28	55.28	0.00	0.00	0.00	0.00
Gelatin	9.72	9.72	9.72	9.72	0.00	0.00	0.00	0.00
Pruteen	0.00	0.00	0.00	0.00	65.0	65.0	65.0	65.0
Calcium carbonate	0.00	3.457	0.00	3.457	0.00	3.457	0.00	3.457
Ferric citrate	0.00	0.00	0.119	0.119	0.00	0.00	0.119	0.119
Polypropylene	5.00	1.543	4.881	1.424	5.00	1.543	4.881	1.424
Premix	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 3.1.2.5. Formulation of the Premix:

Ingredient	% in Premix
Dextrin	28.3871
Alpha-cellulose	19.3548
Carboxy-methyl cellulose	9.6774
Corn oil	22.5806
Cod liver oil	9.6774
Vitamin mix	6.4516
Vitamin E	0.6451
Choline chloride	3.2260
Total:	100.00

Table 3.1.2.6. Formulation of the Vitamin mix:

Ingredient	% in Vitamin mix
Thiamine (HCl)	0.3200
Riboflavin	0.7200
Niacinamide	2.5600
Biotin	0.0080
Calcium pantothenate (D)	1.4400
Pyridoxine (HCl)	0.2400
Folic acid	0.0960
Menadione	0.0800
B12 (Cobalamine 3000ug.g-1)	0.2667
i-Inositol (meso-)	12.5000
Ascorbic acid	6.0000
Para-amino benzoic acid	2.0000
Vitamin D2 (500,000 USP.g-1)	0.0400
Butylated hydroxy anisole (BHA)	0.0750
Butylated hydroxy toluene (BHT)	0.0750
Vitamin A (250,000 IU.g-1)	0.5000
Celite (Inert bulking agent)	73.0793
Total:	100.000

Table 3.1.2.7. Formulation of the Mineral mix:

Ingredient	% in Mineral mix
Calcium biphosphate	13.58
Calcium lactate	32.70
Ferric citrate	2.97
Magnesium sulphate	13.20
Potassium phosphate (dibasic)	23.98
Sodium biphosphate	8.72
Sodium chloride	4.350
Total:	100.00

Table 3.1.2.8. 4% Mineral Mix Supplies:

Mineral	Level in Diet
Na	0.12 (%)
K	0.43 ''
Cl	0.11 ''
Ca	0.30 ''
B	0.34 ''
Mg	520 (ppm.)
S	700 ''
Fe	198 ''

Table 3.1.2.9. Formulation of the Trace Element mix:

Ingredient	% in Trace Element mix
Aluminium chloride	2.885
Potassium iodide	2.885
Cupric chloride	1.92
Manganous sulphate	19.23
Zinc sulphate	57.69
Total:	100.00

Table 3.1.2.10. 0.02% Trace Element Mix Supplies:

Mineral	Level in Dry Diet
Zn	27.3 (ppm.)
Cu	25.7 ''
Mn	69.9 ''
Al	0.67 ''
I	14.09 ''

3.1.3. Individual Experimental Designs:

Experiment II

This experiment was a 2^3 factorial design, testing the effects and interactions of three factors: dietary protein source (F or B), dietary bulk mineral source (M), and dietary trace element source (T). These three factors were combined in eight diets (See Table 3.1.2.1.), which could be described as: F (fishmeal only, or "control"), FM (fishmeal and minerals), FT (fishmeal and trace elements FMT (fishmeal and minerals and trace elements), B (Pruteen only), BM, BT,and BMT. The proximate compositions of the diets as fed are given in Table 3.1.5.2..

Eight fish were randomly allotted to each of 8 tanks (see section 3.1.1.,3.3.2.), and were randomly labelled by freeze branding (see section 3.2.5.). The fish were grown for seven weeks on the experimental diets; at the end of this period four fish were taken for analysis from each treatment. The fish were weighed once per week during the experiment.

Experiment III

Experiment III replicated Experiment II. The same apparatus as Experiment II was used,though a larger number (20 per tank) of smaller fish were grown for a longer period (8 weeks). A commercial diet (E) was given to one treatment as a comparator control. The formulations of the diets are shown in table 3.1.2.1. and their proximate compositions in table 3.1.5.4.

The fish were marked as Experiment II, but during the

experiment the fish were weighed every two weeks. Three fish were taken for analysis from each treatment every two weeks, and six fish from each treatment were retained at the end of the experiment.

Experiment V.

Experiment V tested the effect of the mineral supplement on Pruteen diets and the level of calcium in the environmental water. This experiment was performed using recirculated water (see section 3.3.2.).

Thirty fish were used in each treatment and the experiment lasted 50 days. Fish were weighed and samples taken for analysis after 25 days and at the end of the experiment.

The formulations and proximate analyses of the diet are shown in Tables 3.1.2.2. and 3.1.5.6. Two diets were used, each diet being given to two groups of fish, one group in normal water, the other group in water with calcium concentrations raised by filtration through oyster shell. This design required four treatments (tanks), B0-, B4-, without oyster shell; B0+, B4+, with oyster shell. The experiment therefore studied effects of two factors, dietary mineral supplementation and water calcium elevation. To achieve the difference in water hardness, the water in the systems requiring higher calcium concentrations was recirculated through a filter bed of crushed oyster shell, which gradually dissolved into the water. This increased the total hardness in the water to c. 40ppm. whereas the total hardness in systems with gravel filters was c. 20ppm. Oyster shell has

been recommended by several authors as a suitable material to add to recirculating fish culture system filters,(Bower et al. 1981, Burrows 1972, Atz 1964) in order to maintain a high pH. When nitrogenous waste is oxidised to nitrite and nitrate, the pH of the water falls, and the addition of calcium carbonate (oyster shell) buffers the water by the reaction of carbonate and H+ ions. This forms carbon dioxide, (which dissipates from the system), and water. Thus the pH should be maintained at a higher level than would occur in the absence of the carbonate. In this experiment, however, no differences were observed between the pH of the water in the systems with oyster shell filters and those with gravel filters. The process also introduces calcium into the water, as the calcium carbonate dissolves. This experiment tested the assumption that the increase in Ca was of no consequence. Cuplin (1969) showed no effect of water hardness on the growth rate of trout in American hatcheries. Brown (1946a) reported a correlation between water hardness and growth of trout in natural conditions. It was found that the growth rate of wild trout in soft and hard waters was the same after four years, but before this age trout in soft waters grew more slowly. At four years, wild brown trout are mainly piscivorous, and thus less dependent on the invertebrate fauna for food. Hard waters have been shown to support greater invertebrate populations than soft waters.

Experiment VI.

Experiment VI tested the effect of minerals (M) and trace elements (T) in diets containing Pruteen (B), in part therefore a

repeat of Experiment II, and also the effects of sodium phytate (A), which was included in the diets at levels which would occur if the diet contained 65% soybean meal (0.6%). Diet formulations are shown in Table 3.1.2.3., and the proximate compositions and mineral levels of the diets are shown in Tables 3.1.5.8. and 3.1.5.9. respectively.

In this experiment the fish were not labelled until they were killed for analysis as the growth response was felt to be less important than the mineral analysis. The fish were grown for twelve weeks in recirculated water, 40 fish per tank, of which fifteen were killed for analysis after six weeks. Eight tanks of fish were used. The fish were weighed every two weeks and thirteen fish were retained for analysis at the end of the experiment.

Experiment VII.

Experiment VII examined the effect of particular minerals: calcium supplied as calcium carbonate, (Ca) iron supplied as ferric citrate (Fe), and Pruteen as a protein source (B) tested against a casein/gelatin mixture (C). A factorial design was used to enable the effects of these factors to be examined, and any interactions which occurred between them. Calcium and iron were selected for testing because earlier experiments had shown that an interaction of Pruteen X a bulk mineral mix increased the intake and growth of trout. The bulk mineral mix supplied Na, K, Ca, Mg, S, Fe and P. Of these minerals, it was felt that Ca and Fe were particularly important since neither Ca nor Fe levels in

Pruteen are equal to those in fishmeal.(See Table 2.2.1.). Nose (1976) found that the USP-XII salt mix No. 2 could be replaced in the diet of eels by a mix containing Ca, Mg, Fe and P only. In Pruteen diets, phosphorus levels appear to be adequate without supplementation. Thus probable requirement will be for Ca, Mg and Fe. Magnesium was felt to be just as important as calcium and iron but, because it is accepted as a required mineral for fish, further proof of this requirement is not needed. Ca requirement is less certain, and this experiment offered an opportunity to test the results of Arai et al (1975), who showed that supplementation of trout diets with calcium lactate improved the growth of fish fed yeast. To test the effects of Ca on diets with Pruteen, calcium carbonate, as opposed to calcium lactate, was used as a supplement, because the latter contains some digestible energy, and the carbonate offered a nutritionally "purer" source of calcium with less likelihood of spurious results. Ferric citrate was used as an iron source, rather than an inorganic compound such as Fe (SO_4) since the amounts required were small. The potential influence of the citrate ions was therefore also small. The use of calcium carbonate was disadvantageous in that previous diets (Expts. I-IV) had contained calcium lactate and calcium phosphate in the mineral mix. Nonetheless it was felt that the use of calcium carbonate was preferable, for the reasons given above. A casein/gelatin mixture was chosen as the control protein, against which the effects of Pruteen could be measured. Casein and gelatin contain lower amounts of minerals than fishmeal, and allowed a more realistic comparison of the effects

of Ca and Fe on diets, whether containing Pruteen or not. Moreover, Ogino et al. (1979) have shown that the availability of phosphorus in casein/gelatin mixtures is the same as that in Pruteen, when used in trout diets. Casein/gelatin has a lower phosphorus content than Pruteen, and thus it was expected that the Pruteen diets would provide more phosphorus, given that the amounts of casein/gelatin or Pruteen incorporated would be the same.

Ca and Fe were the only minerals or trace elements supplemented to the diets. This offered both disadvantages and advantages. By supplementing only Ca and/or Fe, no interactions with supplemented trace elements or other supplemented minerals was possible, and thus any effects would be direct (rather than interactions with other inorganic nutrients). Moreover, by analysing the carcasses, the efficiency of Pruteen or casein/gelatin as a source of dietary minerals would be ascertained, since the protein source and the water would be the major sources of all minerals except Ca and Fe. Comparison of the carcass mineral levels with those of trout fed on a commercial diet or "comparator control", would reveal the influence of the dietary factors on uptake of these from the water. The major disadvantage offered by non-supplementation was that the fish would be deficient in essential nutrients and therefore grow slowly, if at all, and would digest and utilise the diets sub-optimally. The advantages were believed to outweigh the disadvantages, and thus the diets were prepared without a mineral or trace element premix, only Ca and Fe being added as

required.

The formulations are shown in Table 3.1.2.4. and proximate and mineral compositions in Tables 3.1.5.10. and 3.1.5.11. The comparator control diet made from moistened trout pellets ('EM') was used to compare the experimental diets with a complete ration.

Fish were held in nine tanks for sixteen weeks and were weighed every four weeks. The fish were not labelled, and at the end of the experiment 18 fish per treatment were retained for whole-carcass analysis and five fish were dissected to provide tissues for analysis.

3.1.4. Diet Preparation and Feeding method:

All diets were prepared and fed to the fish in a semi-moist form, with a moisture content of c.30%. This was achieved by adding water to the dry ingredients in the ratio of approximately two parts by weight of water to five parts by weight of dry mix. The amount of water required to bind the diets to the correct consistency varied.

To produce completely homogeneous pellets, the ingredients were all passed through a 1mm. sieve and then mixed for one hour. To produce homogeneous diets in Experiment III, the Pruteen and white fishmeal were powdered in a liquidiser (Kenwood Ltd. England) before they were sieved. If left as manufactured, the particles were found to be poorly mixed into the feed pellets, causing them to break up easily. In powdered form, the Pruteen acted as an effective binder, and the pellets containing it were found to be very stable in water. However, after experiment III, the Pruteen was only sieved to remove large particles, which avoided the problems of palatability induced by 'liquidising' it (see section 5.2.).

Diets were blended in a "Hobart" commercial food mixer and then extruded through a 2.5mm die. The level of moisture used resulted in long strands of diet which did not adhere to one another, and could be broken into pellets by crumbling them between the hands without drying.

After the diets had been pelleted, they were deep frozen until needed. Fish were fed once per day. Fish of the size used in these experiments are fed several times per day in commercial

units, but as the diets given were experimental and thus probably sub-optimal, feeding frequency was restricted. This has been shown to reduce size variation in fish fed sub-optimal diets (Burrows, 1972). Grayton and Beamish (1977) have shown that fish fed once per day can adapt to this feeding frequency and grow as fast as fish fed three or six times per day.

Before the diets were fed to the fish, they were placed in small containers in a refrigerator at 6 deg. C.

The diet containers were weighed before feeding and if found to be below a certain "refill weight", were filled to a "full weight". The fish were then fed to satiation. The "refill weight" was selected so that if the fish ate sufficient food to take the weight of the container plus contents below a certain level, there would always be some food left, i.e. the weight of food left in the container when the refill weight was reached would always be more than the fish could eat in one day. (4% of the total weight of fish in the tank.)

Water was seen to condense from the atmosphere onto the diet containers when they were removed from refrigeration to feed the fish. If the feed containers had been weighed directly after the fish were fed, the condensation would have caused inaccuracy. By weighing the containers directly after removal from the refrigerator, water build-up on the containers was minimised, and water from the previous day had evaporated.

3.1.5. Proximate and Mineral Compositions of the Diets

The proximate compositions of, and mineral levels in the diets are shown in Tables 3.1.5.2. to 3.1.5.11. The methods of analysis used to obtain the proximate compositions are shown in Table 3.1.5.1.

Table 3.1.5.1. Methods of Proximate Analysis

Nutrient	Method of Analysis
Protein (as Nitrogen)	Micro-Kjeldahl method (A.O.A.C. 1970)
Ether Extractables	Soxhlet extraction with 40:60 petroleum ether (A.O.A.C. 1970)
Ash	Loss of weight after ashing for 24 hours at 525 °C. in a muffle furnace.
Nirogen Free Extract	By difference

Table 3.1.5.2. Proximate compositions of the diets: Expt. II

Diet:	% Protein	% N.F.E.	% Eth. Ext.	% Ash
F	49.048	23.282	17.465	10.205
FM	48.433	23.818	17.015	10.734
FT	50.082	28.076	11.108	10.734
FMT	50.103	24.455	12.212	13.230
B	51.220	27.394	13.615	7.771
BM	51.802	23.362	14.722	10.114
BT	53.513	27.938	10.736	7.813
BMT	54.277	26.313	9.069	10.341

Table 3.1.5.3. Mineral Levels in Dry Diets: Expt II

(Values in ppm. except for P and Ca which are %)

Diet	P	Ca	Mg	Zn	Fe	Cu	Mn
F	1.3	1.73	1050	44.3	132	5.19	5.97
FM	1.64	2.03	1570	44.3	330	5.19	5.97
FT	1.3	1.73	1050	71.6	132	30.89	75.87
FMT	1.64	2.03	1570	71.6	330	30.89	75.87
B	1.50	0.2	1437	13.9	93	4.42	10.0
BM	1.84	0.5	1957	13.9	291	4.42	10.0
BT	1.50	0.2	1437	41.2	93	30.12	79.9
BMT	1.84	0.5	1957	41.2	291	30.12	79.9

Table 3.1.5.4. Proximate compositions of the diets: Expt. III

Diet:	% Protein	% N.F.E.	% Eth. Ext.	% Ash
F	51.067	17.105	18.905	12.908
FM	50.150	11.236	18.523	15.885
FT	51.898	17.234	17.925	12.957
FMT	54.316	12.707	17.009	15.969
B	50.657	26.806	15.435	7.102
BM	55.241	22.421	15.435	9.554
BT	55.146	21.845	15.861	7.148
BMT	52.385	22.124	15.923	9.568
E	54.627	19.853	12.987	12.532

Table 3.1.5.5. Mineral Levels in Dry Diets: Expt III

(Values in ppm. except for P and Ca which are %)

Diet	P	Ca	Mg	Zn	Fe	Cu	Mn
F	1.39	2.82	1170	31.6	159	5.39	5.82
FM	1.73	3.12	1690	31.6	357	5.39	5.82
FT	1.39	2.82	1170	58.9	159	31.09	75.7
FMT	1.73	3.12	1690	58.9	357	31.09	75.7
B	1.50	0.2	1437	13.9	93	4.42	10.0
BM	1.84	0.5	1957	13.9	291	4.42	10.0
BT	1.50	0.2	1437	41.2	93	30.12	79.9
BMT	1.84	0.5	1957	41.2	291	30.12	79.9
E	1.12	0.8	2192	184.4	326	19.1	53.78

Table 3.1.5.6. Proximate compositions of the diets: Expt. V

Diet:	% Protein	% N.F.E.	% Eth. Ext.	% Ash
B0	51.185	21.808	20.561	6.446
B4	50.500	19.883	20.705	8.912

Table 3.1.5.7. Mineral Levels in Dry Diets: Expt V

(Values in ppm. except for P and Ca which are %)

Diet	P	Ca	Mg	Zn	Fe	Cu	Mn
B0	1.5	0.2	1437	13.9	93	4.42	10.0
B4	1.84	0.5	1957	13.9	93	4.42	10.0

Table 3.1.5.8. Proximate compositions of the diets: Expt. VI

Diet:	% Protein	% N.F.E.	% Eth. Ext.	% Ash
B	51.225	27.511	16.427	4.837
BA	50.951	24.557	17.074	7.418
BM	51.840	22.448	16.499	9.213
BAM	54.636	19.131	17.404	8.829
BT	55.564	21.143	16.652	6.641
BAT	54.388	22.095	16.244	7.273
BMT	51.296	23.588	16.354	8.762
BAMT	54.105	23.548	14.746	7.601

Table 3.1.5.9. Mineral Levels in Dry Diets: Expt VI

(Values in ppm. except for P and Ca which are %)

Diet	P	Ca	Mg	Zn	Fe	Cu	Mn
BA	1.2	0.2	1437	13.9	290	4.82	10.0
B	1.0	0.2	1437	13.9	290	4.82	10.0
BAM	1.54	0.5	1957	13.9	388	4.82	10.0
BM	1.34	0.5	1957	13.9	388	4.82	10.0
BAT	1.2	0.2	1437	41.2	290	30.12	79.9
BT	1.0	0.2	1437	41.2	290	30.12	79.9
BAMT	1.54	0.5	1957	41.2	388	30.12	79.9
BMT	1.34	0.5	1957	41.2	388	30.12	79.9

Table 3.1.5.10. Proximate compositions of the diets: Expt. VII

Diet:	% Protein	% N.F.E.	% Eth. Ext.	% Ash
C	58.583	30.02	7.64	3.755
CCa	59.679	27.593	6.76	5.968
CFe	58.495	31.988	6.103	3.414
CaFe	58.715	29.799	5.724	5.762
B	48.423	26.192	17.551	7.834
BCa	46.819	23.897	19.646	9.638
BFe	46.866	24.247	19.689	9.198
BCaFe	46.795	24.147	18.318	10.74
EM	56.785	20.053	11.437	11.725

Table 3.1.5.11. Mineral Levels in Dry Diets: Expt VII

(Values in ppm. except for P and Ca which are %)

Diet	P	Ca	Mg	Zn	Fe	Cu	Mn
C	0.75	0.03	857	50.2	158	4.45	6.74
CCa	0.75	1.38	857	50.2	158	4.45	6.74
CFe	0.75	0.03	857	50.2	356	4.45	6.74
CCaFe	0.75	1.38	857	50.2	356	4.45	6.74
B	1.9	0.2	1437	41.2	290	4.82	10.0
BCa	1.9	1.55	1437	41.2	290	4.82	10.0
BFe	1.9	0.2	1437	41.2	388	4.82	10.0
BCaFe	1.9	1.55	1437	41.2	388	4.82	10.0
EM	2.2	2.96	2721	106.0	329	12.71	52.38

3.2. Fish

3.2.1. Species Used:

Rainbow trout (Salmo gairdneri), were used in all the experiments during this research project. Trout were obtained from several sources, depending on the availability of fish of an appropriate size i.e. fingerlings, c. 3.25g in weight.

3.2.2. Quarantine Procedure:

After delivery to the Fish Culture Unit , trout were placed in the quarantine facility, which consists of a series of 500-litre fibre-glass tanks, supplied with through-flowing mains water at a rate of 5 litres per minute.

During the quarantine period of four weeks, the fish were fed on a commercial trout food (Edward Baker No. 002), which was given ad libitum once each day.

In the second week of quarantine, the trout were bathed in a solution of malachite green as a prophylactic measure against ectoparasites and fungi.

Ten fish were killed during the first two weeks of the quarantine period, for pathological examination. Further post mortem examinations were carried out if any pathogens were detected, or if any mortalities occurred. On one occasion an heavy infestation of the protozoan parasite Octomitis salmonis, (Hexamitis truttae) was identified by microscopic examination of the intestinal tract. Treatment with calomel (mercurous chloride, HgCl) at a level of 0.2% in the diet for four days proved successful. No parasites were seen in the gut or the faeces after

the treatment, and no mortalities occurred. The quarantine period was extended by two weeks in this instance to allow the fish to recover completely before the experiment began.

One problem was caused by the quarantine facility itself. This was the ingestion of rust by the trout. The iron frames which supported the wooden lids of the tanks were considerably corroded by the constant moisture present. When the lids of the tanks were raised particles of rust fell into the water and were eaten by the fish. When the trout were transferred to the experimental tanks they continued to excrete rust particles in the faeces and re-ingest them. Despite regular cleaning of both quarantine and experimental tanks, rust particles were found in the intestines of fish eight weeks later. The gut contents were carefully removed from trout taken for analysis, but even so, the presence of stray particles of rust may have affected the iron determinations in some fish carcasses. (See Sect. 4.2.2.).

3.2.3. Weighing:

After the fish had been quarantined, they were divided into groups by random net sampling and placed in the experimental tanks. The fish were then removed tank by tank and weighed individually in a tared plastic bowl on a top-pan balance. It was found that as the trout were so small, they did not need to be anaesthetised, lying quietly in the bowl whilst being weighed. Anaesthesia (sect. 3.2.4.) was not found by Barton and Peter (1980) to alleviate handling stress in fish. Anaesthesia is itself stressful, and was therefore avoided where possible.

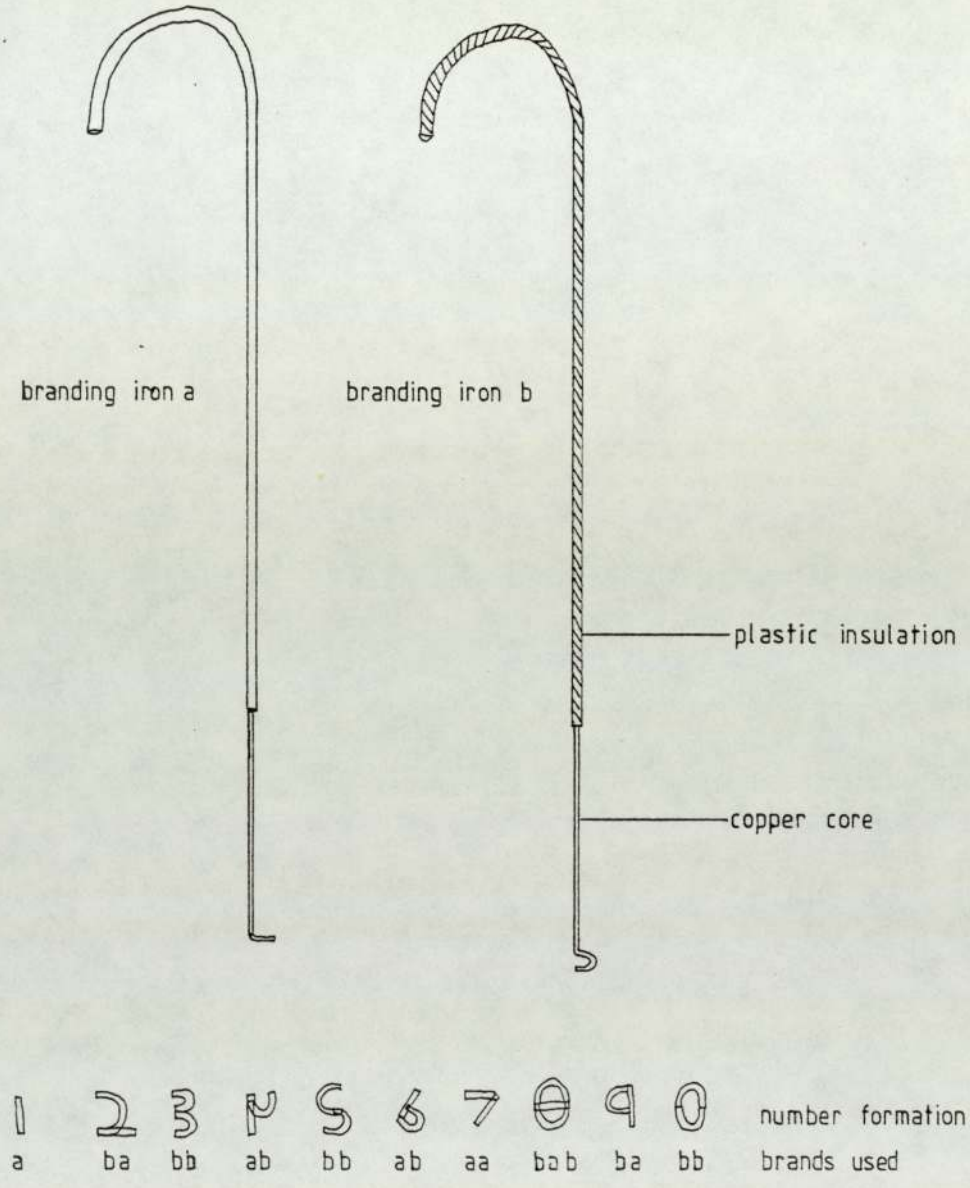
3.2.4. Anaesthesia:

If the fish were to be marked for individual identification they were first anaesthetised. A suspension of 'Benzocaine' (diaminobenzene) in water was used as anaesthetic, at a concentration of 0.05g per litre. The suspension was prepared by dissolving 0.5g of the benzocaine powder in a minimal quantity of acetone, and then mixing this solution into ten litres of water in a bucket. Fish placed in this suspension gradually lost equilibrium, and full anaesthesia, as demonstrated by the loss of the 'optic rotation' reflex was seen after about ten minutes. (The 'optic rotation' reflex is seen if a fish is removed from water and held on its side, when the eye will be seen to 'look down' from the fish's point of view. If the fish is unconscious this reflex is lost, and the eye remains as if the fish were 'upright'.) The fish could then be handled out of water for some minutes with 100% recovery on return to the tanks. Up to 5% mortality occurred in a test to achieve faster anaesthesia by using higher concentrations of benzocaine.

3.2.5. Freeze Branding:

After initial weighing, the trout were either marked or returned directly to the tanks. The technique of freeze branding (Mighell 1969) was used to mark fish with numbers 1 to n where n was the number of fish in the tank.

Fig. 3.2.5.1. Freeze Branding Iron Design and Use.



Two freeze branding irons were required (See Fig. 3.2.5.1.) to mark the fish with numerals 0 to 9. The branding irons were made from 2.5mm single strand copper wire, each with a different coloured insulation. The colours enabled the appropriate brand to be selected when the ends were submerged in liquid nitrogen (the coolant), and the insulation protected the user's hands from the cold metal. The fish were branded by placing the cold ends of the brands against the skin of the upper left side behind the operculum. The orders in which the brands were applied are shown in Fig. 3.2.5.1.. Care was needed to ensure that the branding iron was not in contact with the skin for too long, causing tissue death beneath the epidermis. In practice, a contact period of about one second was found to give clear marks with minimal tissue damage. During contact the skin could be seen to whiten under the branding iron, which enabled the next brand to be placed in correct relation to the first. Once the fish had been returned to the water the brand marks could hardly be discerned for the first day, after which the brands developed as scale free numbers on the skin. The numbers were sufficiently clear to be read from outside the tank, and were still legible at least ten weeks later. (Ten weeks was the longest period over which branded fish were observed, the marks would probably persist for a longer time, depending on the growth rate of the fish. Numbers on fast-growing fish were seen to fade sooner than those on slow growing fish.)

3.2.6. Experimental Procedure:

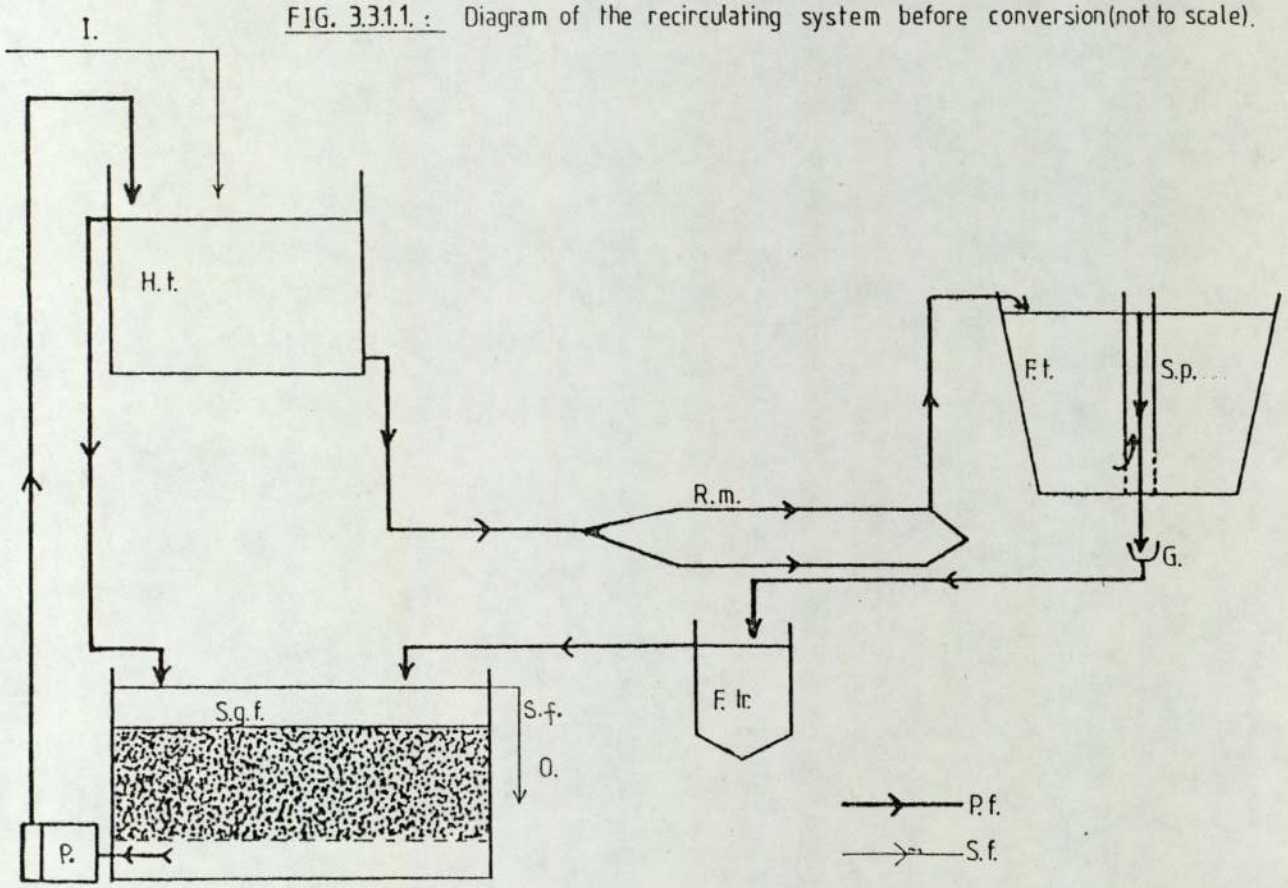
After the fish had been branded or returned directly to the experimental tanks, they were left to recuperate. The next day, the experimental diets were given for the first time. This was taken as being the first day of the experiment. At regular intervals thereafter, the fish were netted from the tanks and weighed. Where necessary, fish were selected using random number tables and killed by a sharp blow across the head behind the eyes. The carcasses were then stored for analysis (See section 3.4.). At the end of the experiment the remaining fish in each tank were weighed and killed as above, and the carcasses preserved for analysis.

3.3. Fish Tank and System Design:

3.3.1. General Considerations:

The first limiting factor in any fish culture enterprise is the supply of water. In this respect, experimental trials are no different from commercial operations. To overcome the restriction of water supply coincident with a city-centre location, the Fish Culture Unit is equipped with recirculating systems of the type shown in Fig. 3.3.1.1.

FIG. 3.3.1.1. : Diagram of the recirculating system before conversion (not to scale).



KEY TO ABBREVIATIONS:

F.t. = Fish tank
G. = Gutter
H.t. = Header tank
I. = Inflow
F.tr. = Faecal trap
O. = Outflow
P. = Pump
P.f. = Pumped flow
R.m. = Ring main
S.f. = Secondary (passive) flow
S.g.f. = Submerged gravel filter
S.p. = Stand pipe

These systems recirculate the water through a biological filter, in which bacteria oxidise the ammonia produced by the fish, and convert it to the less toxic nitrate ion, a build up of which is prevented by constantly passing a small quantity of water through the system. The principal drawback to the use of these systems in experimental trials is the same property which makes them so useful in circumstances where the water supply is limiting. The water is recirculated many times, thus the fish share the same water. Such water sharing may be an advantage in certain experiments, but in studies of mineral requirement, will enable transfer of minerals between treatments via the water.

Section 2. has described the abilities of fish to absorb minerals from the environmental water, thus if a group of fish in one tank were being fed on a mineral deficient diet, and another group on a less deficient diet, the water shared by both groups would provide a potential vehicle for distribution of the minerals. This would reduce the probability of obtaining valid results, because each group could not be considered truly isolated.

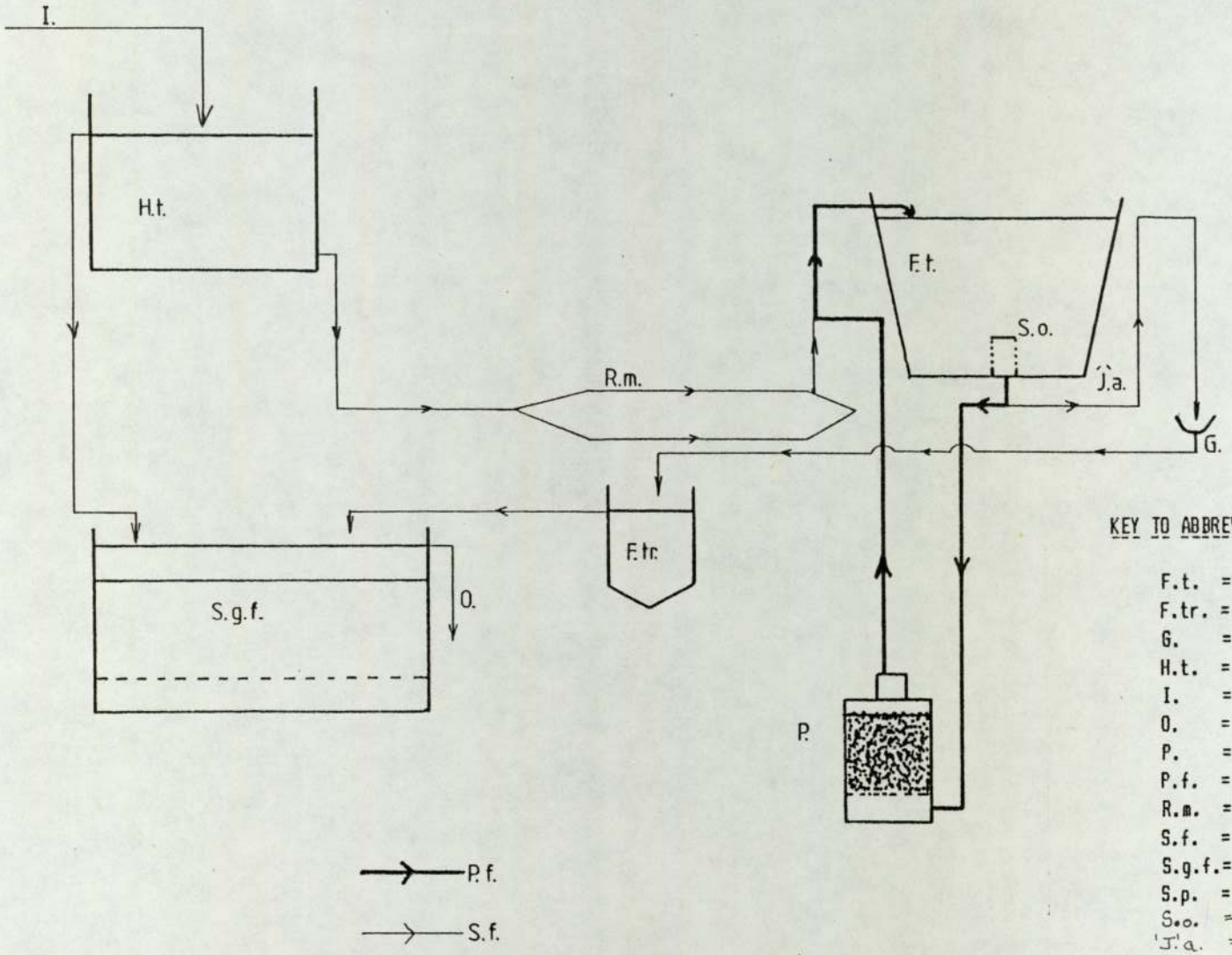
There were two possible solutions to the problems of recirculated water. The first was the use of a separate recirculating system for each group of fish (See sect. 3.3.2.). The second solution involved the use of a "through-flow" system (See sect. 3.3.3.).

3.3.2. The Recirculating System:

The original recirculation system (Fig. 3.3.1.1.) was redesigned and was converted to eight small, separate, recirculating

systems, each with its own power filter and make-up water supply. Make-up water is the term used to describe the small amount of water input to a recirculating system to "flush out" nitrates. This water also replaces that lost by evaporation, splashing, or removed when the filters are cleaned. (See Fig. 3.3.2.1.) The power filters used were Eheim '2021' (Eheim Ltd. Germany).

FIG. 3321: The modified recirculating system



KEY TO ABBREVIATIONS:

F.t. = Fish tank
F.tr. = Faecal trap
G. = Gutter
H.t. = Header tank
I. = Inflow
O. = Outflow
P. = Pump
P.f. = Pumped flow
R.m. = Ring main
S.f. = Secondary (passive) flow
S.g.f. = Submerged gravel filter
S.p. = Stand pipe
S.o. = Screened outlet
'J'a. = 'J' arm

The Eheim 2021 power filter consists of a plastic cylindrical body in which the filter medium is placed, with a close-fitting lid upon which a pump is mounted. The lid is clamped to the body by four spring clips, and a rubber ring seal ensures that the joint between the lid and the body is watertight. The filter material rests on a perforated plastic plate, which is raised above the base by moulded projections. The inlet to the filter is below the perforated plate, so that the water can flow evenly across the bottom of the gravel before passing through it. The suspended solids in the incoming water accumulate in the space below the filter medium, but as this space is fairly large, they do not obstruct the flow until a considerable quantity has built up. The build up of the sediments at the upstream end of the filter increase its efficiency, by reducing the size of particle which is retained.

A second perforated plate is placed across the top of the filter bed preventing it from rising and blocking the filter outlet. This is located in the lid, and is connected to the inlet of the pump by a short length of pipe. The pump operates by a centrifugal impellor, driven by a 50 watt electric motor.

For aquarium use the manufacturers recommend that the space between the filter bed and the outlet be filled with polyester filter mat to increase the retention of suspended solids. This was omitted without adverse effects on the water quality, but the criteria for the quality of water in an aquarium are probably higher than in experimental systems. In the experimental system, the removal of carbon dioxide and ammonia are of primary

importance. De-carbon-dioxidation is the single most important function of the machinery of a recirculating system, as fish are sensitive to the build-up of carbon dioxide in the water rather faster than the build-up of ammonia. Fish may die in well-oxygenated water if the CO_2 level is high. Agitation of the water, either by air stones or an impinging jet from a pump, allows carbon dioxide to escape into the atmosphere.

In use, the filters proved highly effective, giving low ammonia concentrations and efficient filtration of faecal material. The filters were cleaned once each week by emptying the filter material into a bucket and washing it thoroughly with tap water.

The filter material was sharp sand; a fine gravel with particle size 2-4mm as recommended by Spotte (1970). This was used in preference to the activated charcoal generally recommended for small power filters, since activated charcoal granules were not found to improve water quality in a direct comparison, and are more expensive.

In Experiment V the sharp sand was compared with crushed oyster shell as a filter medium.

After six months continuous running, one pump motor bearing had to be replaced. This was the only mechanical failure that occurred during eighteen months of use.

The original recirculating water supply was used to deliver make-up water to the eight units, and the large biological filter and its pump were left unused. Screened outlets replaced the stand pipes at the centre of the tanks, and a "J" arm was attached to carry the overflow from each tank to the waste. This

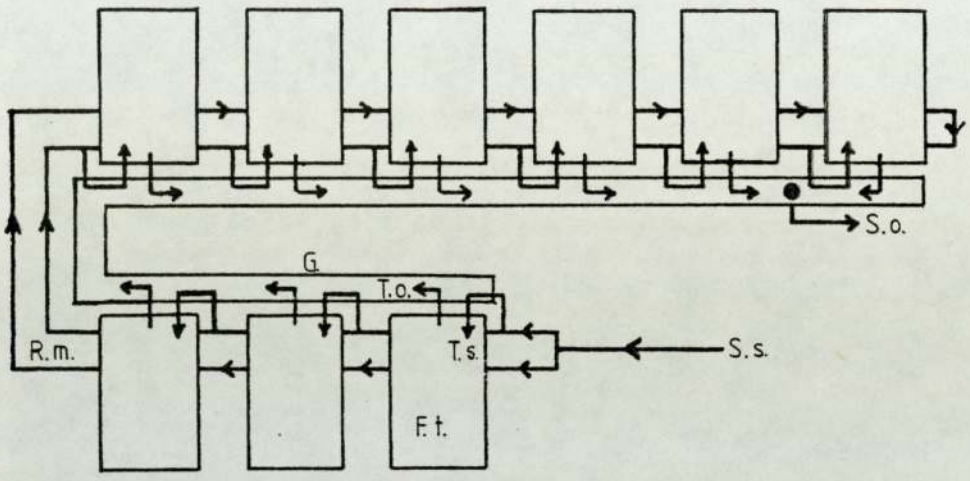
arm was adjustable and allowed variation of the water-level (and thus the volume) in each tank. By placing a thumb over the open end of the "J" arm and swinging it down to one side of the tank, a powerful siphoning action developed which effectively flushed away faeces from the tank to the waste. This was done every day, and the tanks and pipework were thus kept clear of debris. The water lost during cleaning was replaced by make-up water.

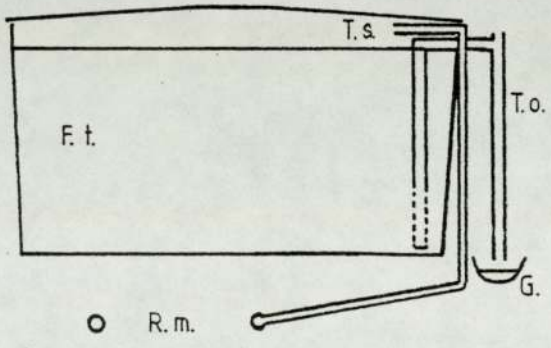
Experiments V and VI were carried out in the modified recirculating system.

3.3.3. The Through-flow Systems:

The problems associated with recirculation were avoided in three experiments by carrying them out in a through-flow system. In such a system the water supplied flows through the tanks and then out into the waste, without being recirculated. This type of system uses far more water to keep alive a given weight of fish than does a recirculating system and thus will hold a lower weight of fish for a given input of water. However, it is ideal for experimental purposes, since all the fish share the same water supply, but each treatment is isolated from the others. An added advantage is that this system is most commonly used in commercial trout farming, and is independent of an electricity supply and pump, (which may fail) for its successful operation.

FIG.3.3.3.1.: Diagram of through-flow system (top: view from above, below: side view)





KEY TO ABBREVIATIONS:

F.t.	=	Fish tank
G.	=	Gutter
R.m.	=	Ring main
S.o.	=	System outlet
S.s.	=	System supply
T.o.	=	Tank outlet
T.s.	=	Tank supply

Experiments II and III were carried out in a small through-flow system, constructed according to the pattern shown in Fig. 3.3.3.1.. The system consisted of eight 7-litre plastic aquaria for Experiment II, and nine aquaria for Experiment III. Water from a single supply was distributed equally to each tank as shown. Both Experiments II and III were done outside the fish culture unit, as no suitable water supply was available in the unit itself. The tanks were generally self-cleaning, but some siphoning of faeces was necessary every two or three days.

As the water had a high oxygen content, and the temperature inside the laboratory was higher than that of the water, there was a tendency for the gas to come out of solution on the sides of the tank and inside the outlet siphons. The gas in the siphons caused them to "air lock" and slow down unless they were cleared every day.

Routine maintenance thus involved siphoning the detritus from the tanks as necessary, and clearing the siphons every day. The faecal traps were also emptied every day. Once set, the control valves on the inlets of the tanks needed no adjustment.

Experiment III repeated part of Experiment II using smaller fish. The system was moved to an outside site for this experiment, where the water was supplied direct from the mains. Flow rates were higher in this situation than in Experiment II.

Experiment VII was also carried out in a through flow system, but in much larger tanks than Experiments II and III (Fig. 3.3.3.1.). When this system was constructed, a water supply of

higher pressure was available than for the previous experiments, sufficient to enable the use of nine 70-litre polythene tanks. The supply to this system was arranged as a "ring" rather than a single feed, and this removed the neccessity for individual control valves. Each tank received the same flow of water as the others, which was supplied direct from the mains. The water was jetted into the tanks from a small nozzle (a plastic hypodermic syringe without a needle) fitted over the inlet pipe. This agitated the water and possibly avoided the risk of supersaturation. This is a problem encountered in water supplied under high pressure. Pressure causes concentrations of dissolved gases to rise above the maximum possible at atmospheric pressure (supersaturation). When the pressure is released (the water leaves the supply pipe), the concentration of gas may not immediately fall to a normal level. Because the gas concentration in fish blood is dependent on the dissolved gas in the water, this can lead to the blood becoming supersaturated. When this occurs, the gas soon comes out of solution as small bubbles, which can block small blood vessels in the gills, causing suffocation. Vigorous agitation of water supplied under pressure avoids this problem.

The problem of flow variation through the outlet siphon experienced in Experiments II and III was overcome by fitting outlets as shown, which drew water from the bottom of the tank to encourage self-cleaning, but which left the tank through the top of one side at the water-line.

Each tank was fitted with a close-fitting black plastic lid.

3.3.4. System Monitoring: Environmental Parameters

Samples of water were drawn from the systems every few days during the experiments, and analysed to ensure that the water quality did not deteriorate. The water temperature was recorded using a thermometer when samples were taken for analysis. Dissolved oxygen and nitrate were estimated using appropriate electrodes. pH was measured with an electrical pH meter. Total hardness was found by titration with EDTA (A.P.H.A. 1972), and ppm. NH_3 assayed by colorimetry using Nesslers reagent (A.P.H.A. 1972). These parameters allowed the progress of any changes in water quality to be monitored, and appropriate action (such as increasing the flow rate) taken if necessary.

3.4. Treatment and Analysis of Samples:

3.4.1. The Nature of Samples:

Samples of fish, diets, fish tissues, fish faeces and environmental water were analysed, primarily for, and in most cases solely, for minerals. This was due to the limited amounts of sample available, which did not allow more than one analysis to be performed. Some fish carcasses were too large for analysis complete, thus to estimate the total mineral content a sub-sample, representative of the whole, was taken, after drying and then grinding the carcass to an homogeneous meal with a porcelain pestle and mortar. Drying prevented the deterioration of the sample during storage. The mineral content of the wet fish was then calculated as

$$Mw = Md.P$$

where Mw = mineral content of wet sample.

Md = mineral content of dry sample.

P = proportion of dry matter in the wet sample.

P was found by the formula

$$P = Wt1 / Wt2$$

where P= proportion of dry matter in the wet sample.

Wt1 = weight of dry sample.

Wt2 = weight of wet sample.

The fish carcasses were dissected to remove the contents of the digestive tract and placed in a labelled Universal Media Bottle (UMB) of known weight, which was then reweighed.

The bottle, with its cap loose, was placed in an oven at 105 deg. C. for 72 hours. It was then removed and the cap sealed whilst still hot. The UMB was allowed to cool in a desiccator and re-weighed. The loss in weight on drying enabled the caculation of the dry matter content of the sample.

3.4.2. Water Sample Analysis:

It has long been known that fish can absorb inorganic nutrients from the water in which they live. In experiments on fish it is therefore important that the nature and availability of minerals in the environmental water is known. Atomic absorption spectrophotometry (AAS) has now become the standard technique for the analysis of metals in water and many other materials, and has replaced many earlier titrimetric and colorimetric determinations. The atoms of the element must be fully dissolved, and present at a concentration above the 'detectable limit' of the atomic absorption spectrophotometer. This limit of sensitivity is the lowest concentration which can be identified as distinct from the 'background noise' of the method of analysis. In the case of certain readily ionised metals e.g. Na, K, the flame emission method of analysis (flame photometry) may be the most accurate technique, but the most sensitive technique is not necessarily the most accurate. The detection limits of the Perkin Elmer 303 atomic absorption spectrophotometer used are shown in Table 3.4.2.1.

Table 3.4.2.1. Detection Limits: Perkin Elmer 303

Metal	Detection Limit (ppm.)
Al	1.0
Ca	0.05
Cd	0.025
Co	0.055
Cu	0.09
Fe	0.12
Mg	0.007
Mn	0.055
Ni	0.15
Pb	0.5
Zn	0.018

The concentrations of metals in water are often below the detectable limits for AAS, and in order to determine such low levels, the sample must first be concentrated. Several techniques for the concentration of water samples before analysis are routinely used, often with a treatment to solubilize the metals present.

Metals may occur in water in many forms,ranging in size from free ionic form to complex organic polymers and even bound into the tissues of living or dead organisms. The concentrations of metals in water are often given, therefore, as 'filtrable', 'non-filtrable' and 'total'. The filtrable portion is taken to represent the atoms present in large complexes, which may be removed from the water by filtration, whilst the non-filtrable is the proportion not removable by filtration, taken to indicate the metals in free ionic or radical form of small diameter.

Fish may be able to absorb minerals which are present in water in forms larger than ionic, but the epithelium will not absorb pieces of dead organisms. The non-filtrable metals are therefore regarded as the most important with regard to their toxicity or availability to aquatic organisms. The estimation of the filtrable fraction is usually accomplished by two analyses, one of the raw sample and and one of the sample after passage through a fine filter paper, and subtraction of the two. The method is accurate but time consuming, and may be further complicated by the necessity to concentrate both the raw and filtered water before analysis. Indeed, the work involved in the taking and preparation of water samples may be so great as to reduce the

number of water samples which can be dealt with in a given time, and samples may have to be combined or taken less frequently than they should. If the samples have to be stored before analysis a bacterial inhibitor must be added. This is often a strong acid such as HCl. Bacteria cannot be allowed to grow in a water sample as they may alter the apparent mineral composition of the sample by absorbing metals and other ions. Addition of an acid may however alter the relative proportions of filtrable and non-filtrable forms present. This may lead to an over- or under-estimation of the non-filtrable metals present and hence an inaccurate assessment of the availability of the elements to fish.

An ideal analytical technique for water samples would be cheap, fast, simple, and result in the accurate determination of both filtrable and non-filtrable species present. The sample would be concentrated and in a suitable form for extended storage without deterioration, and the metal ions would be separate from other species such as sulphate, chloride, phosphate and nitrate.

Samples of water are often concentrated by evaporation, either using heat or freeze-drying. Both methods require expensive equipment and a considerable amount of attention for success. A more recently developed concentration technique uses ion-exchange resin. The properties of synthetic resins for the exchange of dissolved ions are well documented, and the development of specialised resins for particular purposes continues.

Conventional treatment of water with ion-exchange resin uses a column of resin in a vertical cylinder, through which water is

passed. As it flows past the column, ions are exchanged between the water and the resin, and the resin gradually becomes saturated with the ions absorbed. The zone of saturation forms at the 'upstream' end of the column and proceeds along it until all the exchange sites on the resin are occupied with the new ion and the solution passing over the resin emerges from the column unchanged. When the resin is saturated it may be re-generated by passing a concentrated solution of the original resin-bound ion over it, whereupon the exchange sites lose the second ions to the solution and are returned to the primary state. The ions absorbed from the first solution are eluted from the column dissolved in the regenerating solution.

Ion exchange resins are manufactured in four types: strongly cationic, strongly anionic, weakly cationic and weakly anionic. A cationic resin exchanges cations (positively charged ions), whilst an anionic resin exchanges anions (negatively charged ions). Strong resins bind ions more powerfully than weak resins and require correspondingly more concentrated solutions for regeneration.

By correct choice of resin and regenerant it is possible to use resin to absorb ions from water, and then release them into concentrated regenerant solutions with an overall increase in concentration - the ions are eluted in a smaller volume of liquid than that from which they were absorbed.

Ion exchange resins have different affinities for different ions, some ions binding more strongly than others to a given resin. Thus when the resin is regenerated the effluent from the

column will vary in composition with time as the least strongly bound ions are eluted first, succeeded by the other ions in order of ascending affinity. This is the basis for the technique of ion exchange chromatography.

There are two major drawbacks to the use of ion-exchange resins in the separation and concentration of ions from water. The first is the low flow rate required for efficient exchange. The flow rate is determined by the volume of resin used and is typically 0.01 b.v.min-1 where b.v. is the 'bed-volume' or volume of resin in the column. Thus the maximum flow for a resin column of 100ml would be 1ml.min-1 and a solution of one litre would take more than twelve hours to pass over the column. The flow must be kept constant whilst the solution passes over the resin, and requires either continual monitoring by the operator or an expensive pump (Samuelson 1963). The second problem is a consequence of the different solubilities of different ions, for example Ni(II) is soluble in 4M HCl, whilst Fe(III) is not.(Kraus and Moore . 1953). Moreover, some ions are bound so strongly to the resin that their elution is exceedingly difficult. The second problem can be overcome by wet combustion of the resin, which releases all the absorbed ions into solution but destroys the resin. The resin is expensive, as are the reagents for wet combustion, and if the quantity of resin be reduced to minimise the cost, then the first problem is exacerbated.

To overcome these problems, a technique was developed which efficiently removed metal ions from water with an ion-exchange resin and allowed the subsequent removal and concentration of the

ions by wet combustion of the resin. Small quantities of resin were used, not in columns, which avoided the flow restictions described above. The cost of the analysis was low and the attention that the apparatus required was minimal. It was found that a water sample of one litre could be treated each day without acidification or freezing, and that the resin could be stored indefinitely without deterioration, after it had absorbed the metal ions. A concentration factor of 20x the original was obtained, allowing the determination of lead, cadmium, cobalt and nickel levels in water at natural levels below the detection limits of the atomic absorption spectrophotometer.

3.4.3. The Method of Water Sampling and Treatment:

Every day a sample of water was drawn from the supply to the experimental system (see. Fig. 3.3.3.1.). A portion of the sample was stored in a polythene bottle and acidified by adding one part 12M HCl to sixty of water.

A one litre volumetric flask was filled to the mark from the remainder of the sample, rinsing it with some of the sample first to ensure that it was not contaminated. The volumetric flask was then placed on a bench stirrer, and the 'magnetic flea' dropped in. Approximately one gram of wet ion exchange resin (Amberlite IR-120(H) analytical grade, BDH Ltd. England) was then washed into into the flask with a minimal volume of distilled water. The flask stopper was inserted and the stirrer switched on. The speed of the stirrer was set so that the resin was swirled around in the flask, but not so fast that the magnetic flea lost stability. The apparatus was left running for 23 hours and then the flask was removed, and inverted in a clamp-stand. The resin then 'sank' to the top of the flask with the magnetic flea. After the resin had settled in the flask neck against the flask stopper (c. 2 min) the flask was held inverted over a filter funnel fitted with a sintered glass filter plate and a fine fibre-glass filter paper.(The sintered glass plate was preferable to the more conventional perforated porcelain, as the filter paper was fragile, and when used in the latter type of funnel, perforated over the holes.) The flask stopper was carefully removed and the resin and the flea were washed into the funnel. The flea was removed and rinsed, and suction applied for 5 minutes. The filter paper and resin were placed in a UMB and dried in an oven at 105 deg. C. for a week. A sample of the water remaining in the flask was retained for analysis to check for any residual minerals, and the remainder discarded.

The water sample drawn from the experimental supply was thus

stored as three samples: an acidified 'raw' sample, a sample from which the minerals had been absorbed by the resin, and the resin itself. The resin samples were stored dry until the end of the sampling period (130 days) and digested in 1:5 perchloric/nitric acid mixture, after shaking from the dry filter paper.

3.4.4. The Wet Combustion of Samples:

0.5 ± 0.075g of homogenous sample were weighed to ± 0,1 mg. into a tared, clean micro-kjeldahl digestion flask (30ml). The weight of the sample was recorded to within 0.0001g. The flask was then placed on a digestion rack and 10ml of 'Aristar' grade nitric acid was added from a tilt measure.

A vigorous reaction developed, the mixture foaming and giving off brown fumes of nitrogen dioxide. After about five minutes the reaction subsided and the gas burners of the digestion rack were lit. The flame was kept low at first, as the heat accelerated the initial reaction. Care was taken to ensure that the flask contents did not boil over at this stage, which lasted for three minutes, after which the nitrogen dioxide no longer came off, the foaming subsided and the mixture in the flask boiled steadily.

The heating was increased and the opaque brown digest gradually cleared and became paler in colour. The volume of the digest reduced as the nitric acid boiled off, until the flask contents were almost dry. When the last of the acid boiled off a vigorous reaction took place as the residual organic matter was oxidised, the flask contents turning black and solidifying.

After this the flask was removed from the rack and allowed to

cool. When quite cold, 10ml of 1:5 perchloric/nitric acid mixture were added to the flask and it was returned to the rack. It was heated as before, slowly at first and then more strongly. The carbonaceous matter first dissolved, with a foaming reaction, and then the dark solution slowly cleared. After about ten minutes of strong heating, the flask contents were a clear yellow colour and the last of the nitric acid, which has a lower boiling point than perchloric acid, vapourised. The flask contents began to foam again at this point and the mixture turned from yellow to black very quickly (within 5 sec.).(1) The gas burner flame was reduced and the flask was heated gently as this reaction proceeded. After several minutes the dark mixture paled from black to orange to yellow and finally to a water clear solution. When the solution was clear the reaction stopped and further bubbling was sporadic. Further heating merely boiled the flask dry, whereupon the perchlorates which were left caught fire. This was prevented by removing the flask from the heat as soon as the digest cleared and then allowing it to cool. When cold, 10ml of distilled water were added from a wash bottle and the flask returned to the rack. The cooling allowed perchlorates to crystallise out of the solution, and these were difficult to dissolve in cold water. Further heating until the mixture boiled dissolved these

1. This was the most hazardous point of the procedure, as a mixture of perchloric acid and organic matter is highly inflammable and potentially explosive. It was found that provided sufficient perchloric acid was present to dissolve the carbon formed by oxidation of fats and other compounds, then the risk of accident was minimal. If however, solid particles of carbon formed which did not dissolve immediately, there being insufficient acid, then instantaneous combustion occurred.

crystals, which did not come out of the diluted solution when the flask was allowed to cool once more.

The diluted digest was then washed from the micro-kjeldahl flask into a 50ml volumetric flask with three washings of distilled water. The volumetric flask was filled up to the mark with distilled water and shaken vigorously, then emptied into an acid-cleaned 60ml polythene bottle. This solution was then stored for metal analysis by AAS.

The resin samples were digested in the same way as the fish, tissue, diet and faecal samples (Sect 3.4.4.), but one problem peculiar to the digestion of resin was found. It was believed that drying for a week at 105 deg. C. would have removed all the water from the resin samples. During drying the particles of resin shrank and became darker in colour. However, when the resin was placed in the micro-kjeldahl digestion flask and warmed with acid, a temperature was reached at which violent 'bumping' occurred, which initially caused a loss of samples. The resin was found to have retained water, despite the extended drying period, and this water was boiling within the resin particles; water vapour was literally exploding from the resin. The answer to the problem was to ensure that this water had been driven off before the acid was added, and therefore the resin was completely dried by heating it in the digestion flasks without acid. The resin particles lost water rapidly, jumping about at first, as the water boiled out. The water vapour could be seen to condense on the neck of the flask and after about two minutes the particles stopped moving and had turned black. The flasks were allowed to

cool and then acid was added as normal. Holm and Svensson (1954) describe a method for the wet combustion of ion-exchange resins in which concentrated sulphuric acid is added to the resin before nitric acid. This would probably have the same effect as the heating described above, in removing the water from the resin. This method was rejected as the addition of sulphuric acid would form insoluble calcium sulphate and render the analysis of the samples for calcium impossible.

The digested samples were stored in polythene bottles for analysis by AAS.

The same digestion flask was used for each treatment for all the samples from a given experiment. If for some reason a flask was not completely washed out, cross-contamination would thus be within treatments rather than between treatments, the latter being the most serious potential error. In practice, cross-contamination is unlikely to have occurred, for the batch sizes varied slightly (e.g. twenty seven samples where there were nine treatments in the experiment, twenty-five where there were five). The blanks were run in different flasks and no evidence of blank contamination was seen with one exception. This was due to contamination from the supports for the exhaust tube of the digestion rack. These were made of copper and were attacked by the acid vapours produced by the digestion mixture. The crystals of copper nitrate were occasionally knocked off onto the flask necks when the flasks were loaded onto the racks. This was not a problem during the early stages of the analysis as the corrosion took some time to develop, and after the problem was identified,

care was taken to ensure that the flasks were kept well away from the corrosion deposits. However, some samples were contaminated with copper and it was not possible to estimate this element accurately in these samples.

3.4.5. Mineral Analyses

Sodium and Potassium levels in water samples were estimated using an EEL flame photometer, this machine being sufficiently accurate for the purpose.

Phosphorus levels in all samples were analysed using a Technicon autoanalyser running a photometric assay based on the phosphomolybdate reaction (Lundgren 1960). A Perkin Elmer 303 atomic absorption spectrophotometer was used to determine Al, Ca, Cd, Co, Cu, Fe, Mg, Mn, Ni, Pb and Zn in water and digested samples, with the standard conditions set out in Table 3.4.5.1.

Table 3.4.5.1. Standard Conditions used in AAS

Metal	Wavelength (nM)	Oxidant/Fuel	Anti-ioniser
Al	309.3	N_2O:C_2H_2	200ppm Cs
Ca	422.7	''	200ppm K
Cd	228.8	Air:C_2H_2	None
Co	240.7	''	''
Cu	324.8	''	''
Fe	248.3	''	''
Mg	285.2	''	''
Mn	279.5	''	''
Ni	232.0	''	''
Pb	217.0	''	''
Zn	213.9	''	''

The digested samples often contained concentrations of Ca and Mg which were above the range the AA spectrophotometer could analyse directly. Such samples were diluted before analysis, so as to be within the 'linear range' of the machine. The samples were diluted with 200ppm potassium solution in order to prevent ionisation of Ca atoms in the flame. This would have produced errors of estimation. Where the Ca levels were found to lie within the linear range, the 'natural' K present was found to provide sufficient protection against Ca ionisation.

Samples were presented for analysis in batches, both samples within batches and batches being processed in random order. This eliminated the possibility of time-dependent effects occurring due to the order of sample analysis.

When samples were being analysed by AAS, the instrument

occasionally produced a sudden, high value. To prevent these 'kicks' affecting the results, several readings were recorded from each sample. When water samples were analysed, six readings were recorded, of which the first and last were discarded unless a kick had occurred, in which case the 'kick value' was replaced by the last value recorded. When samples other than water were analysed, four readings were recorded, of which the first and last were discarded in the absence of kicks.

3.5. Statistics

3.5.1. Growth Statistics

A one-way analysis of variance and a multiple range test (Duncan 1955) were applied to growth and feeding statistics to enable comparisons to be made between them, and a pooled estimate of the variation was obtained (the standard error about any given mean, the S.E.M.). This value is the same for each mean, as each sample was the same size. In studying the growth of fish, the obvious statistics to compare in order that the effects of different diets may be determined are the mean final weights at the end of the growing period. Unfortunately the growth response of fish to diets given in intensive conditions is highly variable, even among fish given the same diet. This is due to inter-group dominance, genetic variation and other factors. Thus, even if the feeding frequency is restricted to reduce the variation in intake of the fish in a given group, the final weights will generally have a greater variation than the initial weights. Tables 4.1.1.2 and 4.5.1.2 show this, the S.E.M. about the initial weights always being less than that about the final weights.

Where a large number of fish ($n > 30$) can be used, significant differences may be detected despite the variation within the treatments. However, if the numbers of fish are limited, either due to disease, space or cost, the variation in size will considerably reduce the sensitivity of any statistical analyses. Even if significant differences are detected, the variation will always reduce the level of significance of the results. Nevertheless, it is possible to remove much of the variation by using the specific growth rate (SGR) of the fish instead of the absolute weight. The SGR is a statistic which represents the instantaneous rate of weight increase of a fish as a fraction of its body weight in time. The most commonly used units of the SGR are per cent. per day. Thus a fish with a SGR of 1.0 will be seen to increase in body weight by 1% every 24 hours. The weight of the fish is therefore taken to increase exponentially, and the SGR as the 'interest rate' which would apply if the weight of the fish were a sum of money subject to compound interest.

The SGR is calculated using the compound interest formula:

$$SGR = (e^{k} - 1) \times 100$$

where:

e = 2.7183

$k = [(\log_e Wn - \log_e Wn\text{-}d) / d]$

and:

Wn = weight at end of growth period

Wn-d = weight at start of growth period

n = no. of time units in the growing period

d = time unit in the growing period

The SGR is thus given as %increase/d where d may be any unit of time.

The formula above can be seen to differ from the conventional formula (Brown 1946b) used to derive the specific growth rate, or rather to be an extension of it. The conventional formula would give the SGR as k (see above), but this is a log transformation rather than a complete calculation of the instantaneous rate of change. The SGR used here differs but little in magnitude from the statistic k, but is mathematically correct.

If the SGR is recorded for succeeding time intervals, and the values thus obtained are used to calculate a mean rate of increase, the statistic so obtained can be used to predict the final weight of the fish from the initial weight. The SGR can

therefore be seen to be a more robust model of fish growth than is the feed conversion ratio a model of feed conversion (see below). The SGR does however possess some limitations on its value. As the maximum rate of growth of fish decreases with size, so too does the maximum SGR. This limits the length of time over which the SGR can be used to predict the final size of the fish. If too long a period is used, then the SGR will over-estimate the final weight. The main limitation on the use of the SGR occurs when the fish under study are not individually marked. In this case, the SGR must be calculated from the mean weight of all the fish, and thus only one value of SGR is available from any group of fish in a given time interval. This forces the use of time-series data rather than samples drawn at a single time. The SGR may be affected by mortality if the fish are unmarked: the death of one or more fish will generally produce a random shift in the mean weight of the fish in the group, depending on its size. This shift in the mean weight will affect the subsequent SGR which may thus increase or decrease not because of the effect of the diet, but because of fish death. Where fish are marked, then the effect of mortality can be identified, and as each fish can have its individual SGR calculated, the sample size of the SGR's is greatly increased.

3.5.2. Feed Intake and Conversion Statistics

The feed intake of the fish was calculated from the loss in weight of the feed containers each day. The weight of feed given to the fish was calculated and converted to a percentage of the total weight of the fish at the start of the growth period. As

the total weight of feed was integrated with the total weight of fish, the resultant percentage applied equally to one fish as to all the fish in a given group. The major disadvantage created by this method of feeding was the lack of information on the intake of individual fish. This is a problem which could only be overcome by maintaining each fish in a separate tank, which would be rather difficult. Feed conversion ratios (FCR's) could not be compared by statistical analysis. This was a consequence of the sample size: n=1. An attempt was made to obtain an estimate of the variation in feed conversion over time by recording the FCR each week and then calculating a mean value for the entire experiment. This produced a mean from several values about which a standard error could be fitted. However, the definition of feed conversion is as follows: (Feed given/Weight gained) . When the total amount of feed given during the experiment was calculated and divided by the total gain in weight of the fish, the resultant overall known FCR was seen to vary from the 'mean'. This was due to the change in size of the fish. If the fish converted the food to flesh inefficiently at the start of the experiment, when they were small, the food wasted was negligible. The feed conversion resulting from this small wastage would however be very poor. When used to calculate the feed conversion, this poor result would then be given equal importance as later results, when much larger quantities of feed were being given to the (larger) fish. Thus a small weight of food wasted at the start of the experiment would affect the mean FCR by a disproportionate amount. Similarly, if the FCR at the start of

the experiment was high, but then declined with time, a large weight of food would be wasted, but this would not be reflected in the mean value.

The only true measure of FCR is therefore:

(Total Feed given/Total weight gain)

In these experiments, where the fish were the replicates, there was only one true value of FCR for each treatment. The only solution to this problem would be the use of several separate tanks of fish. This was not possible in these experiments, due to the lack of facilities.

3.6. Computer Programming

Three computers were used to process the data generated by this research. The experimental data,i.e. fish weights and feed container weights were integrated using an HP-2000 computer, running a specially written program: FISGAF. The title of this program is an acronym for FISh Growth And Feeding. Statistical analyses of the statistics generated by FISGAF were carried out by two other computer programs: 1ANALV (1-way ANALysis of Variance), and FANALV (Factorial ANALysis of Variance). The calculation performed by these programs are given in Bailey (1959), 'Statistical Methods in Biology'. The program 1ANALV also incorporated a Multiple Range Test (Duncan 1955).

The results of the water sample analysis and the mineral analysis of fish and other samples, were stored and analysed using a Harris 500 computer. This computer operated much faster than the HP-2000, and thus allowed the swift manipulation of the larger quantities of data generated by chemical analysis. The most important program used to integrate the AAS data was MICAL (MIneral CALculation). This calculated the blank value for each batch of samples analysed by AAS, then successively calculated the mean mineral content of the sample solution, and removed the blank value from the sample value. The result was then multiplied by the appropriate dilution factor to give the mineral content of the dry material digested. This mineral content was then multiplied by proportion of dry matter in the original wet sample to give the final concentration of the minerals in the wet sample. The program integrated the concentrations of 7 minerals,

dilution factors, weights and dry matter contents of 1025 samples in less than one minute and then printed out the results for further statistical manipulation. The mineral concentrations produced by MICAL were statistically analysed using 1ANALV and FANALV, which had been transfered from the HP-2000 to the Harris 500 for this purpose. The water analysis data were reduced from the 6 spectrophotometer readings obtained to a single mean value each day. This was done with a small BASIC program. The day numbers and appropriate mean values were then stored and input to a FORTRAN-77 program using GINO (Graphical INput and Output) routines to prepare graphs of water mineral content against time (Figs. 4.6.2.1. to 4.6.2.20.). These graphs were encoded and transferred to an ICL 1904S system which plotted them on a CALCOMP graph plotter.

4. RESULTS

This section contains the results of all experiments and analysis of experimental samples. The tabulated results are presented in two types of table: factorial effects tables, and tables of observed mean levels of the statistics. In the former, symbols are used to indicate the significance level (if any) of the effects identified. The symbols are: N.S. (not significant at P=0.05); +, positive effect (significant P > 0.01, < 0.05); ++, positive effect (significant P < 0.01); -, negative effect (significance as +); --, negative effect (significance as ++). In tables of absolute levels of statistics, superscripts are attached. Means with the same superscripts are not significantly different at P <= 0.05.

4.1. Expt.II

4.1.1. Growth and Feeding

The results of the factorial analysis (Table 4.1.1.1.) showed a highly significant ($P <= 0.01$) suppression of growth when the minerals and trace elements interacted. The Pruteen did not appear to affect intake (Table 4.1.1.1.). The FCR's of fish fed diets containing Pruteen tended to be lower than those given fishmeal, although the proximate compositions of the diets (Table 3.1.5.2.) were similar. Absolute values of these statistics are shown in Table 4.1.1.2.

Table 4.1.1.1. Factorial Effects on Growth & Feed Intake: Expt.II

Statistic:	Final Wt.	SGR	Intake
n	8	56	49
Minerals	N.S.	N.S.	N.S.
Trace Elements	N.S.	N.S.	N.S.
Minerals X Trace Elements	N.S.	--	--
Pruteen	N.S.	N.S.	N.S.
Pruteen X Minerals	N.S.	+	++
Pruteen X Trace Elements	N.S.	N.S.	++
Pruteen X Minerals X Trace Elements	N.S.	N.S.	N.S.

Table 4.1.1.2. Growth and Feed conversion Statistics: Expt.II

Statistic	n	F	FM	FT	FMT	B	BM	BT	BMT	S.E.M.
Initial Weight g.	8	a 13.48	a 14.05	a 12.06	a 11.11	a 13.63	a 12.15	a 11.36	a 13.73	1.25
Final Weight g.	8	a 26.47	a 29.50	a 27.94	a 20.42	a 23.42	a 25.39	a 22.93	a 26.69	3.254
SGR %/day	56	a 1.35	a 1.50	a 1.62	a 1.20	a 1.13	a 1.48	a 1.37	a 1.34	0.133
Intake %/day	49	a 2.33	a 2.32	a 2.56	c 1.63	bc 1.84	a 2.41	ab 2.25	ab 2.20	0.139
FCR	1	1.73	1.55	1.58	1.38	1.63	1.63	1.64	1.64	----

4.1.2. Mineral analyses

No statistically significant differences were observed between the mineral contents of the fish from different treatments. Hence, no significant factorial effects could be identified. This was probably due to the small sample sizes used (n=4). (See Table 4.1.2.1.)

Table 4.1.2.1. Carcass Mineral Contents: Expt.II

Mineral	n	F	FM	FT	FMT	B	BM	BT	BMT	S.E.M.
Ca (%)	4	a 0.510	a 0.540	a 0.559	a 0.587	a 0.473	a 0.537	a 0.643	a 0.574	0.065
Mg (ppm.)	4	a 181.5	a 173.5	a 196.8	a 173.9	a 166.2	a 190.1	a 189.6	a 198.2	11.75
Zn (ppm.)	4	a 26.03	a 20.71	a 38.18	a 16.62	a 27.15	a 24.96	a 22.42	a 24.54	5.586
Fe (ppm.)	4	a 17.90	a 7.391	a 10.94	a 8.363	a 7.624	a 10.79	a 7.786	a 7.873	3.241
Cu (ppm.)	4	a 2.872	a 2.054	a 2.706	a 2.027	a 2.348	a 2.231	a 2.157	a 1.823	0.502
Mn (ppm.)	4	a 2.249	a 1.807	a 3.404	a 11.99	a 1.817	a 2.053	a 1.784	a 1.846	3.517
P (%)	4	a 0.474	a 0.501	a 0.505	a 0.519	a 0.423	a 0.512	a 0.473	a 0.529	0.028

4.2. Experiment III

4.2.1. Growth and Feeding

This experiment replicated Experiment II (Sect. 4.1.) and attempted to determine more accurately the effects of minerals, trace elements and Pruteen on the mineral composition of fingerling trout. The Pruteen suppressed intake and conversion (and hence growth), however, the factorial design permitted the effects of Pruteen to be isolated and the effects of the other factors could then be examined in combination and in isolation. (Table 4.2.1.1.). These results differed from those of Experiment II in that no interaction between minerals and trace elements was seen. In Experiment III, as in Experiment II, the interaction between minerals and Pruteen significantly increased intake. Absolute values of the growth and feed conversion statistics are shown in Table 4.2.1.2. Diet E was the commercial comparator control.

Table 4.2.1.1. Factorial Effects on Growth & Feed Intake: Expt. III

Statistic:	Final Wt.	SGR	Intake
n	6	52	56
Minerals	N.S.	N.S.	N.S.
Trace Elements	N.S.	N.S.	N.S.
Minerals X Trace Elements	N.S.	N.S.	N.S.
Pruteen	--	--	--
Pruteen X Minerals	N.S.	N.S.	+
Pruteen X Trace Elements	-	N.S.	N.S.
Pruteen X Minerals X Trace Elements	N.S.	N.S.	N.S.

Table 4.2.1.2. Growth and Feed conversion Statistics: Expt. III

Statistic	n	F	FM	FT	FMT	B	BM	BT	BMT	E.	S.E.M.
Initial Weight g.	20	a 4.97	a 5.16	a 5.09	a 5.20	a 5.30	a 5.52	a 5.50	a 4.72	a 5.03	0.239
Final Weight g.	6	b 14.70	ab 17.04	a 20.58	ab 18.16	c 5.86	c 5.98	c 6.00	c 4.72	ab 16.81	1.249
SGR %/day	52	a 2.254	a 2.349	a 2.725	a 2.603	b 0.247	b 0.544	b 0.304	b 0.215	a 2.561	0.191
Intake %/day	56	a 4.33	ab 4.12	a 4.43	a 4.41	c 1.59	c 1.98	c 1.77	c 2.20	b 3.55	0.205
FCR	1	1.919	1.753	1.626	1.695	6.421	3.634	5.83	10.25	1.385	----

4.2.2. Mineral analyses

When the mineral compositions during the experiment were examined (Tables 4.2.2.3.-4.2.2.9.), two characteristics emerged. These were that the greatest change in the mineral content occurred before 14 days of the experiment had elapsed, and secondly that the mineral contents of the individual fishes, given the same diet, were very variable. The latter reduced the statistical significance of any differences observed between treatments. The calcium, magnesium and phosphorus in the fish carcasses were seen to increase during the experiment. Zinc increased then decreased at the end of the experiment to levels similar to those at the start, and copper and manganese decreased during the experiment, although the changes with time were only significant for calcium, magnesium and copper. Factorial analysis clarified the results (Table 4.2.2.1.) Phosphorus was highly significantly increased by Pruteen, copper significantly decreased. The trace element factor was shown to increase the calcium and zinc contents of the carcasses, whilst an interaction between Pruteen X trace elements also increased the calcium content. The Pruteen significantly increased the calcium content, and the interaction of Pruteen X trace elements increased calcium even more. Absolute values of carcass mineral concentrations are shown in table 4.2.2.2., with column I representing the initial sample of fish taken at the start of the experiment.

Table 4.2.2.1. Factorial Effects on Mineral Contents: Expt. III

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	6	6	6	6	6	6	6
Minerals	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Trace Elements	+	N.S.	+	N.S.	N.S.	N.S.	N.S.
Minerals X Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen	++	N.S.	N.S.	N.S.	+	N.S.	++
Pruteen X Minerals	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Trace Elements	+	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Minerals X Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 4.2.2.2. Carcass Mineral Contents: Expt. III

Mineral	n	I	F	FM	FT	FMT	B	BM	BT	BMT	E	S.E.M.
Ca (%)	6	d 3.53	c 5.48	c 5.57	c 5.45	c 5.81	a 8.14	a 7.65	b 6.68	ab 7.28	c 5.46	0.30
Mg (ppm.)	6	b 97.0	a 190	a 206	a 194	a 199	a 177	a 204	a 187	a 191	a 202	21.3
Zn (ppm.)	6	b 24.7	b 29.4	b 10.6	b 23.2	b 23.5	b 29.5	ab 34.1	a 65.5	ab 42.6	b 28.0	11.1
Fe (ppm.)	6	a 42.4	a 9.77	a 9.70	a 5.19	a 6.24	a 11.2	a 12.1	a 68.7	a 9.5	a 31.7	21.3
Cu (ppm.)	6	a 13.7	b 2.76	b 3.68	b 4.49	b 2.94	b 1.62	b 1.91	b 3.44	b 4.40	b 3.80	1.42
Mn (ppm.)	6	ab 1.91	b 1.26	b 0.58	b 0.66	b 0.67	ab 1.87	ab 2.24	a 3.58	b 1.56	b 1.47	0.63
P (%)	6	bc 0.32	a 0.50	a 0.52	a 0.50	a 0.52	b 0.65	b 0.62	b 0.61	b 0.60	a 0.53	0.02

Table 4.2.2.3. Table of Carcass Ca Levels (%) During Expt. III

Diet / Time (days)	0	14	28	42	56
F		0.50	0.51	0.46	0.55
FM		0.50	0.52	0.43	0.56
FT	0.35	0.50	0.51	0.47	0.55
FMT		0.51	0.50	0.48	0.58
E		0.49	0.46	0.46	0.55
n	6	3	3	3	6
S.E.M.	0.08	0.02	0.02	0.02	0.03

Table 4.2.2.4. Table of Carcass Mg Levels (ppm.) During Expt. III

Diet / Time (days)	0	14	28	42	56
F		282	1037	266	190
FM		286	777	231	206
FT	97.0	285	882	260	194
FMT		286	291	276	199
E		274	291	271	202
n	6	3	3	3	6
S.E.M.	21.9	8.89	375	11.8	21.3

Table 4.2.2.5. Table of Carcass Zn Levels (ppm.) During Expt. III

Diet / Time (days)	0	14	28	42	56
F		52.5	203	26.5	29.4
FM		97.2	84.1	0.37	10.62
FT	24.7	51.6	181	65.0	23.2
FMT		96.3	50.3	32.7	23.5
E		82.5	94.0	17.7	28.0
n	6	3	3	3	6
S.E.M.	12.1	40.9	69.5	13.9	12.14

Table 4.2.2.6. Table of Carcass Fe Levels (ppm.) During Expt. III

Diet / Time (days)	0	14	28	42	56
F		5.48	228	16.5	9.77
FM		14.1	416	9.86	9.70
FT	42.4	23.0	144	15.0	5.19
FMT		120	366	13.5	6.23
E		221	405	11.0	31.7
n	6	3	3	3	6
S.E.M.	47.2	102	199	3.58	21.3

Table 4.2.2.7. Table of Carcass Cu Levels (ppm.) During Expt. III

Diet / Time (days)	0	14	28	42	56
F		2.26	2.57	1.42	2.76
FM		1.62	2.46	1.26	3.68
FT	13.7	1.92	2.52	1.00	4.49
FMT		1.96	2.36	1.31	2.94
E		1.73	2.10	1.2	3.80
n	6	3	3	3	6
S.E.M.	9.21	0.44	0.31	0.39	1.42

Table 4.2.2.8. Table of Carcass Mn Levels (ppm.) During Expt. III

Diet / Time (days)	0	14	28	42	56
F		2.36	2.86	0.59	1.26
FM		3.49	3.58	0.37	0.58
FT	1.91	1.85	4.25	0.58	0.66
FMT		2.23	0.84	0.62	0.67
E		2.24	1.48	1.06	1.47
n	6	3	3	3	6
S.E.M.	0.66	1.15	1.66	1.73	0.63

Table 4.2.2.9. Table of Carcass P Levels (%) During Expt. III

Diet / Time (days)	0	14	28	42	56
F		0.50	0.48	0.48	0.50
FM		0.51	0.48	0.43	0.52
FT	0.32	0.49	0.49	0.49	0.50
FMT		0.50	0.48	0.51	0.52
E		0.49	0.47	0.48	0.53
n	6	3	3	3	6
S.E.M.	0.06	0.02	0.02	0.02	0.02

4.3. Experiment V

4.3.1. Growth and Feeding

The factorial effects on growth and feeding statistics are shown in Table 4.3.1.1. and the absolute values of these statistics are shown in Table 4.3.1.2. Factorial analyses of the results showed that the oyster shell (increased hardness) factor increased the food intake and the final weight of the fish. The final weight increase was not reflected in the specific growth rate (SGR). The SGR was shown to have been reduced by the presence of dietary minerals, which was in contrast to the results of Experiments II, III, and VI. The dietary minerals were seen to interact with the hardness (oyster shell) to suppress the SGR ($P <= 0.01$) and the feed intake ($P <= 0.01$).

Table 4.3.1.1. Factorial Effects on Growth & Feed Intake: Expt. V

Statistic:	Final Wt.	SGR	Intake
n	10	25	49
Oyster shell	+	N.S.	+
Dietary Minerals	N.S.	-	N.S.
Oyster shell X Minerals	N.S.	--	--

Table 4.3.1.2. Growth and Feed Conversion Statistics: Expt. V

Statistic:	n	B0-	B0+	B4-	B4+	S.E.M.
Initial Weight g.	20	a 8.33	a 8.48	a 8.85	a 8.19	0.305
Final Weight g.	10	b 15.59	a 21.33	b 15.01	b 16.20	1.45
SGR %/day	25	a 1.27	a 1.59	a 1.41	b 0.92	0.123
Intake %/day	49	c 3.41	a 5.72	b 4.60	bc 4.05	0.384
FCR	1	2.69	3.60	3.26	4.40	----

4.3.2. Mineral Analyses

After 25 days the oyster shell had induced an highly significant ($P <= 0.01$) decrease in the copper content of the fish. An interaction between the dietary minerals and the oyster shell induced a significant increase in copper content (Table 4.3.2.1.).

Table 4.3.2.1. Expt. V: Factorial Effects on Mineral Contents After 25 Days

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	4	4	4	4	4	4	5
Oyster Shell	N.S.	N.S.	N.S.	N.S.	--	N.S.	N.S.
Dietary Minerals	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Oyster Shell X Minerals	N.S.	N.S.	N.S.	N.S.	+	N.S.	N.S.

After 50 days the effects previously observed after 25 days, were no longer detectable (Table 4.3.2.2.). Moreover the oyster shell had induced an increase in the zinc contents of the fish carcasses ($P <= 0.05$) and a decrease in the manganese ($P <= 0.05$).

Table 4.3.2.2. Expt. V: Factorial Effects on Mineral Contents After 50 days

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	10	10	10	10	10	10	10
Oyster shell	N.S	N.S	+	N.S	N.S	N.S.	N.S.
Dietary Minerals	N.S.	N.S.	N.S.	N.S.	N.S.	-	N.S.
Oyster shell X Minerals	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Absolute values of the mineral levels in the fish after 25 and 50 days are shown in Tables 4.3.2.3. and 4.3.2.4. respectively. In the first table, column I contains values from the initial sample of fish.

Table 4.3.2.3. Carcass Mineral Contents after 25 days: Expt. V

Diet:	n	I	B0-	B0+	B4-	B4+	S.E.M.
		b	a	a	a	a	
Ca (%)	4	0.58	0.94	0.82	0.92	0.92	0.052
		b	a	a	a	a	
Mg (ppm.)	4	295	472	417	458	464	20.7
		a	a	a	a	a	
Zn (ppm.)	4	9.99	12.0	7.37	9.06	9.49	2.36
		b	ab	ab	ab	a	
Fe (ppm.)	4	17.1	20.5	20.6	22.9	26.4	2.46
		a	a	a	a	a	
Cu (ppm.)	4	7.24	8.41	3.55	5.69	4.98	1.63
		a	a	a	a	a	
Mn (ppm.)	4	1.26	1.77	1.85	1.83	1.57	0.22
		b	a	a	a	a	
P (%)	5	0.53	0.79	0.81	0.87	0.82	0.04

Table 4.3.2.4. Carcass Mineral Contents after 50 days: Expt. V

Diet:	n	B0-	B0+	B4-	B4+	S.E.M.
		a	a	a	a	
Ca (%)	10	0.68	0.67	0.61	0.66	0.029
		a	a	a	a	
Mg (ppm.)	10	342	339	321	342	10.1
		a	a	b	a	
Zn (ppm.)	10	10.8	14.8	8.03	25.7	5.16
		a	a	a	a	
Fe (ppm.)	10	11.2	12.1	9.5	11.4	0.94
		a	a	a	a	
Cu (ppm.)	10	2.69	1.73	2.33	17.5	6.76
		a	a	a	b	
Mn (ppm.)	10	1.50	1.35	1.27	1.13	0.11
		a	a	a	a	
P (%)	10	0.60	0.62	0.57	0.61	0.02

4.4. Experiment VI

4.4.1. Growth and Feeding

The fish were held in recirculating systems in this experiment. The factorial effects on growth and feeding statistics are shown in Table 4.4.1.1., and the absolute values of these statistics are shown in Table 4.4.1.2. The sodium phytate was not shown to affect the specific growth rate significantly, nor to interact with either minerals or trace elements to affect growth. The only factor influencing growth was the mineral mix. Phytate suppressed the intake of food ($P <= 0.05$). The intake was increased by the trace elements, by an interaction between sodium phytate X minerals, and also by an interaction between sodium phytate X minerals X trace elements.

Table 4.4.1.1. Factorial Effects on growth & Feed Intake: Expt. VI

Factor / Statistic:	Final Wt.	SGR	Intake
n	13	6	84
Sodium Phytate	N.S.	N.S.	--
Mineral	N.S.	++	N.S.
Sodium Phytate X Minerals	N.S.	N.S.	+
Trace Elements	N.S.	N.S.	++
Sodium Phytate X Trace Elements	N.S.	N.S.	N.S.
Minerals X Trace Elements	N.S.	N.S.	N.S.
Sodium Phytate X Minerals X Trace Elements	N.S.	N.S.	+

Table 4.4.1.2. Growth and Feed Conversion Statistics: Expt. VI

Diet:	n	BA	B	BAM	BM	BAT	BT	BAMT	BMT	S.E.M.
Initial Weight g.	40	a 4.69	a 4.76	a 4.79	a 4.31	a 4.91	a 4.92	a 4.41	a 4.36	0.26
Final Weight g.	40	a 6.92	a 6.30	a 7.35	a 8.63	a 7.29	a 8.45	a 7.92	a 7.21	0.85
SGR %/day	6	ab 0.70	ab 0.76	ab 0.98	a 1.31	b 0.58	ab 0.70	ab 1.07	ab 1.05	0.21
Intake %/day	84	c 1.81	bc 2.05	c 1.89	bc 2.14	bc 1.97	a 2.69	ab 2.22	bc 2.16	0.14
FCR	1	2.59	2.70	1.93	1.63	3.40	3.80	2.07	2.06	----

4.4.2. Mineral Analyses

After 42 days, the sodium phytate had produced an highly significant increase in calcium content (Table 4.4.2.1.), and had increased the phosphorus concentration. The minerals suppressed the calcium, magnesium and phosphorus . The trace elements increased the amounts of Ca, Mg, Cu, Mn and P in the carcasses.

Table 4.4.2.1. Expt. VI: Factorial Effects on Mineral Contents After 42 days

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	15	15	15	15	15	15	15
Sodium Phytate	++	N.S.	N.S.	N.S.	N.S.	N.S.	+
Minerals	--	-	N.S.	N.S.	N.S.	N.S.	--
Sodium Phytate X Minerals	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Trace Elements	++	++	N.S.	N.S.	+	++	++
Sodium Phytate X Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Minerals X Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Sodium Phytate X Minerals X Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

After 84 days (Table 4.4.2.2.), the mineral contents of the fish had changed relative to those at 42 days (Table 4.4.2.1.). After this time the sodium phytate had no effect on calcium, and had suppressed magnesium and phosphorus . The effect of the interaction between sodium phytate X minerals was significant causing an increase in magnesium where before there was no effect. Absolute mineral levels after 42 days are shown in Table 4.4.2.3., and after 84 days in Table 4.4.2.4. In Table 4.4.2.3., the column 'I' shows the concentrations of minerals in the carcasses at the start of the experiment, that is, in the 'Initial sample' of fish.

Table 4.4.2.2. Expt. VI: Factorial Effects on Mineral Contents After 84 days

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	13	13	13	13	13	13	13
Sodium Phytate	N.S.	-	N.S.	N.S.	N.S.	N.S.	N.S.
Minerals	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Sodium Phytate X Minerals	N.S.	++	N.S.	N.S.	N.S.	N.S.	++
Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Sodium Phytate X Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	
Minerals X Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Sodium Phytate X Minerals X Trace Elements	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 4.4.2.3. Carcass Mineral Contents After 42 days : Experiment VI

Diet:	n	I	BA	B	BAM	BM	BAT	BT	BAMT	BMT	S.E.M.
Ca (%)	15	f 0.38	d 0.51	de 0.47	de 0.48	e 0.43	a 0.64	b 0.58	bc 0.57	cd 0.52	0.02
Mg (ppm.)	15	d 230	c 261	c 272	c 265	c 260	a 355	a 342	ab 329	b 309	8.98
Zn (ppm.)	15	a 37.1	a 25.0	a 22.4	a 19.8	a 16.3	a 31.8	a 27.0	a 35.6	a 34.2	8.41
Fe (ppm.)	15	a 11.6	a 12.5	a 25.7	a 13.5	a 13.4	a 18.4	a 26.1	a 59.9	a 15.7	15.3
Cu (ppm.)	15	a 7.36	a 5.52	a 4.50	a 5.85	a 4.53	a 6.18	a 6.98	a 7.19	a 5.60	1.02
Mn (ppm.)	15	bc 0.90	c 0.84	bc 0.89	c 0.78	bc 0.93	ab 1.26	ab 1.12	ab 1.16	abc 1.05	0.09
P (%)	15	e 0.38	d 0.47	d 0.47	d 0.46	d 0.45	a 0.62	ab 0.58	bc 0.56	c 0.52	0.02

Table 4.4.2.4. Carcass Mineral Contents After 84 days : Experiment VII

Diet:	n	BA	B	BAM	BM	BAT	BT	BAMT	BMT	S.E.M.
Ca (%)	13	ab 0.54	a 0.58	ab 0.55	b 0.52	ab 0.55	a 0.59	ab 0.57	ab 0.57	0.02
Mg (ppm.)	13	b 287	a 320	b 298	b 294	b 292	ab 304	ab 306	ab 305	6.41
Zn (ppm.)	13	ab 26.9	ab 33.0	ab 31.7	ab 31.6	ab 40.1	a 63.0	ab 53.3	b 23.57	42.6
Fe (ppm.)	13	a 11.7	a 15.3	a 12.6	a 14.2	a 14.4	a 14.7	a 14.6	a 13.8	1.46
Cu (ppm.)	13	a 6.69	a 6.58	a 5.26	a 4.46	a 4.49	a 4.88	a 5.83	a 5.80	1.08
Mn (ppm.)	13	abc 1.22	a 1.34	bc 1.04	abc 1.05	abc 1.05	abc 1.18	ab 1.28	c 0.97	0.09
P (%)	13	b 0.48	a 0.53	ab 0.50	b 0.48	b 0.49	ab 0.50	ab 0.51	ab 0.51	0.01

4.5. Experiment VII

4.5.1. Growth and Feeding

The results of the factorial analysis of the growth and feeding statistics are shown in Table 4.5.1.1. This shows that none of the dietary factors influenced the SGR. However, in this experiment, the fish were not marked, and thus the sample size for this statistic was n=4. As more fish were used in this experiment than previously, the mean final weights could be calculated from large samples of fish (n=46). In this experiment, the mean final weight statistic has the most degrees of freedom and provides the most sensitive indicator of differences in growth. The growth of the fish (as expressed by the final weights) was found to have been increased (see Table 4.5.1.2.) by dietary iron (Fe) and decreased by an interaction of Pruteen X calcium (Ca). Intake was unaffected by these factors, but was highly significantly increased by dietary Pruteen (B). This result implies that the FCR of the fish fed Pruteen was higher than that of fish fed casein/gelatin (C), but as the sample size for FCR was n=1, statistical confirmation of this hypothesis was impossible. Table 4.5.1.2. shows the absolute growth and feed statistics. Diet EM is the commercial comparator control.

Table 4.5.1.1. Factorial Effects on Growth & Feed Intake: Expt. VII

Factor / Statistic:	Final Wt.	SGR	Intake
n	44	4	112
Calcium	N.S.	N.S.	N.S.
Iron	+	N.S.	N.S.
Calcium X Iron	N.S.	N.S.	N.S.
Pruteen	N.S.	N.S.	++
Pruteen X Calcium	--	N.S.	N.S.
Pruteen X Iron	N.S.	N.S.	N.S.
Pruteen X Calcium X Iron	N.S.	N.S.	N.S.

Table 4.5.1.2 Growth and Feed Conversion Statistics: Expt. VII

Diet:	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM	S.E.M.
Initial		a	a	a	a	a	a	a	a	a	
Weight g.	64	5.23	4.80	4.90	4.95	4.66	5.00	5.13	4.82	5.15	0.18
Final		bc	b	bc	bc	bc	c	b	bc	a	
Weight g.	46	9.91	12.89	11.85	12.55	10.83	8.97	12.96	10.54	26.86	1.19
SGR		a	a	a	a	a	a	a	a	a	
%/day	4	0.54	0.85	0.76	0.78	0.73	0.50	0.81	0.70	1.42	0.37
Intake		d	abcd	cd	bcd	ab	abc	abc	a	ab	
%/day	112	1.10	1.53	1.39	1.42	1.81	1.78	1.78	1.85	1.81	0.13
FCR	1	2.19	1.8	1.83	1.82	2.48	3.56	2.20	2.64	1.28	-----

4.5.2. Mineral analyses

4.5.2.1. Fish Carcasses

At the end of the experiment (16 weeks), 16 fish were randomly selected from each treatment for carcass mineral analysis (Tables 4.5.2.1.1. and 4.5.2.1.2.). The results showed a number of effects on whole-body Ca, Mg, Zn and Mn. Ca was significantly increased by an interaction of calcium X iron, and highly significantly increased by the presence of Pruteen in the diet, but was reduced ($P <= 0.01$) by Pruteen X Ca X Fe.

Mg was significantly reduced by dietary calcium and raised by Pruteen. The interaction Pruteen X Fe significantly raised the Mg content.

Zn was highly significantly suppressed by dietary Fe.

Mn was highly significantly reduced by Fe added to the diet, and also by the interaction Pruteen X Ca X Fe. Pruteen significantly reduced the manganese content. Ca X Fe increased the Mn content ($P <= 0.01$), as did Pruteen X Fe. Pruteen X Ca induced a significant increase in Mn content ($P <= 0.05$).

The amount of phosphorus in the fish carcasses was reduced ($P <= 0.01$) by dietary Ca and by Fe, and increased by Pruteen, Pruteen X Ca, and Pruteen X Fe ($P <= 0.01$). The interaction Pruteen X Ca X Fe suppressed phosphorus levels ($p <= 0.05$).

Table 4.5.2.1.1. Expt. VII: Factorial Effects on Mineral Contents After 112 days

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	16	16	16	16	16	16	16
Calcium	N.S.	-	N.S.	N.S.	N.S.	N.S.	--
Iron	N.S.	-	--	N.S.	N.S.	--	--
Calcium X Iron	+	N.S.	N.S.	N.S.	N.S.	++	N.S.
Pruteen	++	++	N.S.	N.S.	N.S.	-	++
Pruteen X Calcium	N.S.	N.S.	N.S.	N.S.	N.S.	+	++
Pruteen X Iron	N.S.	+	N.S.	N.S.	N.S.	++	++
Pruteen X Calcium X Iron	--	N.S.	N.S.	N.S.	N.S.	--	-

Table 4.5.2.1.2. Carcass Mineral Contents After 112 days : Experiment VII

Diet:	n	I	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	S.E.M.
Ca (%)	16	ab 0.54	ab 0.52	c 0.39	c 0.38	b 0.47	ab 0.54	a 0.58	a 0.55	a 0.55	0.03
Mg (ppm.)	16	a 282	a 291	b 258	b 251	b 251	a 293	a 292	a 298	a 288	7.76
Zn (ppm.)	16	a 31.1	b 25.1	b 23.6	b 21.6	b 19.3	b 23.9	b 24.8	b 22.7	b 21.2	1.94
Fe (ppm.)	16	ab 15.8	ab 14.7	ab 13.3	a 22.8	ab 14.5	b 12.4	ab 14.8	ab 13.9	ab 14.4	3.16
Cu (ppm.)	16	a 6.28	ab 5.9	b 3.83	b 3.79	ab 4.18	ab 5.27	a 6.35	ab 4.29	ab 4.12	0.75
Mn (ppm.)	16	a 1.42	a 1.47	bc 0.89	c 0.67	b 0.98	bc 0.84	b 1.03	bc 0.87	bc 1.42	0.09
P (%)	16	a 0.51	a 0.49	b 0.39	b 0.39	b 0.37	a 0.50	a 0.53	a 0.52	a 0.51	0.02

4.5.2.2. Bone

Factorial analysis (Table 4.5.2.2.1.) showed that dietary calcium reduced Ca and P in bone ($P <= 0.01$), and also magnesium . Dietary iron highly significantly increased the iron content of the bones. Pruteen highly significantly increased the calcium, Mg and P content of the bone. Pruteen X Ca also increased the Ca and magnesium content of the bones, and as this analysis is not additive, indicates that this highly significant increase was above that induced by Pruteen alone. Pruteen X Ca induced a highly significant increase in the Mn and P contents of the bone. Absolute values of the mineral contents in bone are shown in Table 4.5.2.2.2.

Table 4.5.2.2.1. Factorial Effects on Minerals in Bone: Expt. VII

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	5	5	5	5	5	5	5
Calcium	--	--	N.S.	N.S.	N.S.	N.S.	--
Iron	N.S.	N.S.	N.S.	++	N.S.	N.S.	N.S.
Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen	++	++	N.S.	N.S.	N.S.	N.S.	++
Pruteen X Calcium	++	++	N.S.	N.S.	N.S.	++	++
Pruteen X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 4.5.2.2.2. Tissue Mineral Contents: Bone

Mineral	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM	S.E.M.
		ab	cd	a	d	abc	ab	ab	ab	bc	
Ca (%)	5	3.31	2.69	3.66	2.44	3.17	3.51	3.32	3.39	3.02	0.16
		b	c	ab	c	ab	a	ab	ab	b	
Mg (ppm.)	5	705	551	753	559	758	824	741	757	698	28.9
		abc	abc	a	abc	abc	ab	abc	bc	c	
Zn (ppm.)	5	37.4	38.7	48.9	36.3	42.8	46.8	34.8	32.6	29.8	4.55
		b	b	a	b	b	b	b	b	b	
Fe (ppm.)	5	18.9	18.7	33.4	23.0	18.8	20.4	23.3	25.0	24.2	2.82
		a	d	ab	cd	d	abc	cd	bcd	ab	
Mn (ppm.)	5	4.82	2.39	4.21	2.59	2.44	3.82	2.74	3.44	4.19	0.42
		a	b	a	b	a	a	a	a	a	
P (%)	5	2.09	1.71	2.25	1.59	2.10	2.25	2.12	2.14	2.11	0.09

4.5.2.3. Eye

Dietary calcium induced a highly significant increase in the Ca content of the eye, whilst Ca X Fe interacted to increase the zinc content of the tissue. Pruteen appeared to suppress the Ca and Mn levels and the interaction Pruteen X Ca induced further reduction of the Fe content ($P <= 0.01$). Ca and Pruteen X Ca also induced an increase ($P <= 0.01$) in the P, whilst Pruteen X Fe appeared to reduce the P levels (Table 4.5.2.3.1.). Absolute values of minerals in the eyes of fish are shown in Table 4.5.2.3.2.

Table 4.5.2.3.1. Factorial Effects on Minerals in Eye: Expt. VII

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	5	5	5	5	5	5	5
Calcium	++	N.S.	N.S.	-	N.S.	N.S.	N.S.
Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Calcium X Iron	N.S.	N.S.	+	N.S.	N.S.	N.S.	N.S.
Pruteen	--	N.S.	N.S.	N.S.	N.S.	--	++
Pruteen X Calcium	--	N.S.	N.S.	++	N.S.	N.S.	++
Pruteen X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	--
Pruteen X Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 4.5.2.3.2. Tissue Mineral Contents: Eye

Mineral	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM	S.E.M.
		bc	c	ab	c	ab	a	ab	ab	bc	
Ca (ppm.)	5	427	310	462	322	487	596	470	498	440	43.8
		ab	ab	ab	ab	ab	a	bc	ab	c	
Mg (ppm.)	5	119	115	119	117	119	126	109	116	99.0	4.20
		ab	ab	abc	a	bc	abc	abc	c	c	
Zn (ppm.)	5	89.2	94.1	74.2	96.4	71.2	74.9	76.0	63.9	61.5	7.10
		a	a	a	a	a	a	a	a	a	
Fe (ppm.)	5	26.3	22.9	30.0	23.1	26.4	26.7	21.3	23.3	27.1	3.75
		a	a	a	a	a	a	a	a	a	
Mn (ppm.)	5	0.77	0.40	0.61	0.64	0.71	0.48	0.51	0.04	0.30	0.266
		abc	d	ab	cd	a	a	bcd	ab	d	
P (%)	5	0.14	0.12	0.15	0.13	0.16	0.16	0.13	0.15	0.12	0.005

4.5.2.4. Kidney

The results of the factorial analysis of kidney minerals are shown in Table 4.5.2.4.1. Pruteen decreased the Ca content of the kidney ($P <= 0.05$), and no other significant effects were observed. Mn could not be determined absolutely (as opposed to relatively), because tissue samples were small. Absolute mineral levels are shown in Table 4.5.2.4.2. Kidney tissue had the lowest weight of all the tissues analysed. Factorial analysis could be used to examine the relative Mn levels, even when the absolute values were negative. Negative values occurred when sample readings fell below the blank Mn level during spectrophotometric analysis. A negative value was, in itself, meaningless.

Table 4.5.2.4.1. Factorial Effects on Minerals in Kidney: Expt. VII

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	5	5	5	5	5	5	5
Calcium	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen	-	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 4.5.2.4.2. Tissue Mineral Contents: Kidney

Mineral	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM	S.E.M.
		b	a	b	ab	b	b	b	b	b	
Ca (ppm.)	5	191	338	163	221	195	137	101	169	128	45.5
		a	a	a	a	a	a	a	a	a	
Mg (ppm.)	5	218	226	217	250	258	214	186	248	200	25.5
		a	a	a	a	a	a	a	a	a	
Zn (ppm.)	5	58.7	36.8	8.04	12.3	6.75	53.8	79.4	39.2	27.1	30.3
		a	a	a	a	a	a	a	a	a	
Fe (ppm.)	5	96.3	96.7	138	119	105	155	158	118	150	22.3
		b	b	b	ab	a	b	b	b	b	
Cu (ppm.)	5	3.67	2.52	2.58	4.71	8.90	2.90	2.69	1.27	3.14	1.67
		a	a	a	a	a	a	a	a	a	
P (%)	5	0.32	0.37	0.33	0.37	0.44	0.37	0.36	0.40	0.36	0.05

4.5.2.5. Muscle

The results of the factorial analyses of muscle mineral levels are shown in Table 4.5.2.5.1. Calcium in the diet decreased the magnesium content of the muscle, ($P <= 0.05$), and the phosphorus content ($P <= 0.01$). Iron increased the zinc content of the muscle ($P <= 0.05$). Ca X Fe significantly reduced the zinc content, whilst Pruteen highly significantly decreased Mg and Zn. Pruteen X Ca further suppressed the Mg and P . Absolute mineral levels are shown in Table 4.5.2.5.2.

Table 4.5.2.5.1. Factorial Effects on Minerals in Muscle: Expt. VII

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	5	5	5	5	5	5	5
Calcium	N.S.	-	N.S.	N.S.	N.S.	N.S.	--
Iron	N.S.	N.S.	+	N.S.	N.S.	N.S.	N.S.
Calcium X Iron	N.S.	N.S.	-	N.S.	N.S.	N.S.	N.S.
Pruteen	N.S.	--	--	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium	N.S.	-	N.S.	N.S.	N.S.	N.S.	-
Pruteen X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 4.5.2.5.2. Tissue Mineral Contents: Muscle

Mineral	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM	S.E.M.
		ab	ab	ab	ab	ab	ab	a	ab	b	
Ca (ppm.)	5	208	166	213	1874	176	184	234	201	154	22.7
		ab	ab	a	a	ab	c	a	bc	abc	
Mg (ppm.)	5	291	291	303	295	289	253	295	259	273	10.5
		ab	ab	a	ab	b	b	ab	b	ab	
Zn (ppm.)	5	4.03	4.05	5.88	4.41	2.13	2.92	3.78	2.55	3.69	0.77
		a	ab	ab	ab	ab	ab	b	b	b	
Fe (ppm.)	5	5.02	3.60	3.23	3.10	2.89	3.96	2.13	2.07	1.57	0.79
		a	a	a	a	a	a	a	a	a	
Cu (ppm.)	5	0.68	0.48	0.62	0.39	0.37	0.26	0.42	0.22	0.80	0.20
		ab	ab	a	ab	a	b	a	ab	a	
P (%)	5	0.26	0.26	0.27	0.25	0.27	0.23	0.28	0.25	0.27	0.01

4.5.2.6. Liver

Ca increased the calcium content of the liver (P <= 0.01), iron increased the Fe content of the liver (P <= 0.01) (Table 4.5.2.6.1.). Pruteen highly significantly reduced the Mg and Zn levels. Pruteen X Ca further decreased the Mg level. Pruteen X Ca X Fe reduced the copper content (P <= 0.01). Table 4.5.2.6.2. shows that the Cu levels in the livers of fish fed diet EM were significantly higher than those of the experimental fish.

Table 4.5.2.6.1. Factorial Effects on Minerals in Liver: Expt. VII

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	5	5	5	5	5	5	5
Calcium	++	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Iron	N.S.	N.S.	N.S.	+	N.S.	N.S.	N.S.
Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen	--	++	N.S.	N.S.	+	N.S.	N.S.
Pruteen X Calcium	N.S.	++	N.S.	+	+	N.S.	N.S.
Pruteen X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium X Iron	N.S.	N.S.	N.S.	N.S.	--	N.S.	N.S.

Table 4.5.2.6.2. Tissue Mineral Contents: Liver

Mineral	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM	S.E.M.
Ca (ppm.)	5	abcd 86.2	ab 107	bcd 72.0	a 118	d 55.4	bcd 73.9	cd 68.2	abcd 91.9	abc 94.7	11.3
Mg (ppm.)	5	ab 171	c 131	ab 176	bc 152	ab 167	ab 180	ab 175	a 188	ab 174	11.0
Zn (ppm.)	5	a 28.8	a 18.4	a 23.1	a 18.0	a 16.5	a 21.1	a 20.2	a 20.8	a 27.3	4.93
Fe (ppm.)	5	abc 60.3	c 28.5	a 101	bc 47.3	bc 44.8	ab 85.5	ab 86.9	abc 70.0	ab 81.7	14.3
Cu (ppm.)	5	b 42.0	c 17.2	bc 35.1	bc 32.9	bc 34.1	b 53.3	b 44.0	bc 34.9	a 80.9	6.39
Mn (ppm.)	5	a 1.00	a 1.09	a 1.76	a 1.35	a 0.52	a 0.67	a 0.61	a 1.18	a 1.12	0.56
P (%)	5	a 0.37	b 0.28	a 0.34	ab 0.36	a 0.34	a 0.35	a 0.35	a 0.32	ab 0.36	0.02

4.5.2.7. Skin

Factorial analysis (Table 4.5.2.7.1.) showed that dietary calcium reduced the levels of Ca, Mg, P and Fe in the skin, whilst iron reduced Fe . Pruteen highly significantly increased Ca, Mg and P, and the interaction Pruteen X Ca increased the Fe content. Pruteen X Fe decreased the levels of Fe ($P <= 0.05$). Pruteen X Ca X Fe reduced the iron content further. Absolute levels of minerals are shown in Table 4.5.2.7.2.

Table 4.5.2.7.1. Factorial Effects on Minerals in Skin: Expt. VII

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	5	5	5	5	5	5	5
Calcium	-	--	N.S.	-	N.S.	N.S.	--
Iron	N.S.	N.S.	N.S.	-	N.S.	N.S.	N.S.
Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen	++	++	N.S.	N.S.	N.S.	N.S.	++
Pruteen X Calcium	N.S.	N.S.	N.S.	+	N.S.	N.S.	N.S.
Pruteen X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium X Iron	N.S.	N.S.	N.S.	-	N.S.	N.S.	N.S.

Table 4.5.2.7.2. Tissue Mineral Contents: Skin

Mineral	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM	S.E.M.
		bcd	cd	abc	d	a	abc	ab	ab	abc	
Ca (%)	5	0.76	0.58	0.85	0.49	1.13	0.88	0.98	1.07	0.91	0.10
		cd	d	bc	d	a	abc	abc	ab	bc	
Mg (ppm.)	5	408	332	430	328	547	469	490	507	433	26.9
		a	a	a	a	a	a	a	a	a	
Zn (ppm.)	5	44.3	47.5	41.5	45.0	55.9	46.2	172	42.5	34.7	42.9
		a	b	b	b	b	b	b	b	b	
Fe (ppm.)	5	22.4	6.56	7.58	6.29	11.7	12.0	10.9	8.61	9.17	3.15
		ab	b	ab	b	ab	ab	a	ab	ab	
Mn (ppm.)	5	2.52	1.00	1.85	0.97	1.50	1.52	3.06	1.55	1.79	0.58
		bc	cd	bc	d	a	ab	ab	ab	ab	
P (%)	5	0.71	0.56	0.74	0.50	0.98	0.80	0.87	0.91	0.81	0.07

Plates 4.5.2.7.1.-9. show scanning electron micrographs of scales from the fish. The scale of each micrograph is X80, and the reference bar 1mm. in each case.

Plate 4.5.2.7.1. Scanning Electron Micrograph of Scale from Fish Fed on Diet C

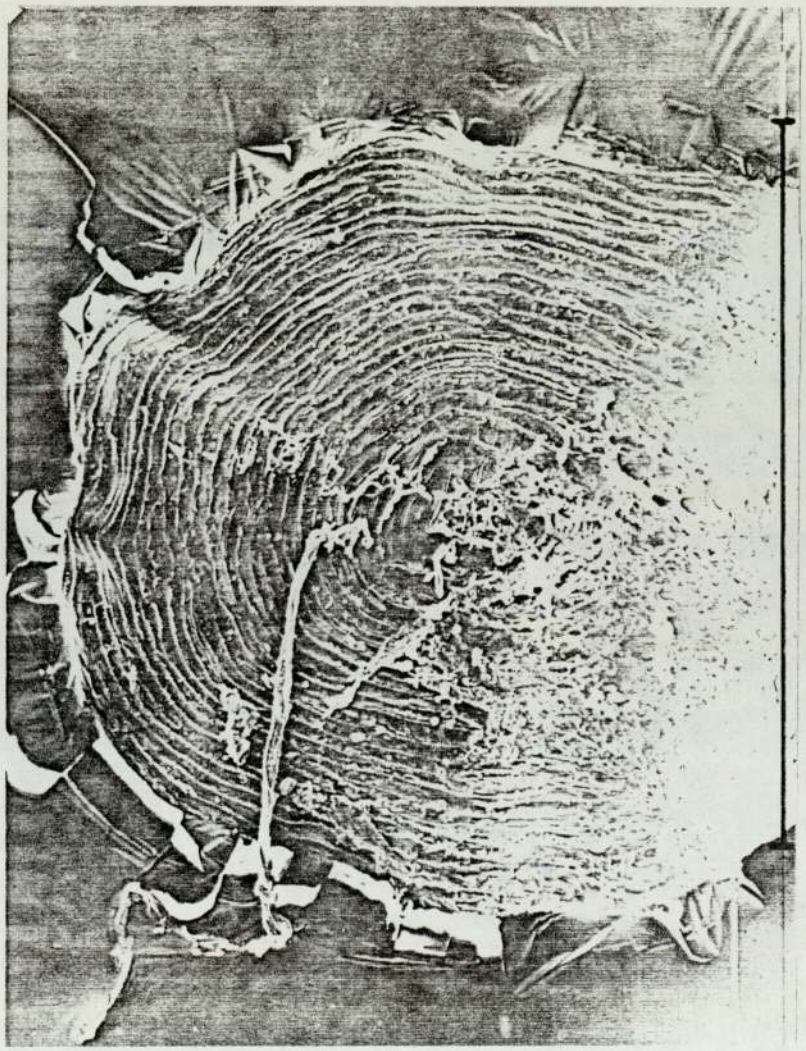


Plate 4.5.2.7.2. Scanning Electron Micrograph of Scale from Fish Fed on Diet CCa

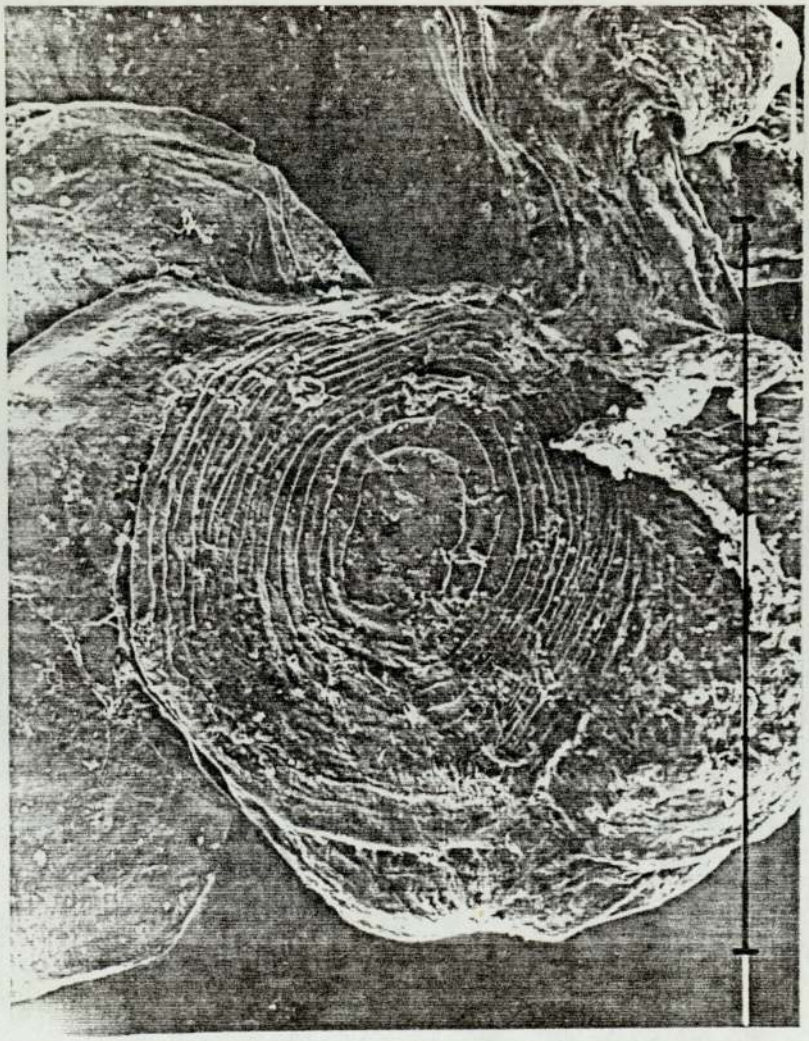


Plate 4.5.2.7.3. Scanning Electron Micrograph of Scale from Fish Fed on Diet CFe

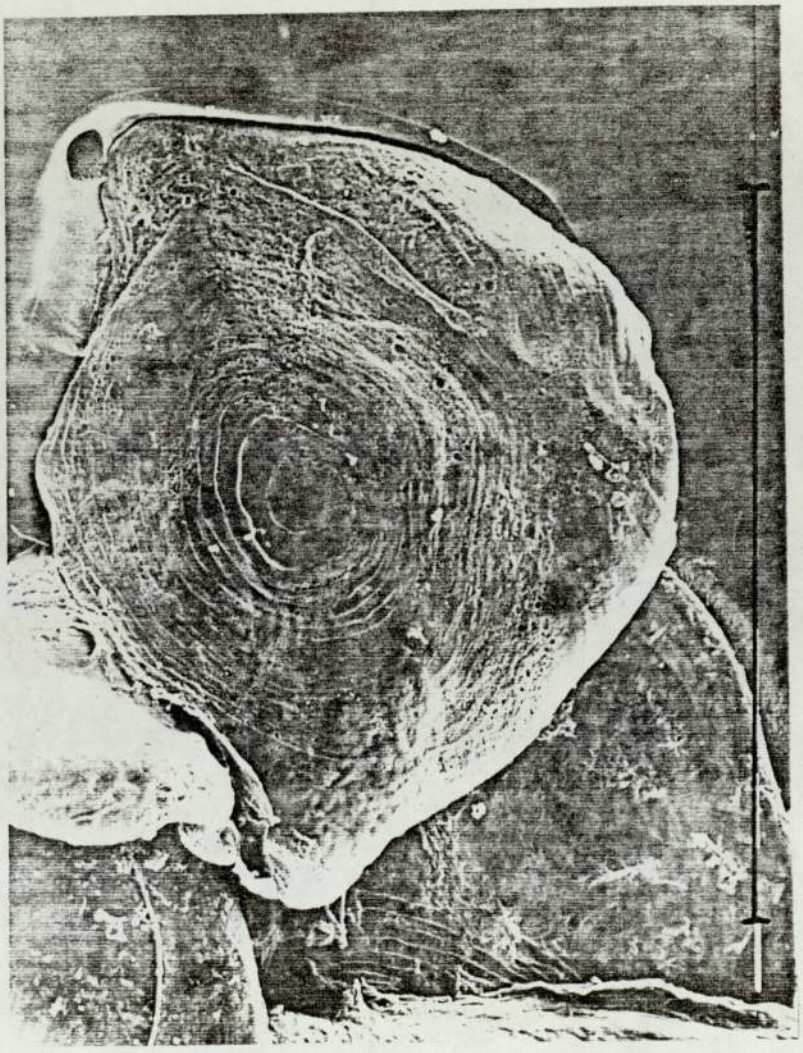


Plate 4.5.2.7.4. Scanning Electron Micrograph of Scale from Fish Fed on Diet CCaFe

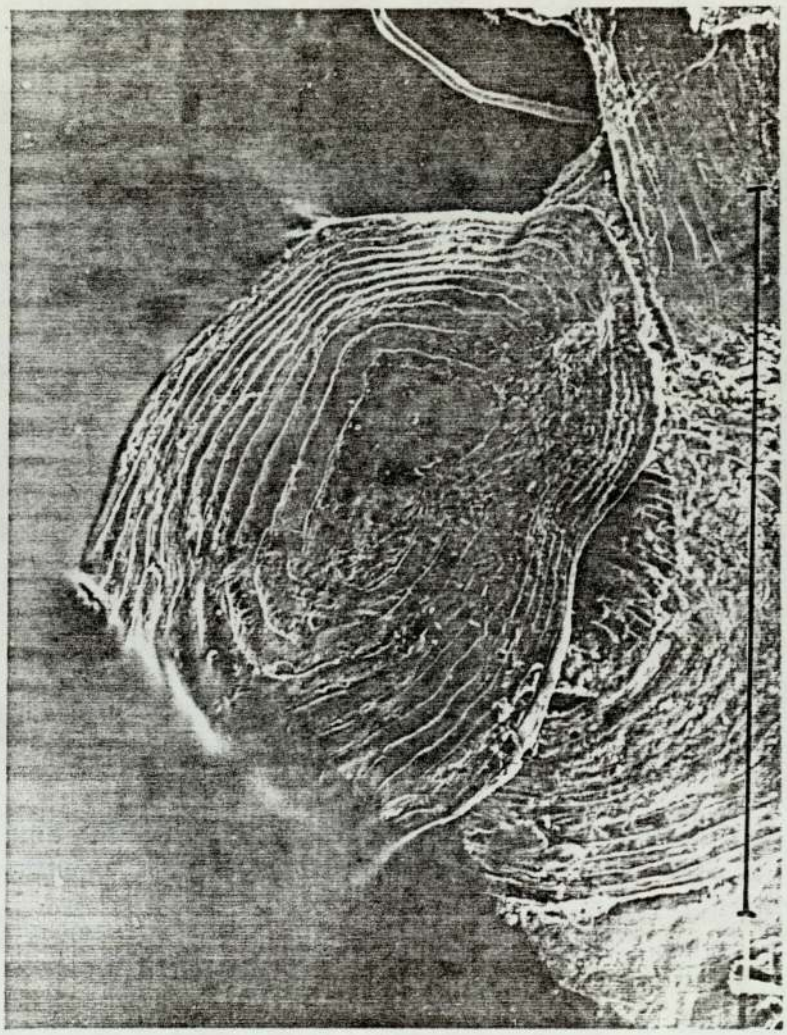


Plate 4.5.2.7.5. Scanning Electron Micrograph of Scale from Fish Fed on Diet B

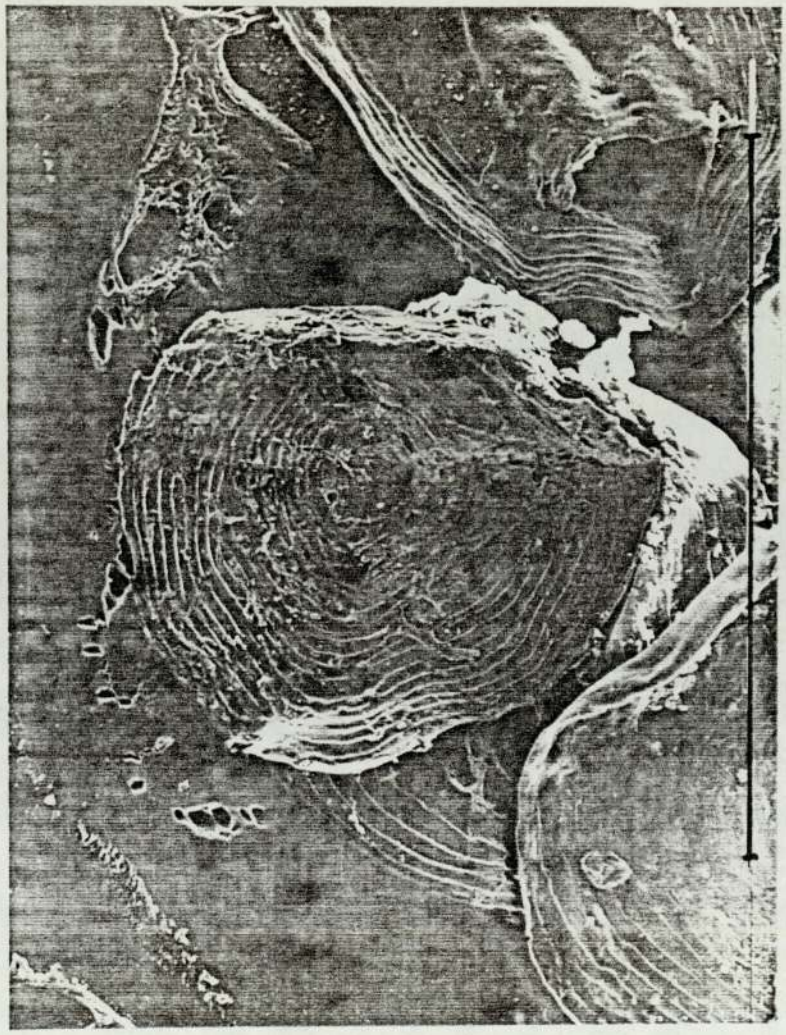


Plate 4.5.2.7.6. Scanning Electron Micrograph of Scale from Fish Fed on Diet BCa

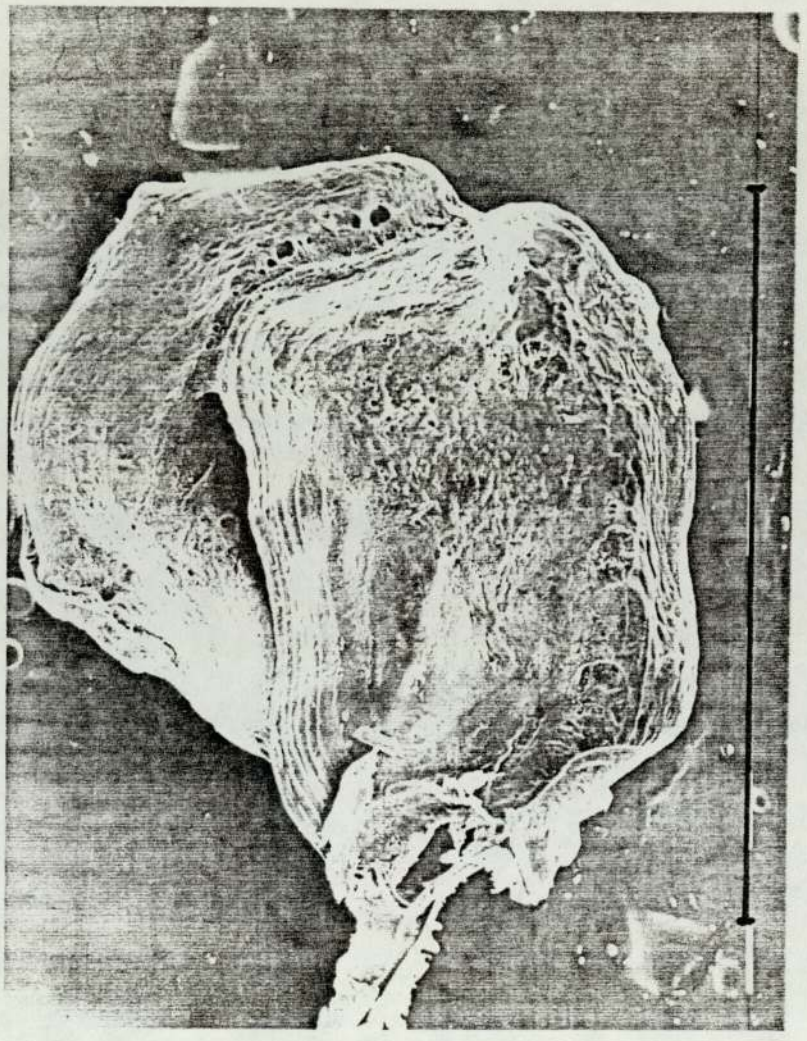


Plate 4.5.2.7.7. Scanning Electron Micrograph of Scale from Fish Fed on Diet BFe

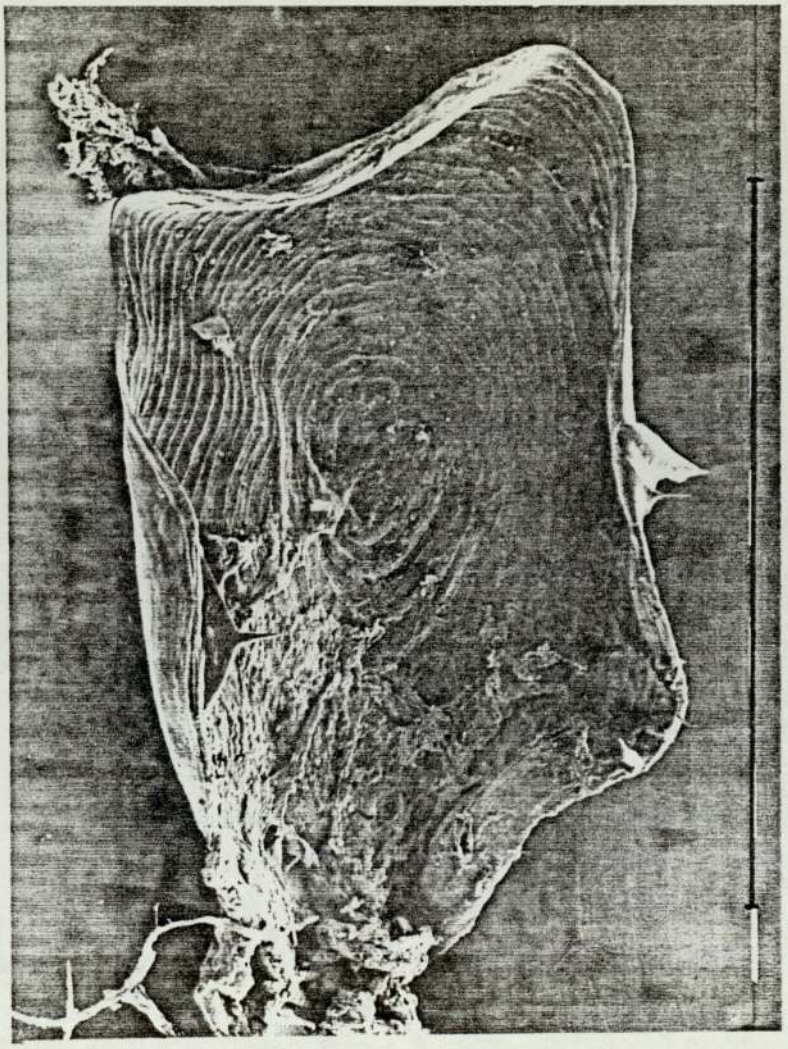


Plate 4.5.2.7.8. Scanning Electron Micrograph of Scale from Fish Fed on Diet BCaFe

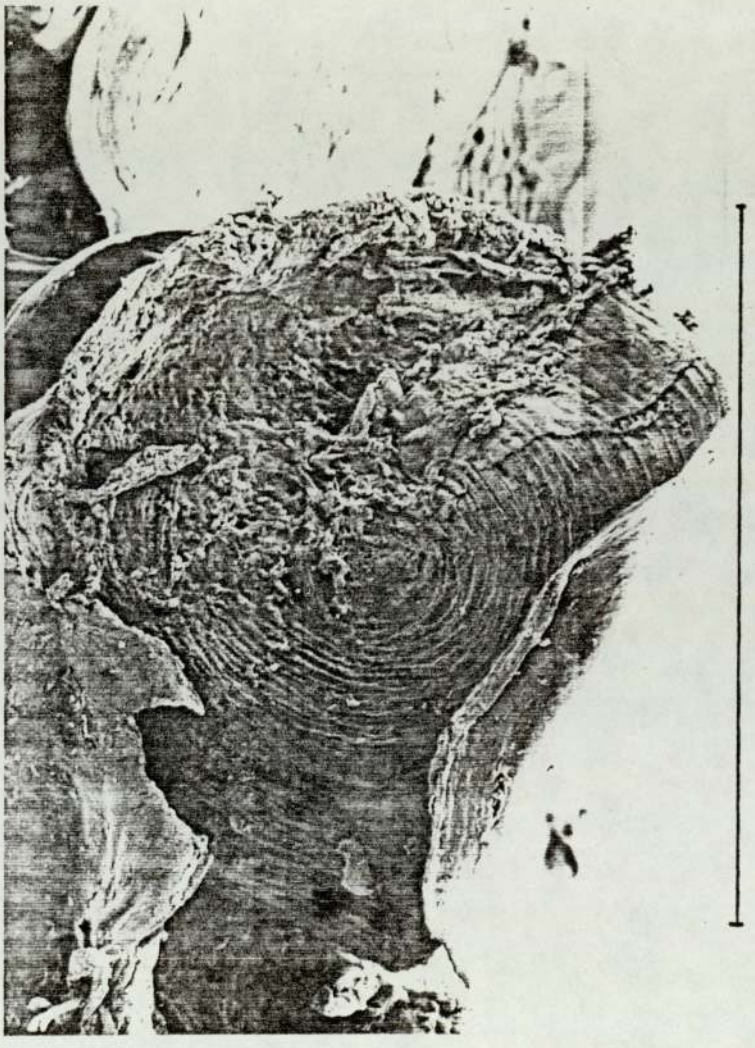


Plate 4.5.2.7.9. Scanning Electron Micrograph of Scale from Fish Fed on Diet EM

4.5.2.8. Viscera

Pruteen highly significantly increased the Ca content of the viscera, and reduced the zinc content. Pruteen X Ca significantly increased the Ca content ($P <= 0.05$) (Table 4.5.2.8.1.). Absolute mineral levels are shown in Table 4.5.2.8.2.

Table 4.5.2.8.1. Factorial Effects on Minerals in Viscera: Expt. VII

Mineral:	Ca	Mg	Zn	Fe	Cu	Mn	P
n	5	5	5	5	5	5	5
Calcium	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen	++	N.S.	--	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium	++	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen X Calcium X Iron	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 4.5.2.8.2. Tissue Mineral Contents: Viscera

Mineral	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM	S.E.M.
		b	a	b	a	b	b	b	b	b	
Ca (ppm.)	5	222	714	168	657	132	158	109	216	166	68.9
		a	ab	ab	ab	ab	b	b	ab	b	
Mg (ppm.)	5	208	164	167	177	168	151	155	161	143	15.3
		a	a	a	a	a	a	a	a	a	
Zn (ppm.)	5	30.0	57.5	42.6	53.6	49.8	41.9	36.9	37.6	52.0	8.90
		a	c	ab	bc	bc	bc	bc	ab	ab	
Fe (ppm.)	5	52.6	12.9	40.5	26.8	29.6	25.6	25.4	39.1	39.7	6.24
		a	a	a	a	a	a	a	a	a	
Cu (ppm.)	5	1.38	1.46	0.97	1.10	3.32	2.27	1.11	1.02	1.83	1.07
		a	ab	ab	ab	b	b	ab	b	a	
Mn (ppm.)	5	1.53	0.98	1.08	0.86	0.33	0.37	0.50	0.26	1.47	0.33
	b		a	ab	ab	ab	ab	ab	b	b	b
P (%)	5	0.25	0.21	0.21	0.22	0.20	0.23	0.23	0.23	0.23	0.10

4.5.2.9. Faeces

The mineral levels observed in the single sample of faeces available from each treatment are shown in Table 4.5.2.9.1. No statistical analyses were possible due to the sample size (n=1).

Table 4.5.2.9.1. Faecal Mineral Levels

Mineral	n	C	CCa	CFe	CCaFe	B	BCa	BFe	BCaFe	EM.	S.E.M.
Ca (ppm.)	1	463	7751	1070	7966	982	4252	1284	11335	17692	****
Mg (ppm.)	1	763	537	1261	668	666	566	943	2693	653	****
Zn (ppm.)	1	5.17	8.69	6.53	5.93	0.7	5.33	0.95	0.7	18.4	****
Fe (ppm.)	1	232	138	555	323	103	98	620	603	265	****
Cu (ppm.)	1	2.52	2.0	1.36	1.73	1.17	1.61	2.33	3.11	5.32	****
Mn (ppm.)	1	2.84	4.63	5.99	4.96	1.3	4.53	6.65	10.2	3.7	****
P (ppm.)	1	126	1450	109	1466	1401	1290	1654	2407	5661	****

4.5.2.10. Ca:Mg Ratios in tissues

Calcium increased the Ca:Mg ratio in the liver and viscera. Ca X Fe increased the carcass and bone Ca:Mg ratio. Pruteen increased the ratio in the carcass and eye, and decreased it in bone, kidney, liver and viscera. Pruteen X Ca increased the Ca:Mg ratio of the eye and decreased it in the liver. Pruteen X Ca X Fe decreased the Ca:Mg ratio of the carcass.

Table 4.5.2.10.1. Factorial Effects on Ca:Mg Ratios in Tissues: Expt. VII

Tissue:	Carc.	Bone	Eye	Kid.	Liv.	Mus.	Skin	Vis.
Ca	N.S.	N.S.	N.S.	N.S.	++	N.S.	N.S.	++
Fe	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Ca X Fe	++	+	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pruteen	++	--	++	--	--	N.S.	N.S.	--
Pruteen X Ca	N.S.	N.S.	++	N.S.	-	N.S.	N.S.	N.S.
Pruteen X Ca X Fe	--	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

4.6. Water Analysis

4.6.1. Water quality

The water quality in each experiment was monitored and records maintained of important parameters. The plots of temperature, dissolved oxygen, pH, total hardness, total nitrite and nitrate and dissolved ammonia during each experiment are shown in Figs. 4.6.1.1. to 4.6.1.4.

No water quality data were recorded for Experiment II.

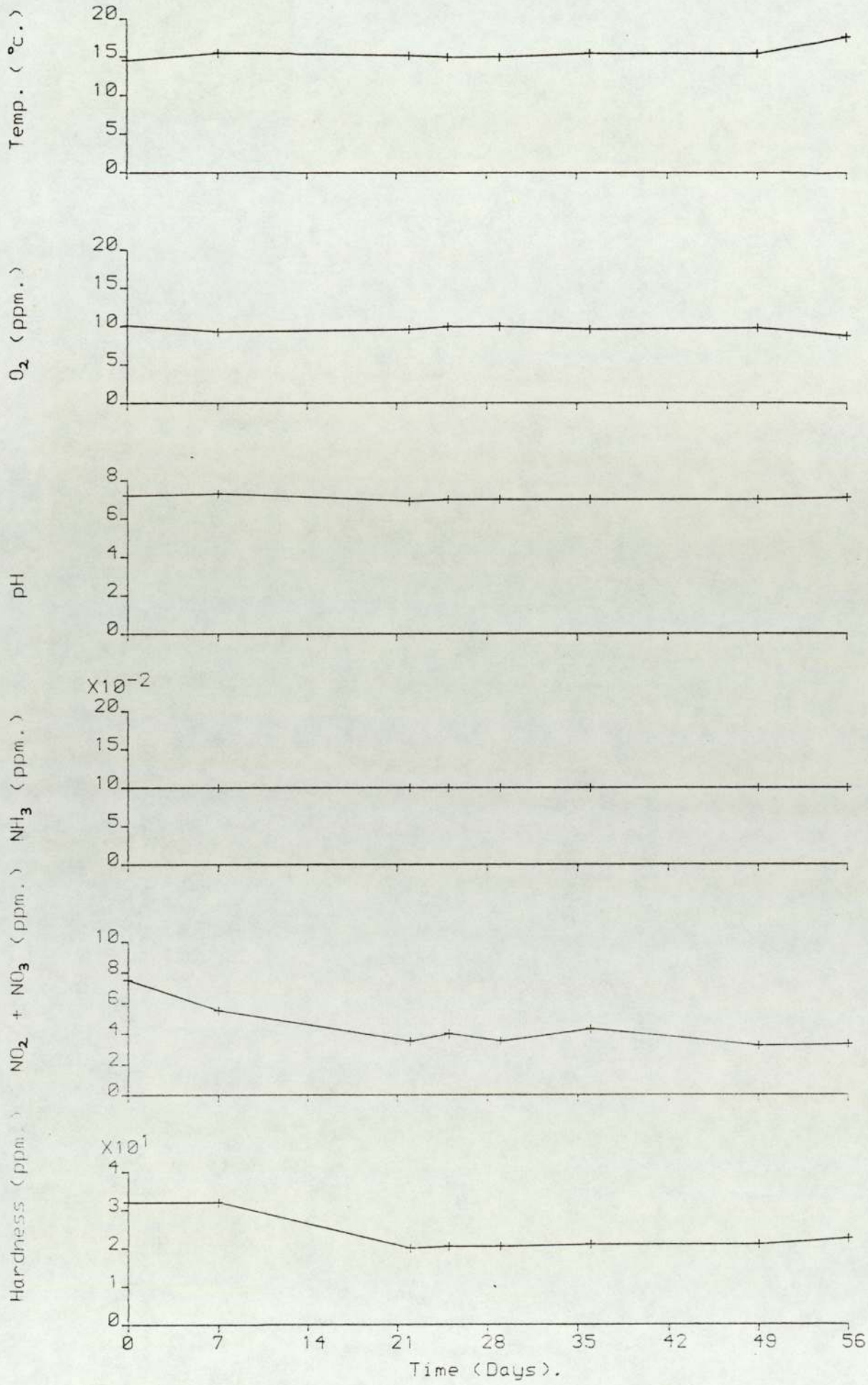


Fig.4.6.1.1. : Graphs of Water Environmental Parameters (Expt. III).

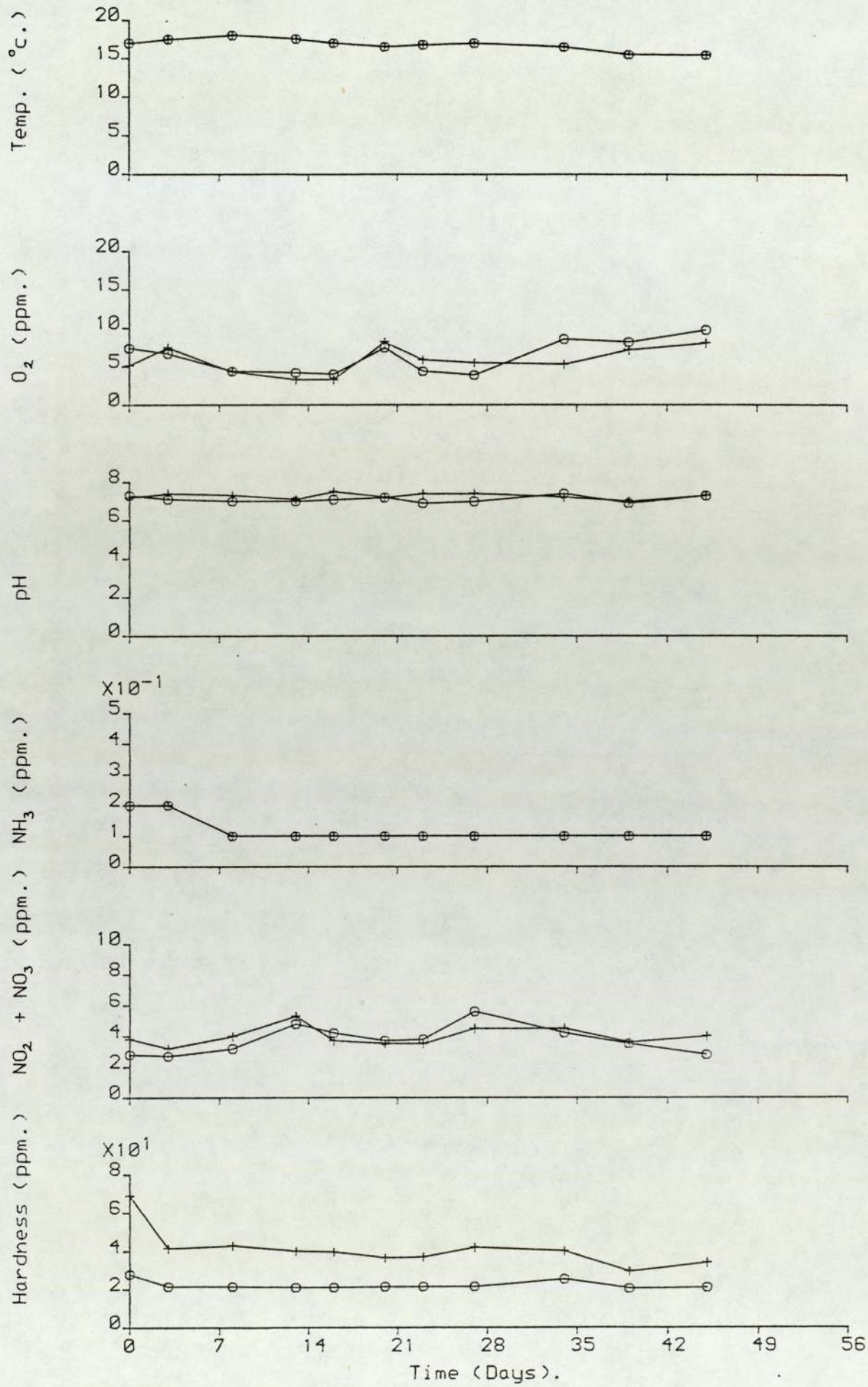


Fig.4.6.1.2. : Graphs of Water Environmental Parameters (Expt. V).

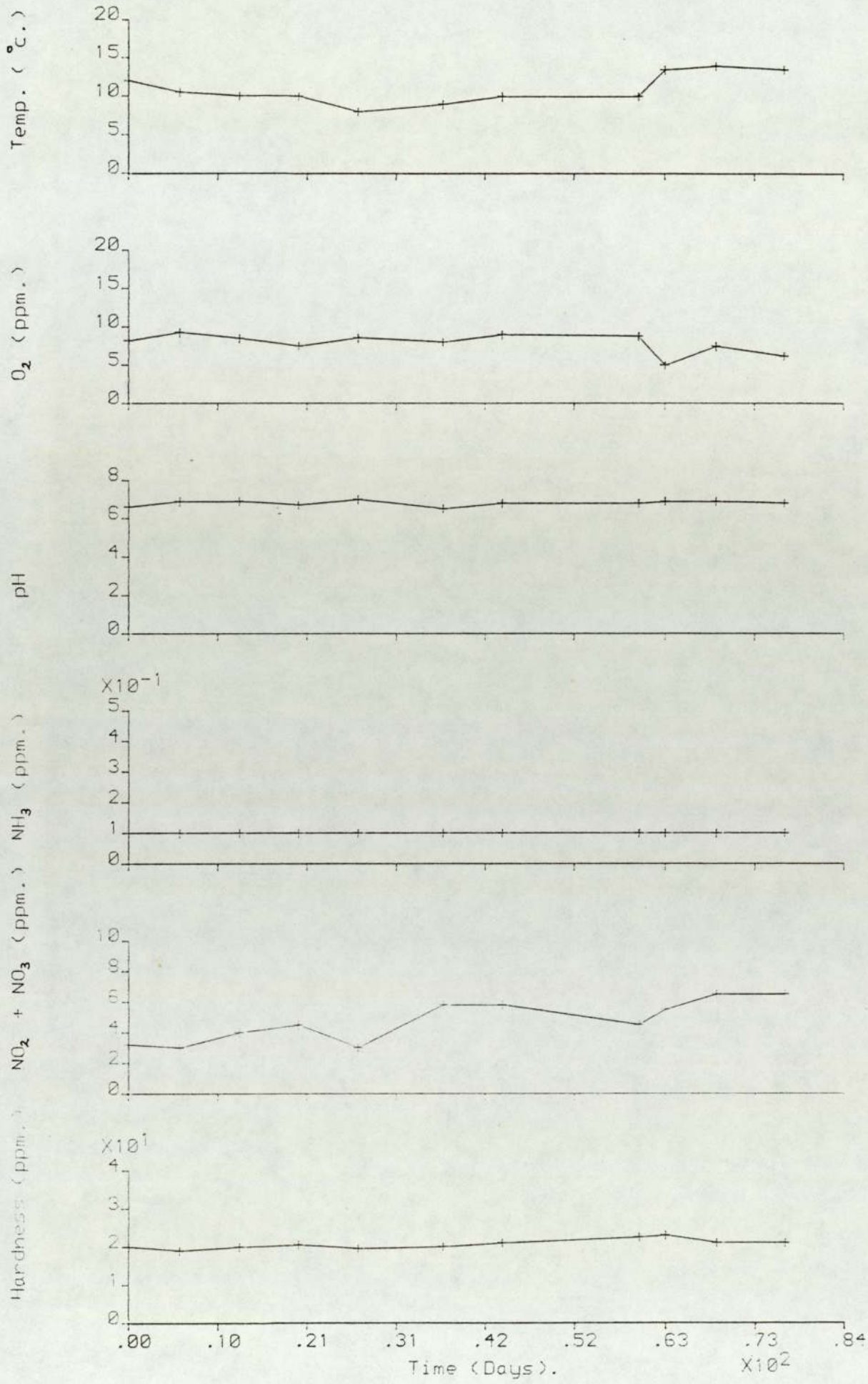


Fig. 4.6.13. : Graphs of Water Environmental Parameters (Expt. VI).

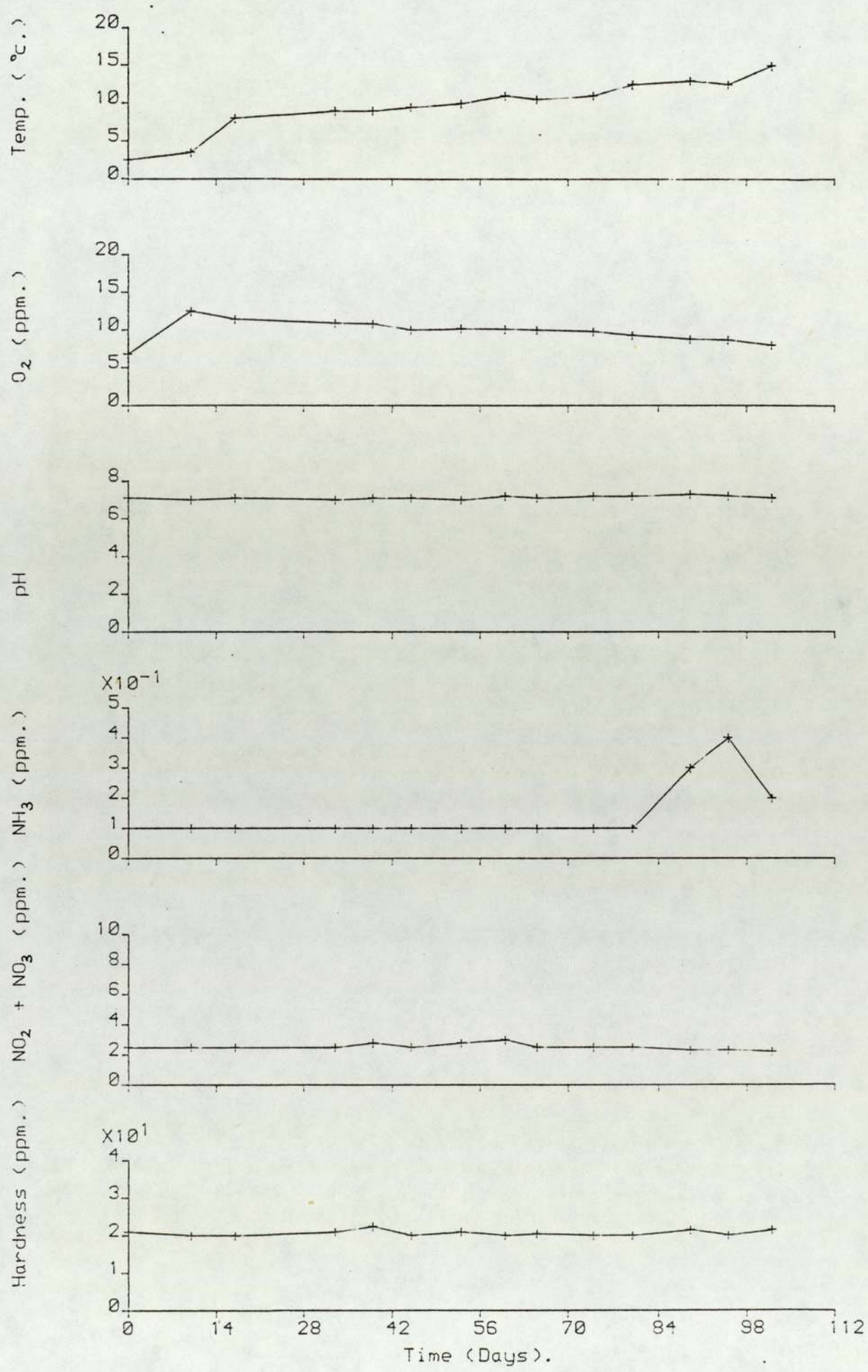


Fig.4.6.1.4. : Graphs of Water Environmental Parameters (Expt. VII).

4.6.2. Water Minerals

During Experiments VI and VII the supply of water to the fish culture unit was analysed for minerals. The concentrations of metals determined in raw water and by the use of ion exchange resin to concentrate the minerals are shown in Figs. 4.6.2.1. to 4.6.2.20. The detection limits of the spectrophotometer are shown on each graph, to indicate the greater sensitivity that concentration of water samples allowed.

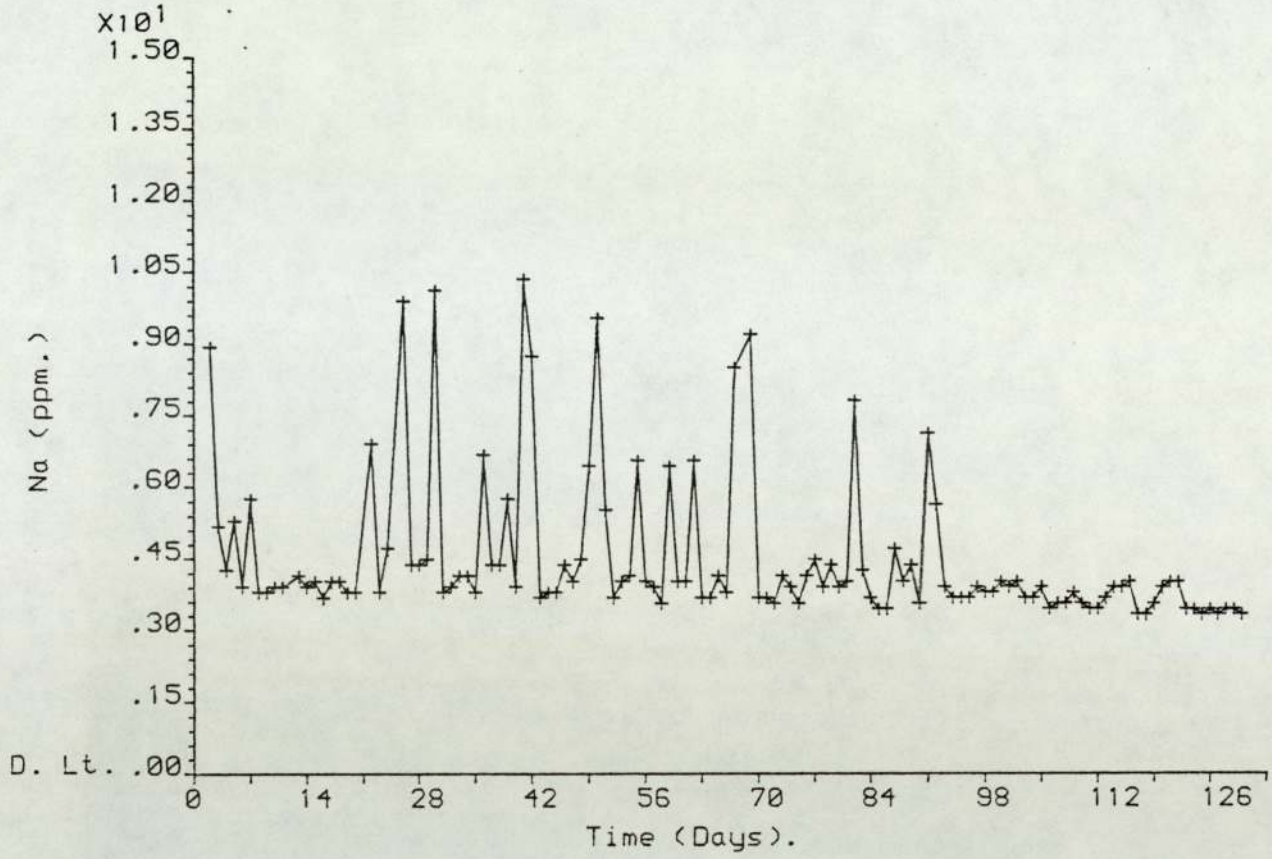


Fig.4.6.2.1. : Graph of Water Na Levels v. time.

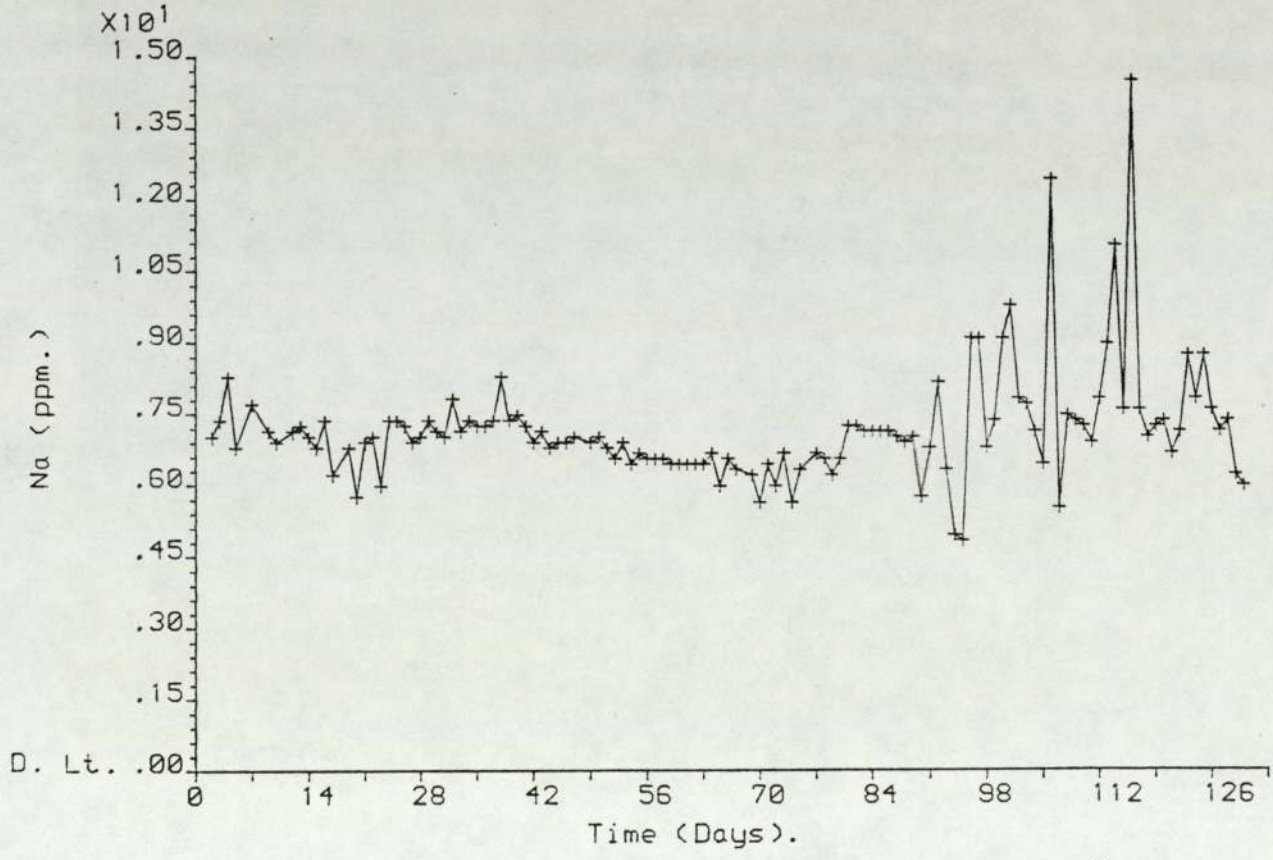


Fig.4.6.2.2. : Graph of Water Na Levels v. time. (By digestion of resin.)

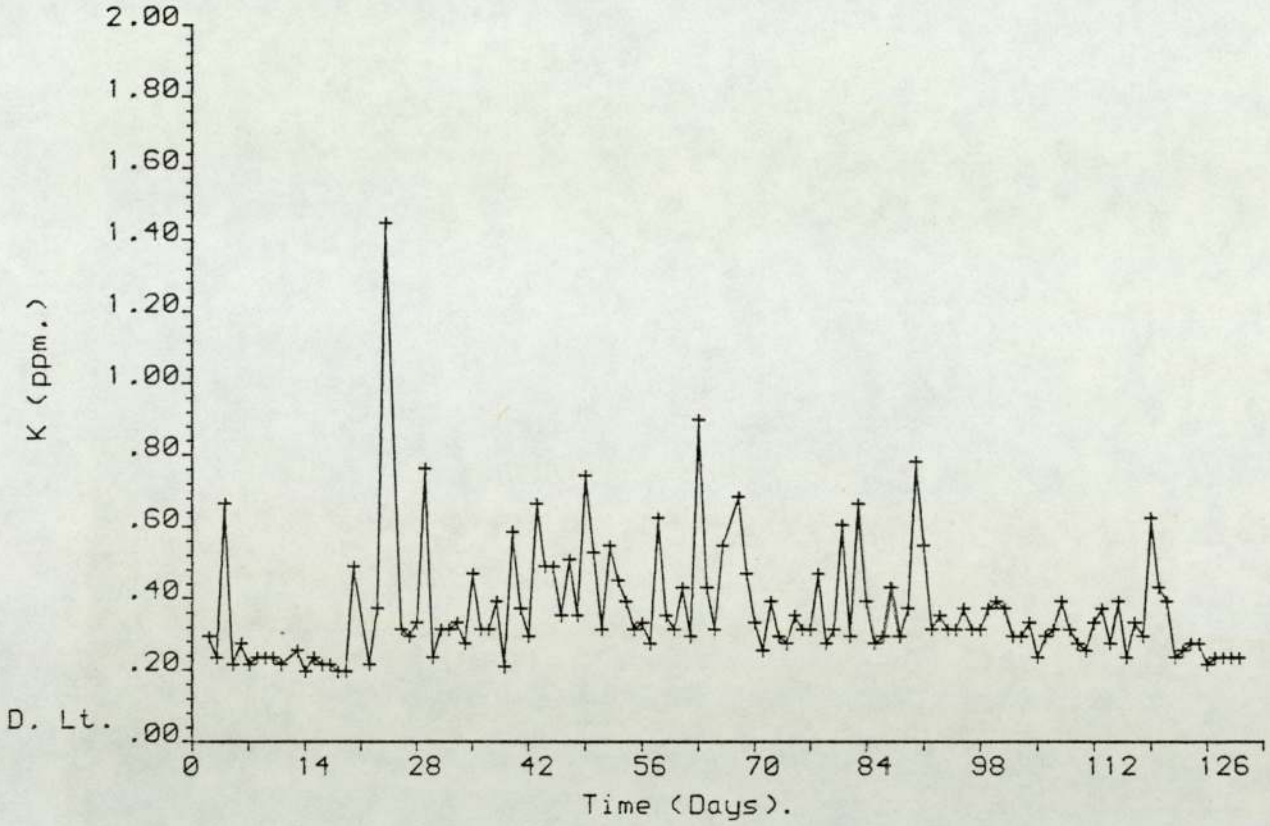


Fig.4.6.2.3. : Graph of Water K Levels v. time.

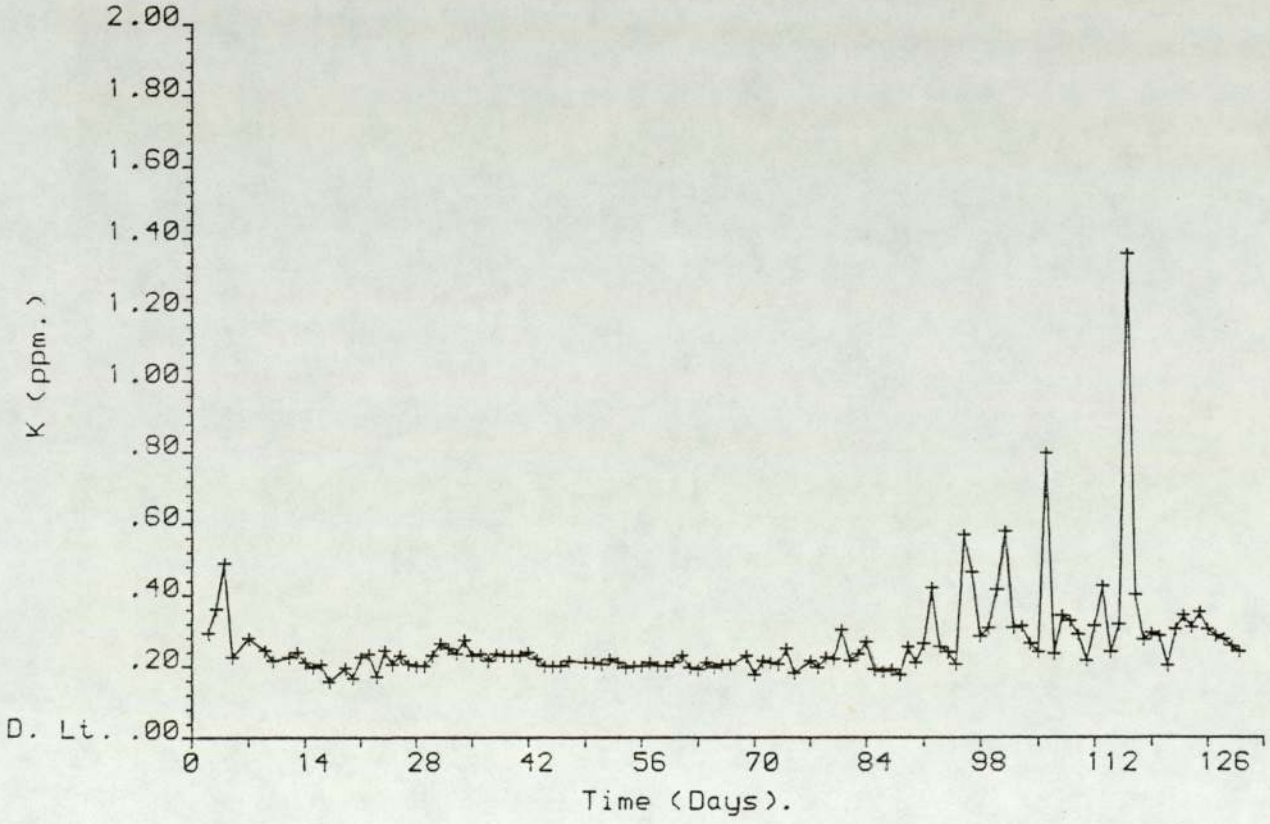


Fig.4.6.2.4. : Graph of Water K Levels v. time. (By digestion of resin.)

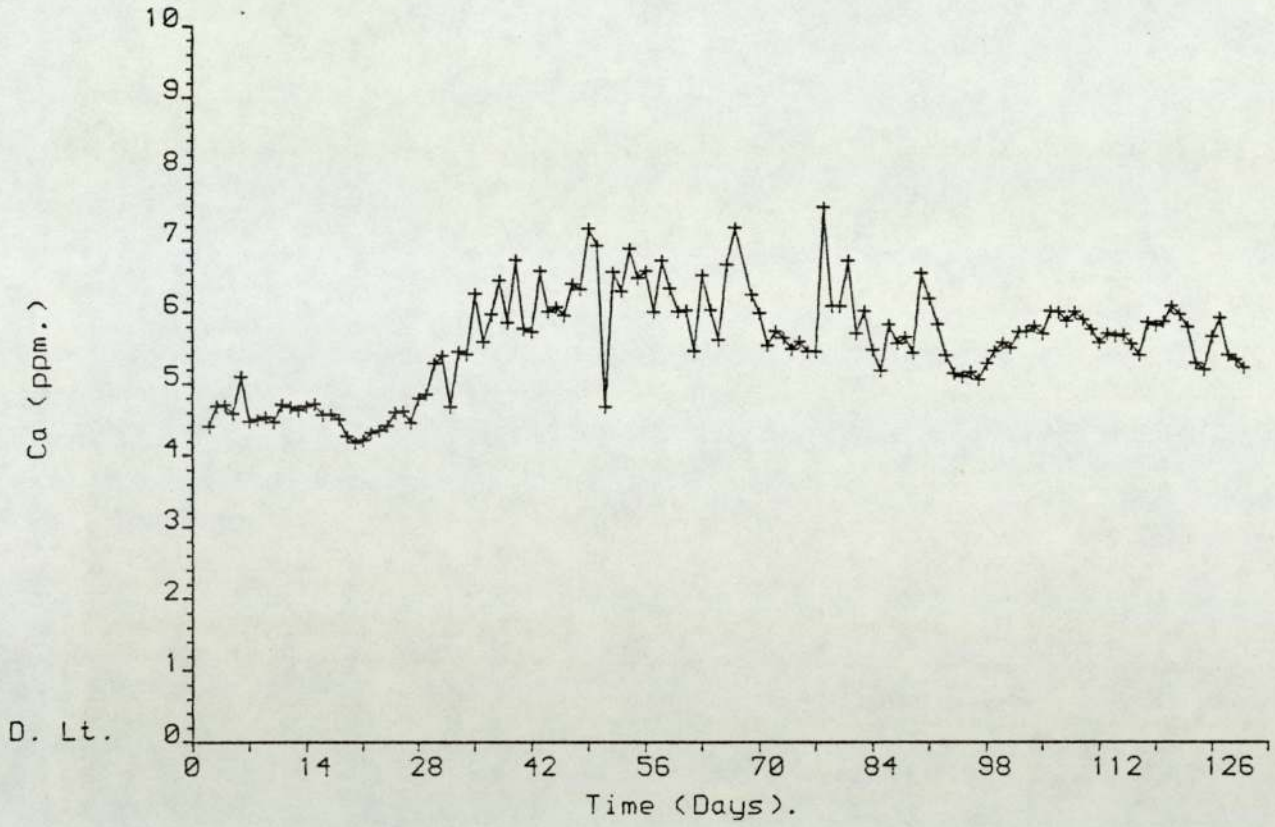


Fig.4.6.2.5. : Graph of Water Ca Levels v. time.

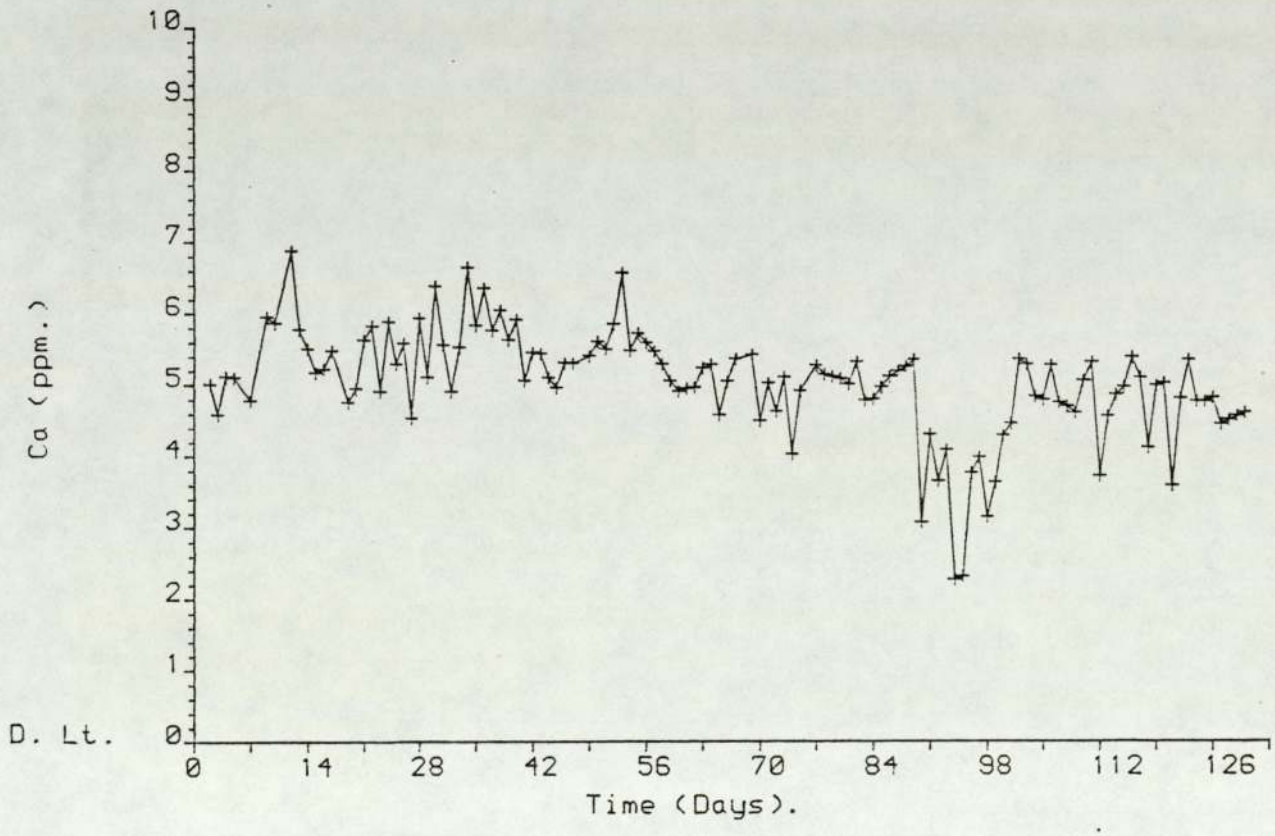


Fig.4.6.2.6. : Graph of Water Ca Levels v. time. (By digestion of resin.)

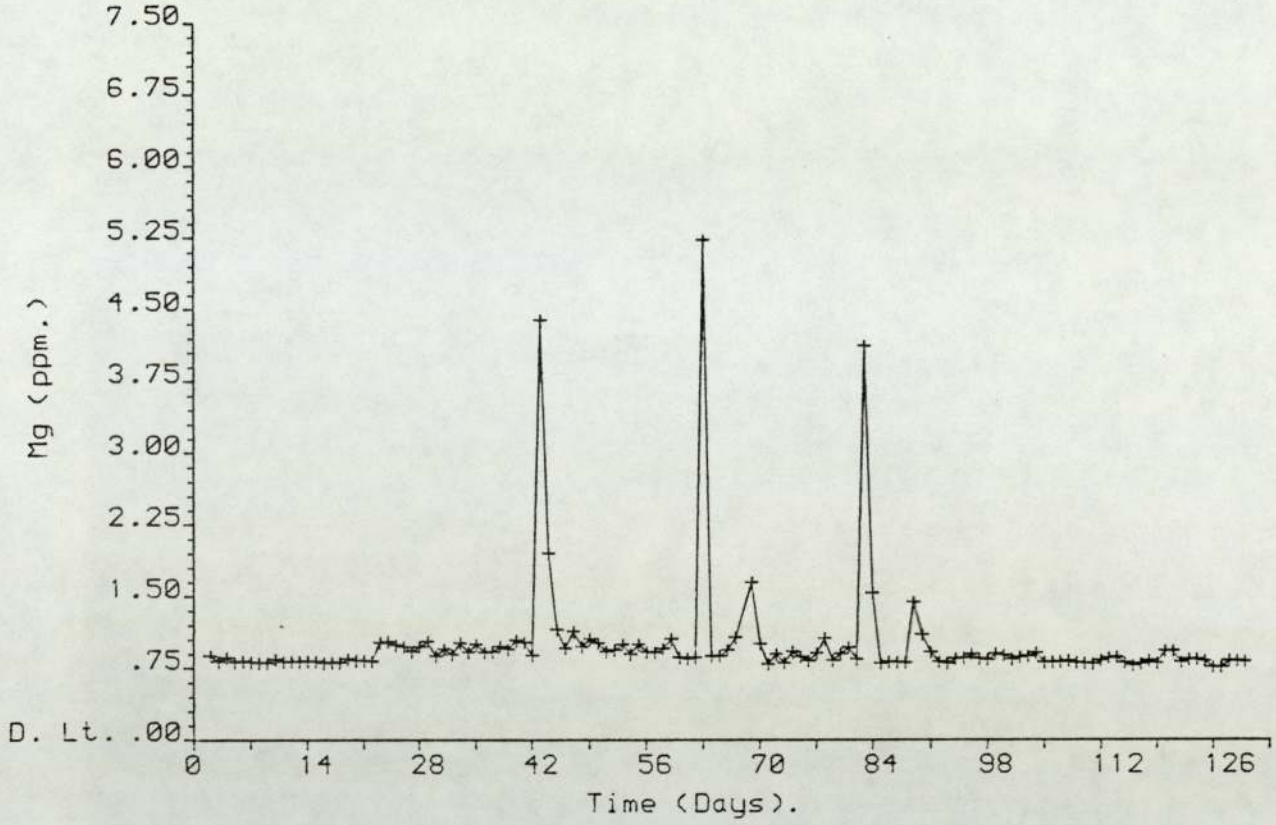


Fig. 4.6.2.7. : Graph of Water Mg Levels v. time.

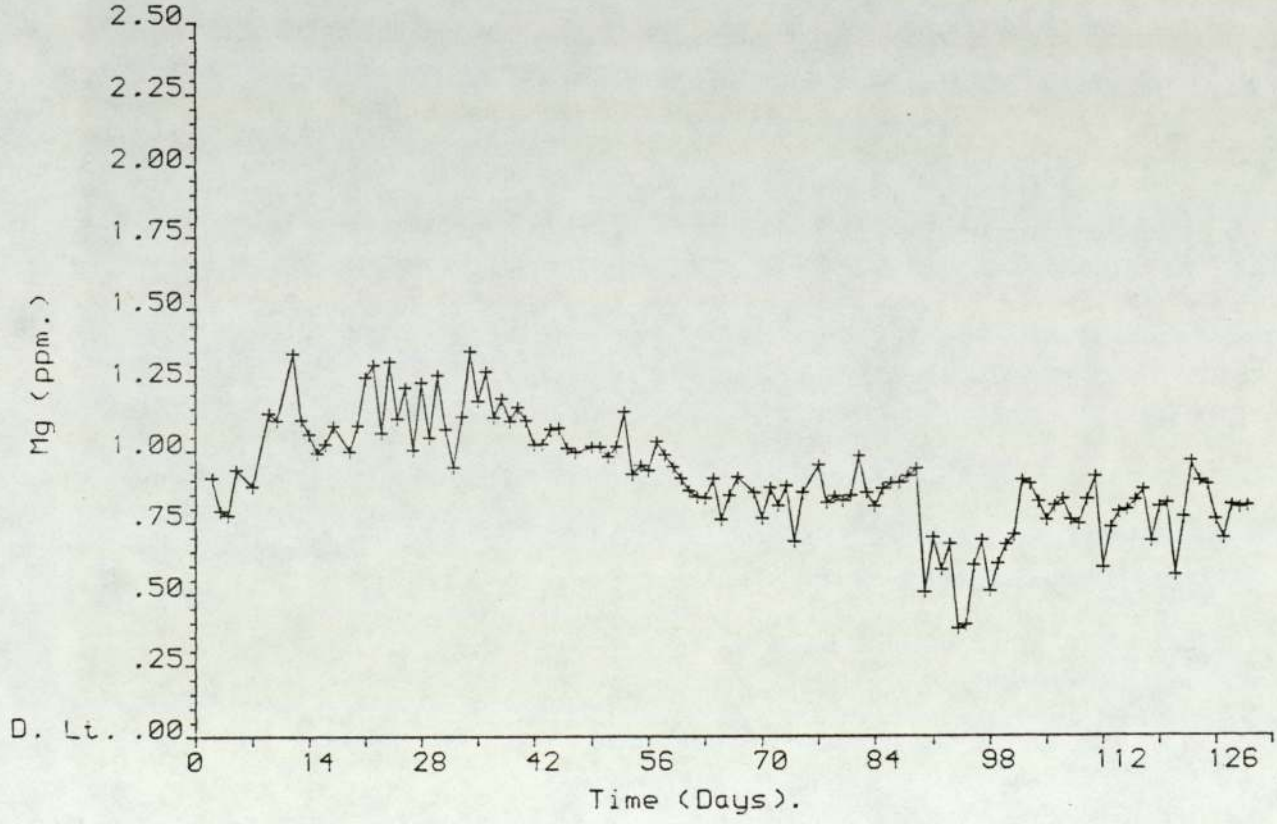


Fig.4.6.2.8. : Graph of Water Mg Levels v. time. (By digestion of resin.)

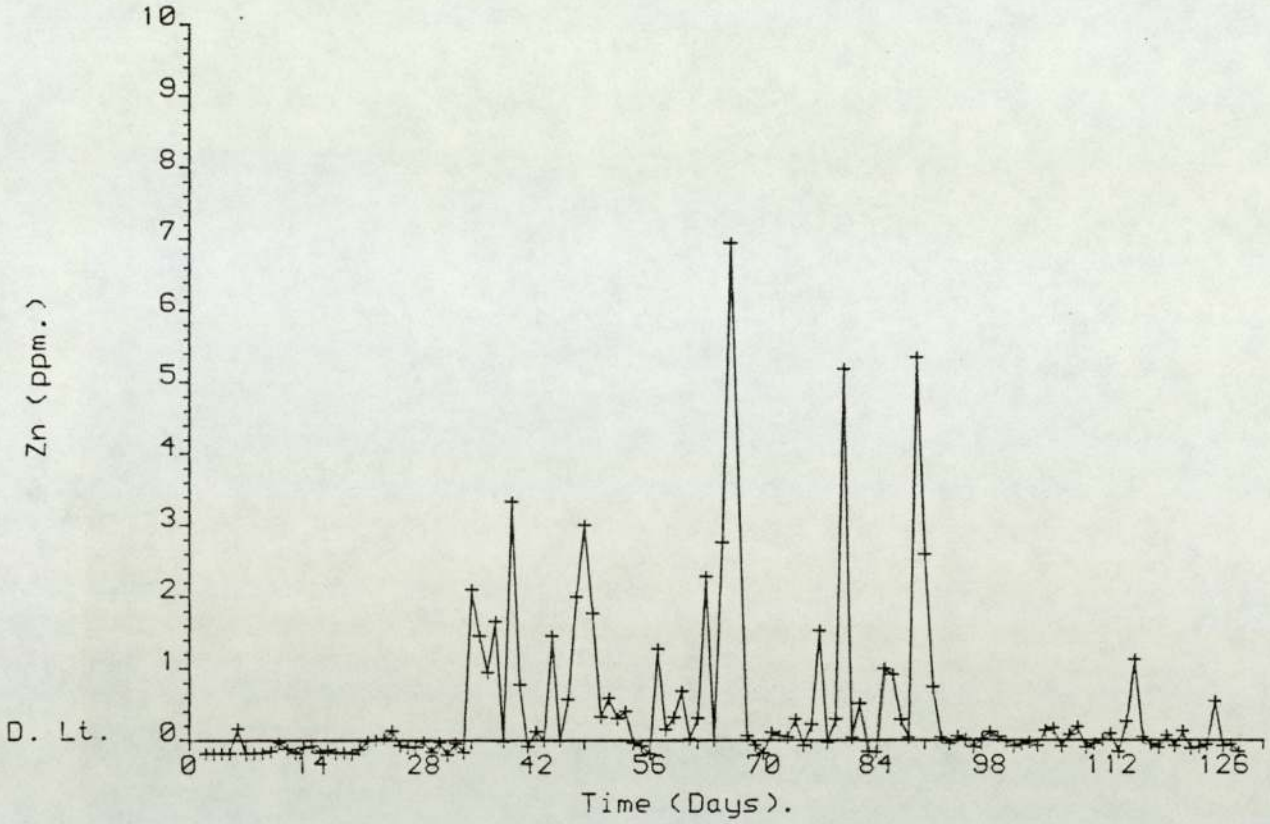


Fig.4.6.2.9. : Graph of Water Zn Levels v. time.

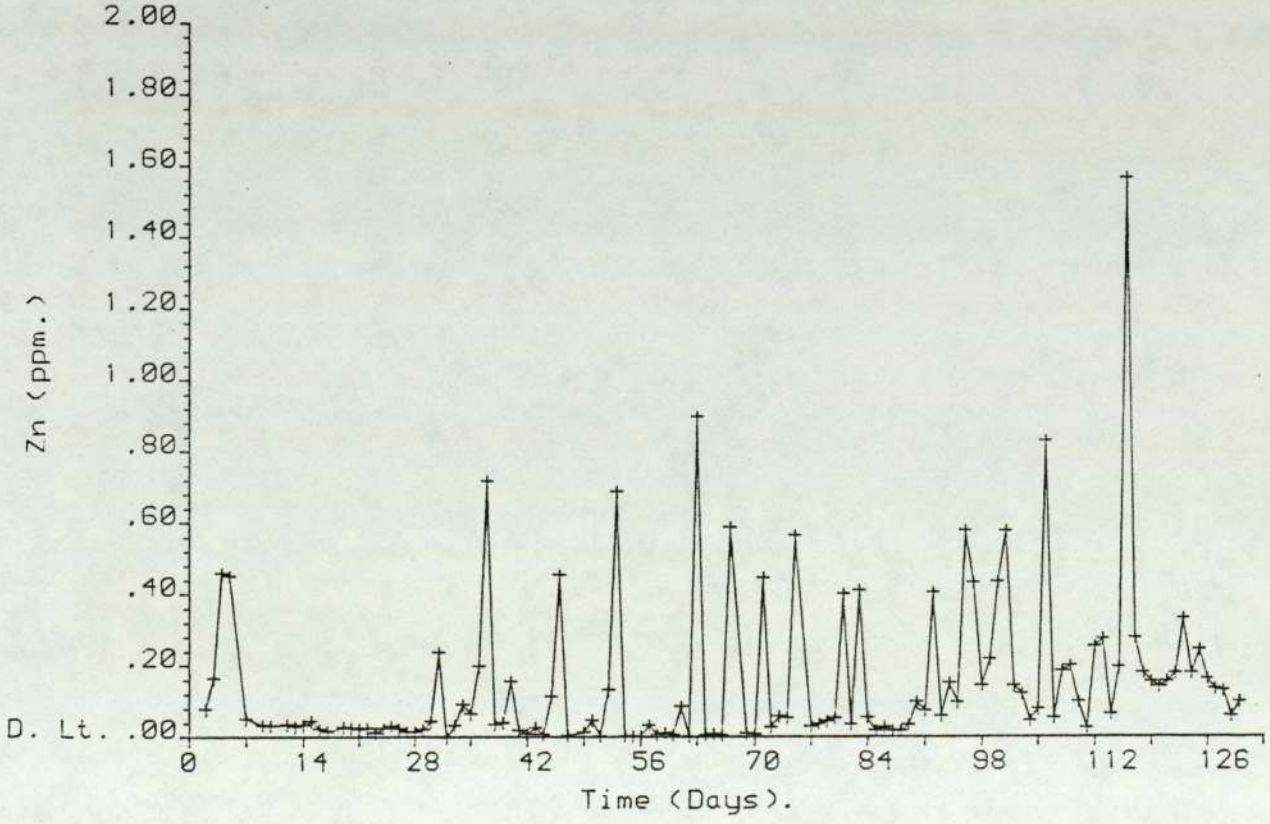


Fig.4.6.2.10. : Graph of Water Zn Levels v. time. (By digestion of resin.)

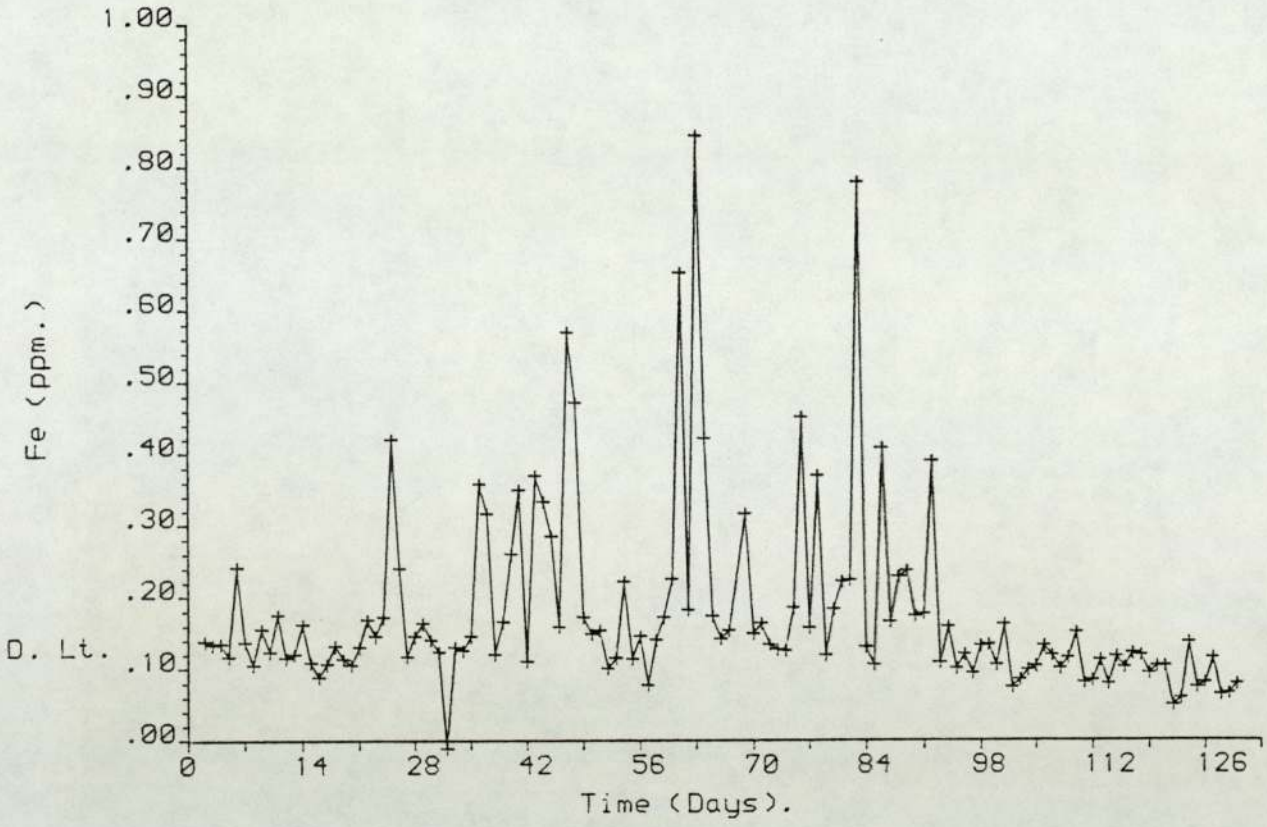


Fig.4.6.2.11. : Graph of Water Fe Levels v. time.

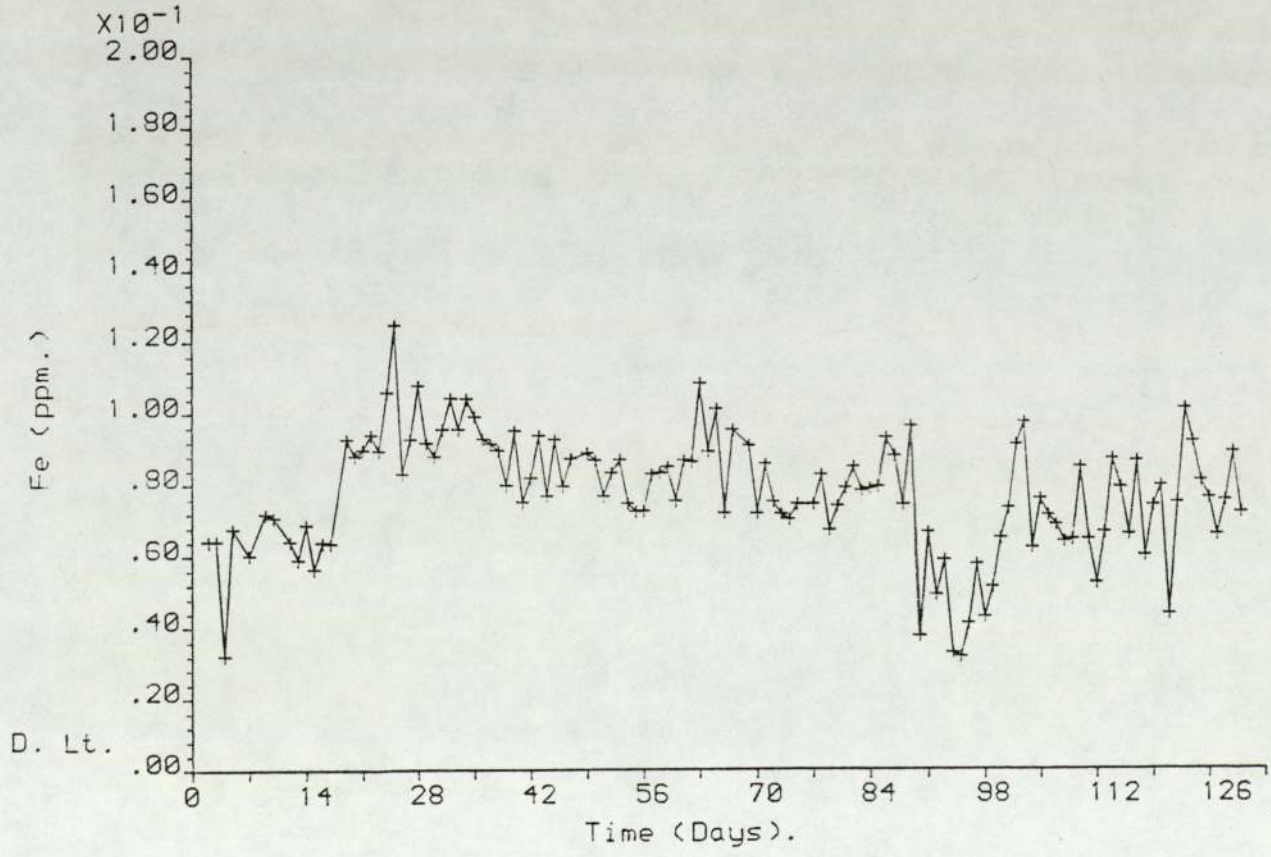


Fig.4.6.2.12. : Graph of Water Fe Levels v. time. (By digestion of resin.)

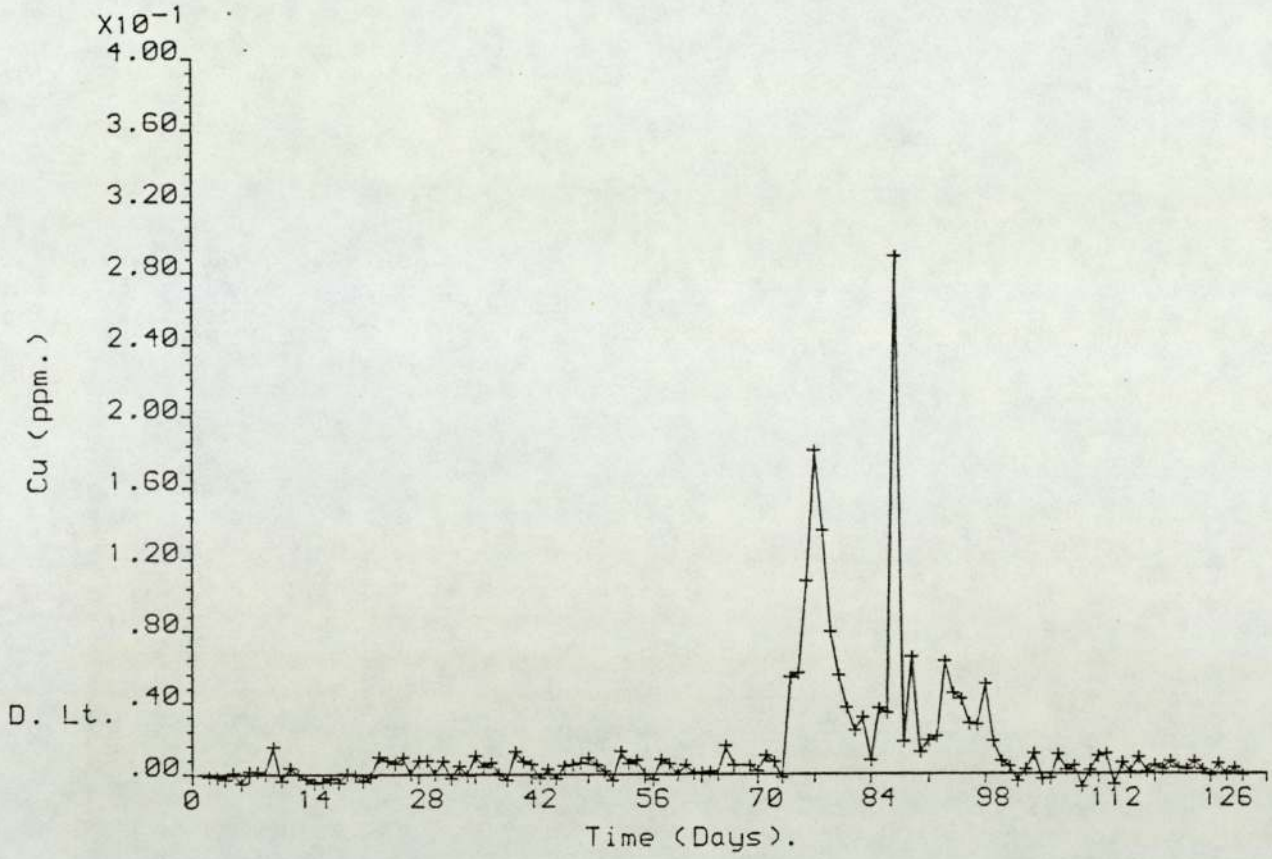


Fig.4.6.2.13.: Graph of Water Cu Levels v. time.

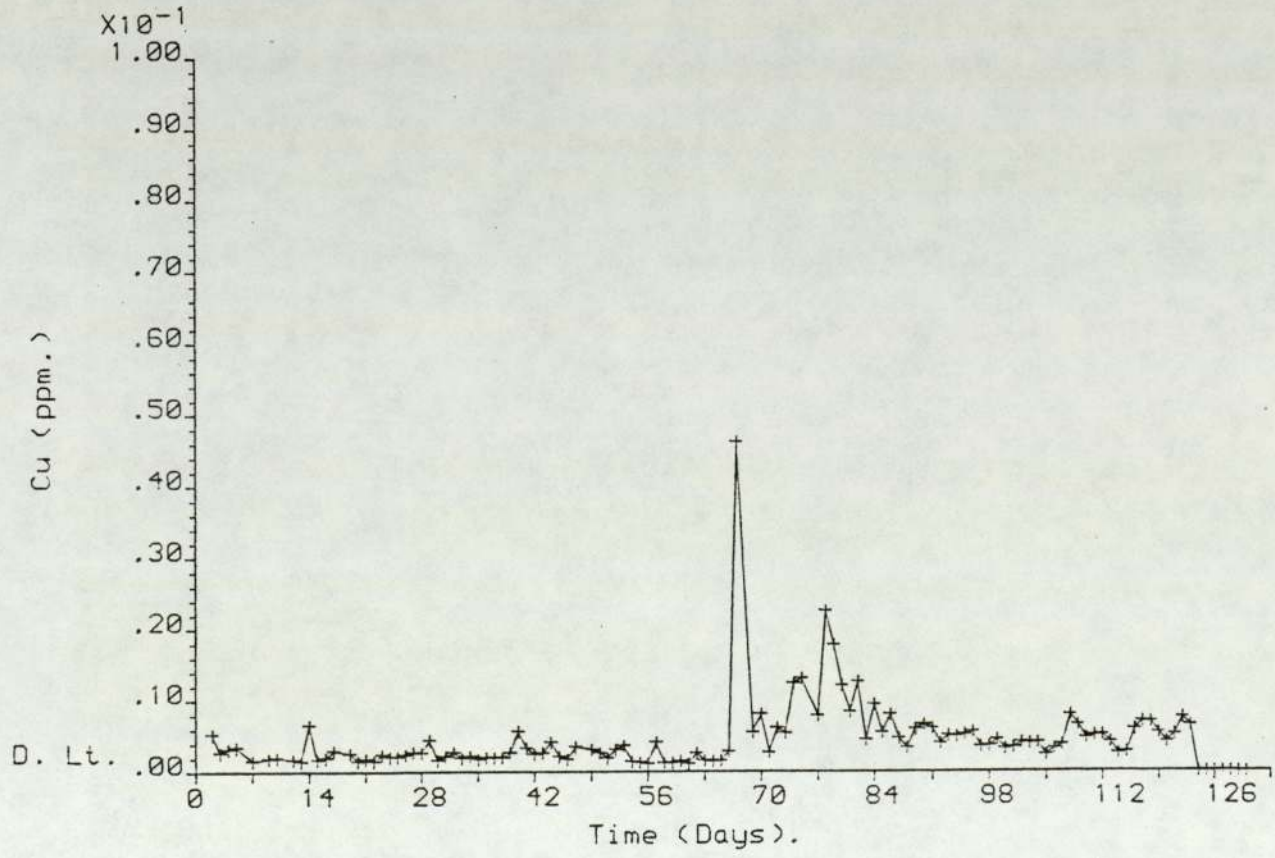


Fig.4.6.2.14.: Graph of Water Cu Levels v. time. (By digestion of resin.)

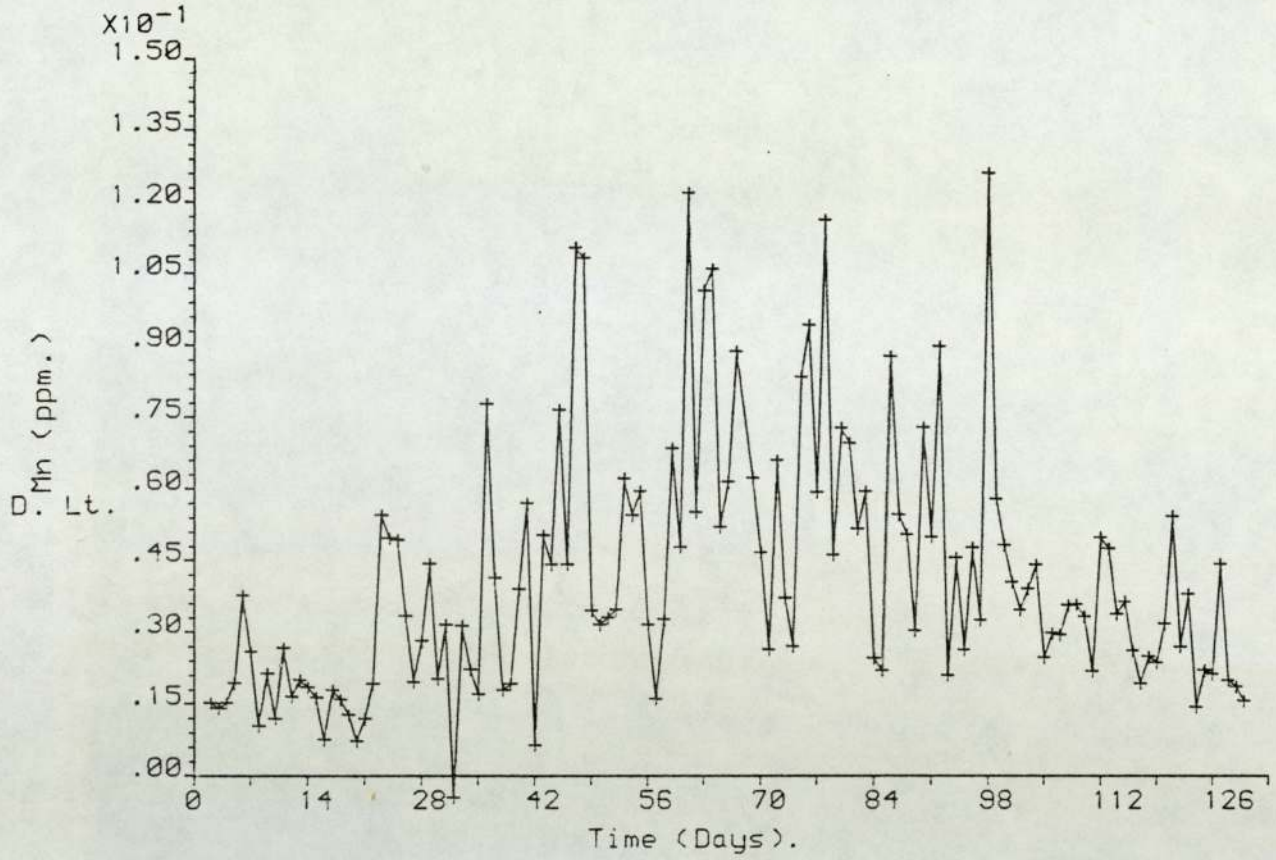


Fig.4.6.2.15.: Graph of Water Mn Levels v. time.

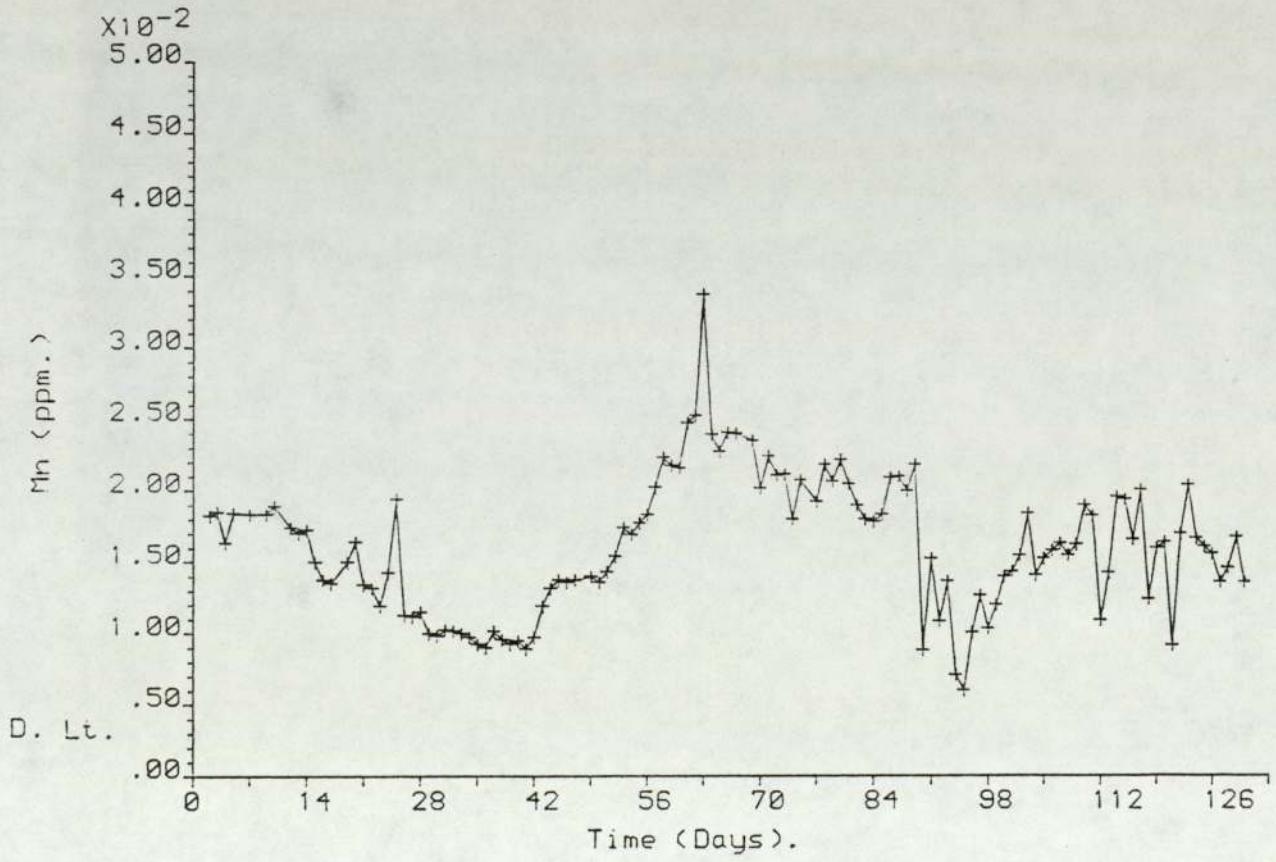


Fig.4.6.2.16.: Graph of Water Mn Levels v. time. (By digestion of resin.)

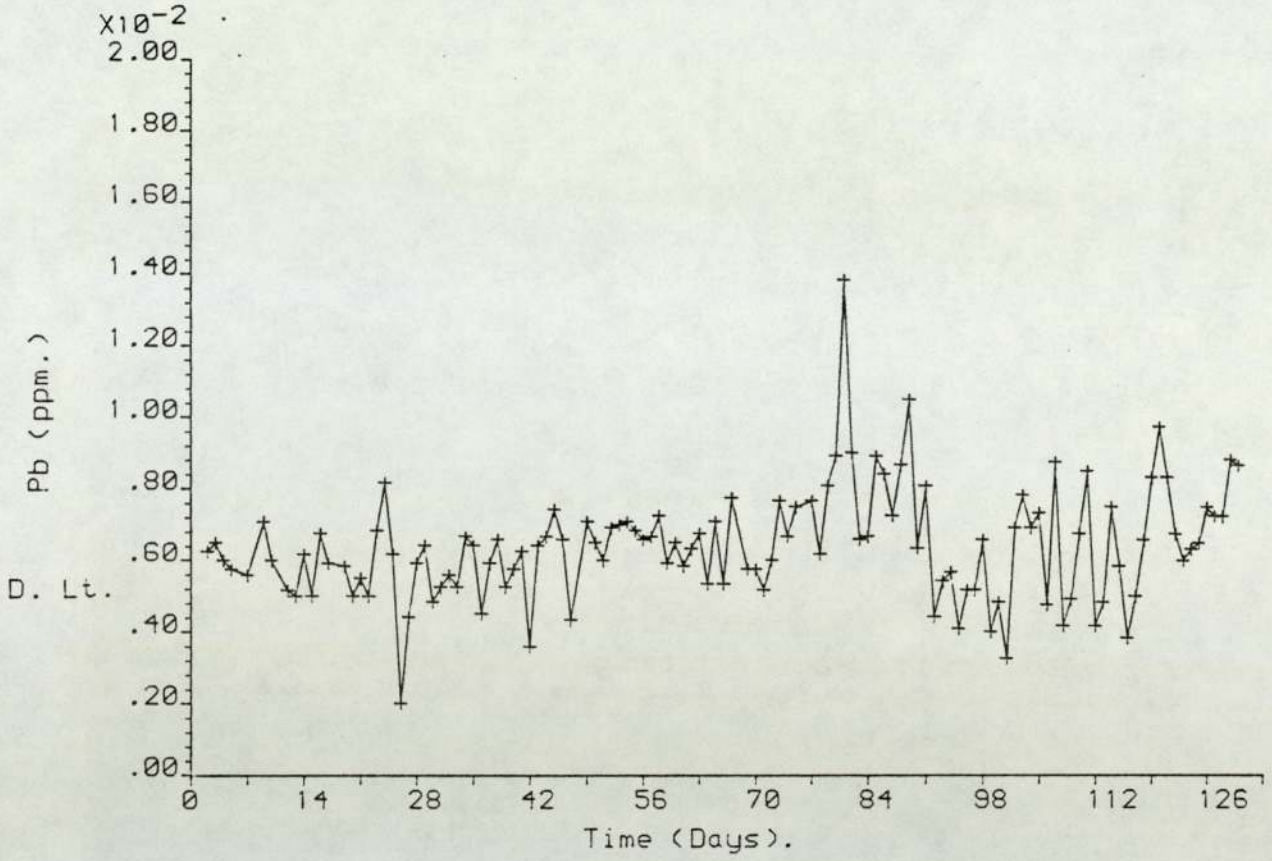


Fig.4.2.6.17. : Graph of Water Pb Levels v. time. (By digestion of resin)

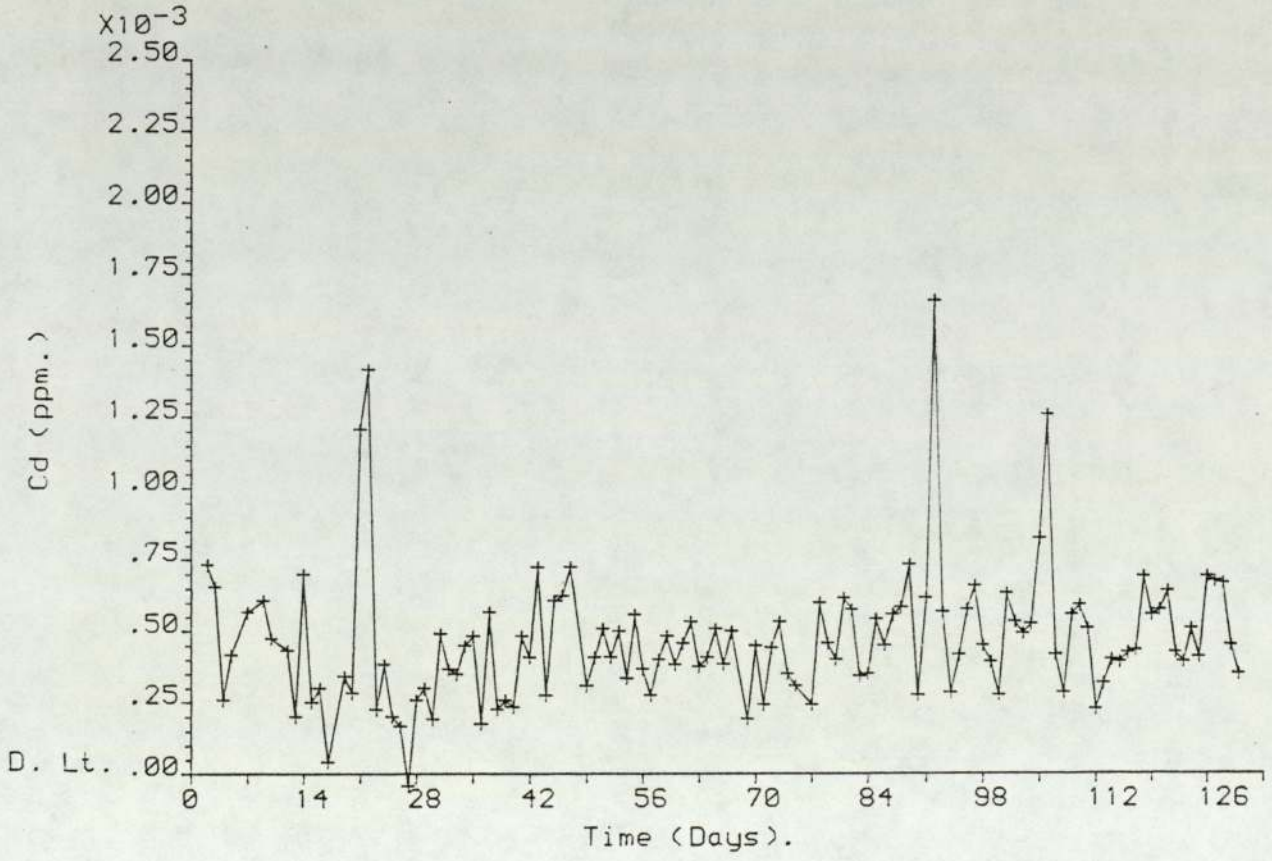


Fig.4.6.2.18. : Graph of Water Cd Levels v. time. (By digestion of resin.)

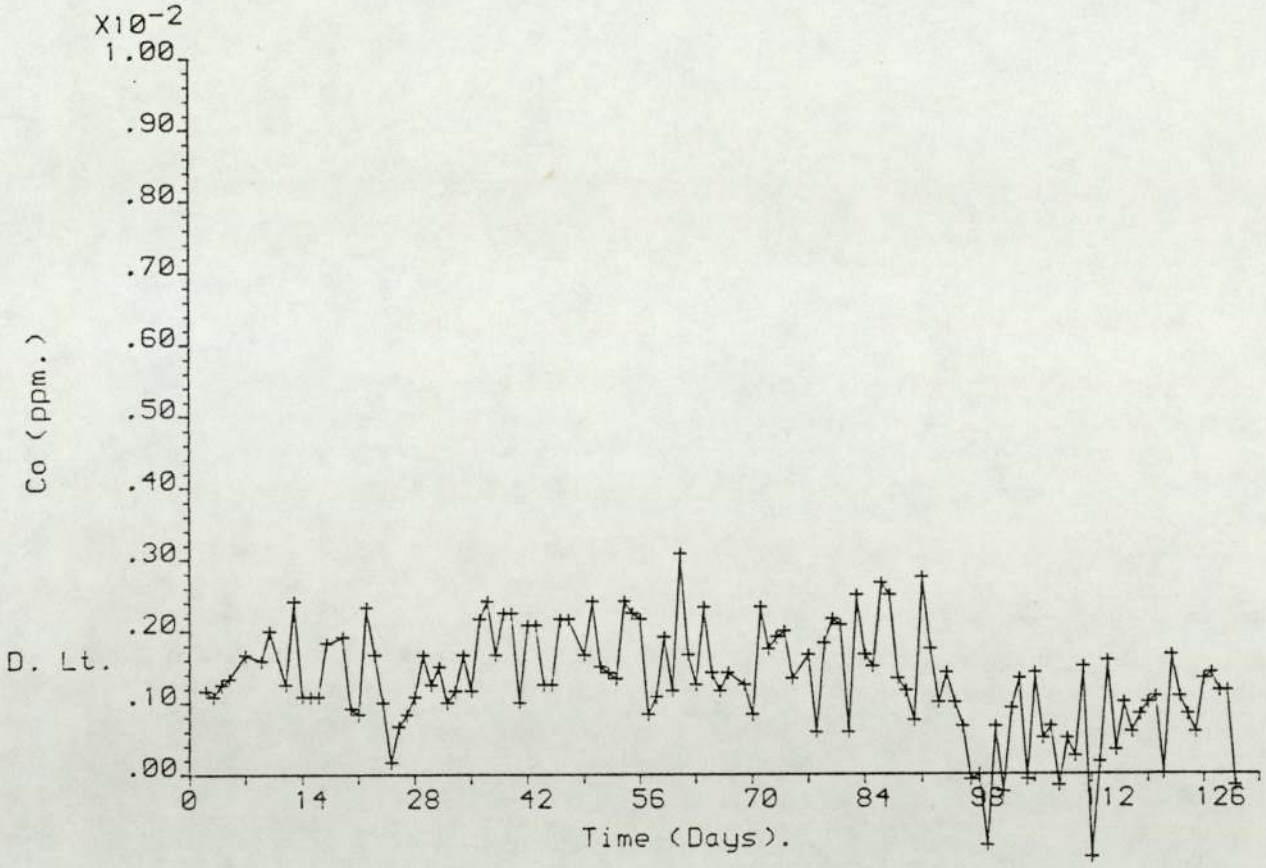


Fig.4.6.2.19. : Graph of Water Co Levels v. time. (By digestion of resin)

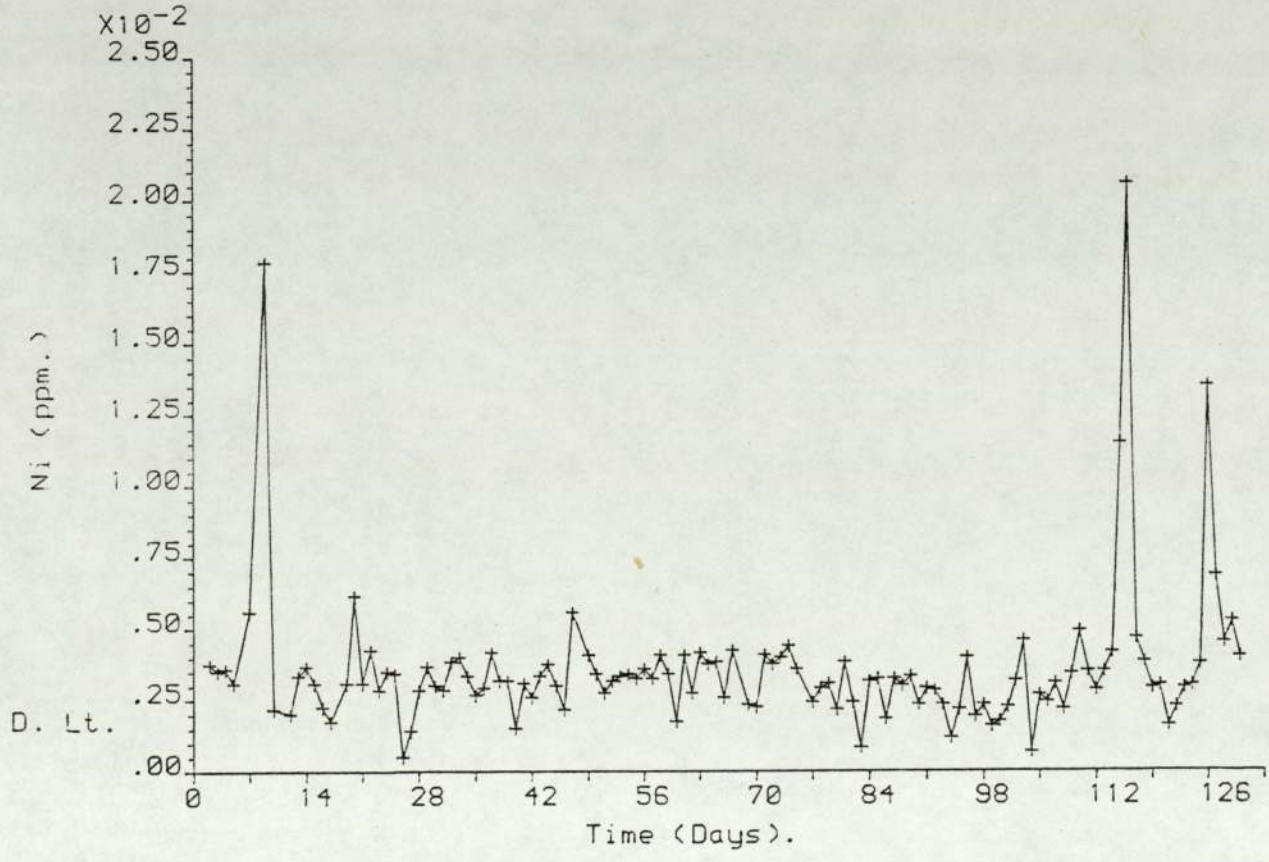


Fig.4.6.2.20 : Graph of Water Ni Levels v. time. (By digestion of resin.)

These figures show that the mineral content of mains water may vary considerably over a period of 24 hours, and that there is little association between levels of elements. Regression analyses revealed that the closest association of metals in the water was between iron and manganese ($P <= 0.01$).

Table 4.6.1.. Mean Levels of Minerals in Water

Mineral	Mean	+/- S.D.	% Absorbed by resin
Na (ppm.)	4.48	1.55	85.8
K (ppm.)	0.36	0.17	54.5
Ca (ppm.)	5.64	0.72	100.0
Mg (ppm.)	1.01	0.59	95.4
Zn (ppm.)	0.60	1.10	69.93
Fe (ppm.)	0.25	0.13	82.79
Cu (ppm.)	0.015	0.036	100.0
Mn (ppm.)	0.045	0.026	100.0
Al (ppm.)	0.146	0.069	N.A.
Pb (ppb.)	6.4	1.6	N.A.
Ni (ppb.)	3.6	2.6	N.A.
Co (ppb.)	1.3	0.7	N.A.
Cd (ppb.)	1.0	5.9	N.A.

5. DISCUSSION

5.1. Experiment II

Since fishmeal contains significant quantities of minerals, and fish can take up minerals from the water, the mineral supplementation of fish diets initially received little research effort. In 1972 Cowey and Sargent published a major review of the 'state of the art' of fish nutrition, and made no mention of mineral nutrition. An updated review by the same authors (1979) included a section on mineral nutrition, but in comparison with proteins, fats, vitamins and carbohydrates, minerals have not been fully investigated. The dietary mineral supplements in common use are the USP-XII salt mix No. 2 (Halver and Coates 1957, Arai et al. 1975) and the McCollum salt mix (Ogino and Kamizono 1975); neither was designed for use in fish diets, being intended respectively for poultry and laboratory rats (Cowey and Sargent 1979). The experimental supplementation of fish diets with these mixes has shown that growth is optimal with c. 4% of the dry diet composed of the mineral mixture. It would appear however that a number of the ingredients of these mixes are redundant in fish diets. Sakamoto and Yone (1978b) found that the growth of red sea bream in sea water was not improved by the addition of trace elements to the diet. These workers (1979c) also showed that the USP-XII salt mixture No. 2 could be replaced in diets for red sea bream by a supplement of phosphorus and iron, and that the other constituents were dispensable.

Experiment II was designed to test the effects the USP-XII salt mix No. 2 in trout diets, and to identify the relative merits of

the trace element and bulk mineral fractions. By supplementing either the trace elements or minerals or both in diets containing fishmeal or Pruteen as protein sources, the effect of the supplements on different dietary proteins would be ascertained, with greater opportunity to identify general principles of mineral nutrition.

5.1.1. Growth and Feeding

All the fish grew fast and fed aggressively during the experiment. No fish died, and the water quality remained good throughout. Although the fish were rather variable in size, individual marking allowed the growth to be studied in detail.

The growth of the fish was markedly depressed by an interaction of minerals X trace elements. This interaction appeared to have different effects in different diets depending upon whether fishmeal or Pruteen was the protein source. In fishmeal diets, the interaction suppressed intake. (Table 4.1.1.1.) This may have been due to the formation of a compound or compounds having an unpleasant flavour, thus decreasing the palatability of the diets. However, palatability is not the only factor to regulate intake, and the rate of gastric evacuation is also an important influence on appetite. This is in turn regulated by the rate of digestion (Windell et al. 1969). The growth of the fish given diet FMT was poor, but the feed conversion ratio (FCR) was low. Where Pruteen was the protein source, an interaction between the trace elements and the minerals may have reduced the availability of one of

these,(possibly a digestive enzyme cofactor), and hence reduced conversion. In diet BMT, intake was high but FCR was also high (i.e. not good). The Pruteen did not appear to affect intake. This result was unexpected, as other workers (Beck et al. 1978) described a suppression of intake by bacterial proteins. The palatability of Pruteen here may be due to the semi-moist diets. The consistency of the fishmeal and Pruteen diets was the same, although the Pruteen diets appeared to be better bound and more water stable. Pruteen appears to contain substances which bind the ingredients of a diet and produce very water stable pellets in the moist state. This is advantageous, because diets which are poorly bound will tend to disintegrate in the water and lose nutrients. The better bound the diet, therefore, the less potential wastage (Wood et al. 1954). Binders must be used with restraint, as over-bound diets may cause intestinal blockage or deposits, and affect digestion. Halver (1957) described the deposition of carboxy-methyl cellulose around the pyloric caecae of fingerling chinook salmon fed large amounts of this binder. The natural binding agents in Pruteen are probably muco-polysaccharides (MPS's) which form a large part of the cell wall of the bacterium from which Pruteen is made. If such pellets are dried they become very hard and possibly unpalatable. Shimma and Nakada (1974a) found that diets made with yeast produced very hard and unpalatable pellets when dry, and attempted to soften them with fats and oils. In this experiment water was used to maintain an acceptable consistency (semi-moist preparation). Burrows (1972) has emphasised the need

to ensure that the physical properties of diets do not influence the results of nutritional trials unless by design. The semi-moist preparation may have altered the response of the fish to Pruteen diets, but Poston (1974) found that feeding rainbow trout a fishmeal diet formulated in dry or moist form did not affect growth or conversion, although more of the wet diet was eaten. The assumption was therefore made that in the absence of effects of hardness, moist would be as acceptable as dry for trout. Beck et al. (1978) used dry diets, which were possibly harder and less palatable when made with SCP than with fishmeal. The feed conversions in fish fed diets containing Pruteen tended to be higher than fish fed fishmeal, although the proximate compositions of the two groups of diets (Table 3.1.5.2.) were similar. This similarity may have been due to an over-estimation of the protein content of the diets containing Pruteen, in turn caused by the relatively large amounts of non-protein nitrogen present in the bacterial protein. This non-protein nitrogen (mainly nucleic acids) may comprise up to 10% of SCP's, and is not available for growth, but may be an excellent source of phosphorus (see section 5.2.2.). If the true protein, as opposed to nitrogen content of Pruteen is lower than that of fishmeal, isonitrogenous substitution (as here) of fishmeal by Pruteen will result in diets of lower protein content and increased FCR's will be the result.

5.1.2. Mineral analyses

No significant differences were found between the carcass mineral contents. This may have been due to the small sample size

(n=4), rather than the absence of 'real' differences. Later experiments revealed significant differences between the mineral content of fish fed Pruteen and fish fed fishmeal diets.

5.2. Experiment III

This experiment was designed to repeat and extend Experiment II to obtain a larger sample of fish for mineral analysis, and to allow a comparator control (commercial diet ,E) to be used for comparison with the experimental diets. The experiment replicated experiment II to attempt to determine more accurately the effects of minerals, trace elements and Pruteen on the mineral composition of fingerling trout. Although water quality remained good throughout the experiment, some fish died. Mortalities in the groups of fish given Pruteen diets were more frequent than fish given fishmeal diets. The experiment was terminated after 8 weeks, as only six fish remained in treatment BT. Nine fish from each treatment were killed during the experiment for carcass mineral analysis. At the end of the experiment six fish were randomly selected from each treatment (except BT, in which there were only 6 survivors) for mineral analysis.

5.2.1. Growth and Feeding

The fish deaths (see above) appear to have been due more to the diets than overcrowding, as the fish fed fishmeal diets grew rapidly, and there was always a greater weight of fish in these tanks than in the others, but mortalities in groups fed fishmeal were few. The suppression of intake by the presence of Pruteen resulted in poor growth and FCR. This highly significant ($P <= 0.01$) effect was believed to have been caused by the process used to reduce the Pruteen granules to a fine powder. During this process the Pruteen was mixed in a liquidiser (See sect. 3.1.4.), and although this rapidly reduced the size of the particles, it also caused friction heating of the material, to the extent that some smoke was observed. The Pruteen was therefore 'scorched', and this may have produced an unpleasant taste. A similar reduction in palatability of Pruteen was observed by I.C.I. in a trial on pigs (J. C. Cansick pers. comm.). In this instance the feed was pelleted in a mill with insufficient water to lubricate the die. Here again, friction caused slight burning of the Pruteen and the pigs rejected the feed. The relatively large amounts of nitrogen free extract (NFE) in Pruteen may be the cause of this thermal sensitivity, as the sugars in the NFE may caramelise. The effect was not seen in fishmeal, although this was given the same treatment. The greater oil content of fishmeal may reduce friction by self-lubrication. This observation may be of importance to feed manufacturers. After this experiment Pruteen was sieved to remove large particles, and was not 'liquidised'. No palatability problems were seen in other experiments.

In this experiment, the significant interaction of minerals X trace elements seen in Experiment II was not observed. However, table 4.2.2.2. shows that the growth of the fish was improved by both diets FM and FT relative to diet F, but diet FMT produced slightly lower growth than FT. This suggests that although not significant, some interaction of minerals and trace elements took place. If each improved growth independently, then the addition of both should improve growth by more than each alone. The interaction may be produced by the calcium in the mineral mix complexing and reducing the availability of one or more of the trace elements (O'Dell et al. 1972).

5.2.2. Mineral Analyses

Fish were analysed at intervals during the experiment to attempt to discern the rate at which changes in mineral content occurred. (see Tables 4.2.2.3. to 4.2.2.9.) The greatest variation was seen in the iron content of the fish (Table 4.2.2.6.), and this may have been due to the ingestion of rust particles by the fish during quarantine (see Sect. 3.2.2.). Zinc contents also showed considerable variation, possibly because the particles of rust originated from galvanised metal. The Pruteen may have had a direct effect on the mineral content due to its different mineral composition when compared to fishmeal. Alternatively there may also have been an indirect effect, as the fish given Pruteen were all smaller than those given fishmeal, because they grew more slowly. Fish have been shown to change in proximate composition as they grow although the ash content of the carcasses of Sockeye salmon remained a constant fraction of the wet weight of the fish

as they grew (Brett et al. 1969). Similar results were reported by Elliott (1976) and Tiews et al. (1973). This hypothesis does not appear, therefore, to account for the differences observed. Trace elements increased the calcium content of the carcass, an interesting result, since this trace element mix contained no calcium, which may have been caused by the presence of Mn in the trace element mix. Knox et al. (1982) showed that Mn was essential for adequate bone mineralisation. However, no significant difference in Mn content was observed, possibly because of the variability in mineral content of the fish carcasses.

Fish given trace elements had higher zinc levels. Adequate dietary zinc may have increased the absorption of calcium. It is possible however that the calcium increase was produced as a pathological symptom of hypomagnesia. A deficiency of magnesium has been shown to induce hypercalcaemia and renocalcinosis (Cowey et al 1977). Interference of the trace elements with the absorption of magnesium might have induced the effect observed. As zinc deficiency has been shown to induce high Ca and P in vertebrae (Knox et al. 1981), the increase in Ca produced by trace elements (which included zinc) may have been induced by increased phosphorus availability. Zinc is a digestive enzyme cofactor, and may improve digestibility of feeds, making more P available. This hypothesis is supported by the interaction of Pruteen X trace elements, which also induced an increased calcium

content. The greatest influence on Ca content is probably the availability of phosphorus. Ca is primarily stored in the carcass as phosphorus compounds, and thus a lack of P will result in a reduction of carcass Ca. Pruteen is known to provide abundant dietary phosphorus in the form of nucleic acids, and this was confirmed by the highly significant increase in carcass P induced by Pruteen (Table 4.2.2.1.). The increase in Ca and Mg produced by the Pruteen can therefore be assumed to have followed as a consequence of the adequate phosphorus supply.

5.3. Experiment V

The results of Experiments II and III showed that Pruteen diets for trout were significantly improved by the addition of a bulk mineral supplement. Trace element supplements were not found to be necessary in Pruteen diets. As the mineral mix contained calcium, it was believed that this was probably one of the minerals effecting the growth improvement, especially since Pruteen has a low calcium content. This hypothesis accorded with the results of Arai et al. (1975) who showed that trout growth was improved by the addition of calcium to a yeast diet.

Nevertheless, the water in which Experiments II and III were carried out had a low calcium content (c. 20 ppm total hardness). It was thought that the low availability of calcium in the water may have exaggerated the effect of the mineral supplements, as the fish would be more dependent on the diet as a mineral source than in harder water. Testing the effect of water hardness on mineral supplements would thus indicate the potential use of

Pruteen and the value of mineral supplements in water of different composition. The supplementation of the diet with minerals may not be the best way to supply them, especially calcium. Dietary Ca has been shown to increase the requirement for other minerals, and to bind and reduce the availability of trace elements (O'Dell 1960).

5.3.1. Growth and Feeding

A problem arose as a consequence of the removal of fish at 25 days. Although the fish killed were selected by random numbers, the mean weight of the fish which remained was not the same as that before sampling. Thus the weight change of the fish during the experiment was an unreliable estimate of growth. As the fish were individually marked, the growth of each could be recorded and expressed as a specific growth rate (SGR). This proved the more sensitive estimate of growth differences. When the results were analysed, the oyster shell was shown to have interacted with the mineral supplement in the diet to reduce the SGR. This result was unexpected, since the unsupplemented diets were deficient in calcium, and the water without the oyster shell in the filters was soft: about 20 ppm. total hardness. The addition of both should have improved growth.

It is possible that water hardness affects the appetite of fish. The use of oyster shell as a filter material increased intake of food, but did not affect growth. This implied an increased FCR, however, as in the other experiments, this could not be confirmed statistically. The growth suppression by the interaction of oyster shell X minerals was probably due to the

suppression of intake. It is difficult to suggest the reason for this, but it may be that if the food contains minerals which are also available in the water, the fish eat less. This suggests that fish will feed to maintain a mineral balance, in the same way that they feed to maintain a balance of other nutrients. Intake is thus not regulated by appetite alone. The rate of gastric evacuation also affects intake, and this may in turn be affected by feed digestibility, or by the response of the gut to the diet.

5.3.2. Mineral analyses

Analysis of the carcasses revealed but few significant effects on mineral levels. The only effect observed after 25 or 50 days of growth, produced by an interaction of oyster shell X minerals, was an increase in Cu content after 25 days, which was not observed after 50 days. The suppression of growth by the dietary minerals may have been related to the Mn concentration reduction induced after 50 days. Cotzias (1960) reported that diets high in Ca and P exacerbated Mn deficiency. Although the oyster shell had no detectable effect on the growth of fish, it reduced the copper content (25 days) and increased the zinc content (50 days). These observations are of considerable importance to those who aim to detect alterations in mineral balance from a single sample. The decrease in copper concentration may have been caused by the increased water calcium levels, Waiwood and Beamish (1978) having shown that hard water may protect fish from copper toxicity. In this case, hardness would have reduced the availability of water-borne copper by complexation, (Pagenkopf et al. 1974) and may

have induced a temporary deficiency. The increased zinc content associated with the oyster shell filters may have been caused by the zinc in oyster shell dissolving into the water and being absorbed by the fish. When analysed, the oyster shell was found to contain significant amounts of zinc, although no differences in the zinc content of the water could be detected. The levels of zinc found in the fish were lower than those of fish in Experiment III, although after 50 days in this experiment they were slightly above those at the start, prior to which the fish had been fed on a commercial feed. The levels of zinc in the fish were all below those in Experiments III, VI and VII, although some of the diets were comparable with diets in Experiment V. (Pruteen diets with and without minerals.) The different carcass zinc levels were probably caused by differences in the zinc concentrations of different batches of Pruteen. The batch used in Experiments II, III and V was low in zinc, but in Experiments II and III, the uptake of zinc from the water would have allowed the fish to compensate for dietary deficiency. In Experiment V, the recirculating system with a low input of fresh water was used, and thus the role of the water as a source of minerals would be reduced.

In conclusion, this experiment showed that the water hardness may affect the response of the fish to diets, and may cause different responses, as measured by intake, to different diets. Differential intake of diets, caused by differing water hardness, may lead to different growth rates. The mineral balance of the fish does not appear to be permanently affected by either water

hardness or dietary mineral levels. This observation accords with that of Umehara and Oguri (1978) who observed no change in the plasma Ca levels of goldfish transferred to water of high Ca concentration. It must be emphasised that this experiment was performed in recirculating water, and thus may have made minerals excreted by one fish available to others in the same tank. Transfer of minerals between treatments via the water could not occur as each system was isolated from the others. The fish in each treatment shared a common water filter so if minerals were accumulated and released by the filter, the apparent availability of minerals in the water could have been altered. Rundquist (1977) showed that Fe in a hatchery effluent was removed from the water by a biological filter.

5.4. Experiment VI

The results of Experiments II and III showed that in certain circumstances the mineral and trace element fractions of the USP-XII salt mix No.2 may interact and reduce the benefits each provide when added separately to fish diets. Experiment VI further investigated this phenomenon, which had previously occurred in through-flowing water.

5.4.1. Growth and Feeding.

The fish grew well and fed aggressively during the experiment, with few mortalities. The results of the growth and feeding study are shown in Tables 4.4.1.1. and 4.4.1.2. When the results of the growth study were analysed, the only factor to have affected the SGR was the mineral mix. This confirmed the results of Experiments II and III, which had shown an improvement in the growth of fish given Pruteen diets supplemented with minerals. When the voluntary intake of food was considered, however, the fish proved to have been significantly affected by the sodium phytate and trace elements. The phytate suppressed the intake of food ($P <= 0.05$), whilst the trace elements induced an highly significant increase in intake. It is possible that the intake was not influenced by palatability alone, but also by the rate of passage of the diet through the gut. This could be the underlying cause of the changes in feed intake observed. Holt et al.(1979) showed that the ingestion of viscous carbohydrates increased gastric emptying time in man. If trace elements reduce gut

passage time, possibly by increasing the digestibility of the feed, then the increase in intake caused by this factor is explained. The increased intake induced by the trace elements was offset by an increase in FCR (Table 4.4.1.2.), although this could not be statistically confirmed. This suggests that trace elements reduced digestibility. Gut passage time and digestibility could have been decreased if the trace elements irritated the gut, which would respond by increasing the rate of evacuation. The feed conversion of fish given the diet BM approached that of fish given the commercial feeds in other experiments, with the SGR approaching that of diet EM (commercial feed), in Experiment VII. The increased growth resulting from mineral supplementation apppears to be due to improved conversion rather than improved intake. If the minerals are adsorbed by the phytate, the potential adsorption of trace elements by minerals, notably calcium (Oberleas et al. 1966, O'Dell 1960), would be reduced. Thus the presence of phytate may increase the activity of trace elements. The results of this experiment revealed no significant interaction between minerals and trace elements, suggesting that the interaction in Experiment II was due to the mineral composition of one batch of fish meal and thus is of little general significance; if so, it illustrates the problems which may be caused by the variation in mineral content of fishmeal. Commercial fish diets are highly variable in mineral content (Tacon and Da Silva, in press), and may be so because of the fishmeal content, among other factors.

The results of this experiment differed from those of previous

experiments in that the minerals, although increasing growth, did not appear to do so by increasing the intake of food. This is in contrast to the effect shown in Experiments II and III, possibly because this experiment was performed in a recirculating system. In such a system, the minerals, once ingested, would tend to be held in the system, and be available to the fish for longer periods. In this case (Experiment VI) the fish did not need to eat to achieve an adequate mineral intake, as the rate of mineral loss from the system as a whole was probably reduced. Water mineral levels did not, however change during the experiment. If this were so then fish may feed to maintain mineral balance, just as they are believed to feed to a 'fixed energy level' (Rozin and Meyer 1961, Palohaemo and Dickie 1966, Hastings and Dickie 1973).

5.4.2. Mineral analyses

When a sample of fish were killed and analysed after 42 days, a number of effects were revealed. The phytate caused an increase in Ca and P concentrations, possibly as a result of its digestion by the fish. The phosphorus from phytate has not been shown to be available to fish prior to this experiment, but it is possible that it was an artificial effect, since after 84 days no effect of phytate was observed. It is possible that the phosphorus from the phytin was more available than the phosphorus from the Pruteen, and so the fish took longer to adapt to the Pruteen. This is unlikely, because in Experiment V the fish fed Pruteen diets significantly increased in P content in only 25 days. Moreover, Ketola (1975a) found that the phosphorus in soybean meal (mainly phytate) was not available to Atlantic salmon. Ketola also showed

that the growth of trout fed soybean meal was improved by dietary minerals, which included inorganic phosphate. The minerals had caused a decrease in Ca, Mg and P content after 42 days (c.f. Ca in Experiment VII), but after 84 days these effects were not detectable. In Experiments V and VI, the samples of fish taken during the earlier part of the experiment showed greater differences between treatments than those taken at the end of the experiment. This indicates that fish may take some time to adapt to experimental diets, and that some transient effects may occur before the fish stabilise. Indeed, it could be argued that all these experiments were terminated before the fish had stabilised their response to the diets. The trace elements had induced increases in Ca, Mg, Cu, Mn and P after 42 days, but after 84 days the effect was no longer detectable. This further indicates that effects produced by diets may be transient and of no long-term importance, however interesting they may be in the short-term. After 42 days, the phytate X minerals interaction had produced no significant effects. After 84 days, the interaction had caused P and Mg levels to increase ($P <= 0.01$). The reason for this interaction may have been a synergism in phosphorus release from phytate by the presence of minerals, which possibly increased the digestibility of the phytin. The coincident increase in P and Mg content is seen in Experiment VII as a consequence of feeding Pruteen, as opposed to a casein/gelatin mixture of lower phosphorus content. The reduction in Mg by phytate after 84 days may be due to the binding of Mg by the phytin in the gut, reducing its availability. Phytate has been

shown to reduce zinc availability (O'Dell et al. 1972). The presence of calcium was found by Oberleas et al. (1962, 1966) and Likuski and Forbes (1966) to exacerbate the reduction of zinc availability by phytate. The results show that the increase in growth produced by the minerals could be due to a physiological effect, rather than a simple improvement of digestibility. This physiological effect may be the reduction of an hyperphosphataemia produced by an excessive intake of phosphorus from Pruteen with insufficient dietary magnesium. By providing more magnesium, the mineral mix may have enabled the fish to utilise efficiently the phosphorus absorbed from the Pruteen.

5.5. Experiment VII

Experiments II and III showed that minerals added to Pruteen diets for trout improved growth. Calcium, iron and magnesium were believed to be the effective minerals. This experiment tested the effects of calcium and iron supplements on trout growth in through flowing water.

5.5.1. Growth and Feeding

Some of the fish fed Pruteen and calcium in their diet (BCa, BCaFe) died, apparently of bacterial kidney disease, although this was unconfirmed. When the results were analysed, it was immediately apparent that the experimental treatments had suppressed the growth of the fish. This was expected because the diets were, as described, incomplete. The growth of all the fish on the experimental diets (C - BCaFe) was much less than that of the comparator control treatment EM (commercial feed). At the end of the 16 week experiment, the fish given the commercial diet had

increased in weight by a factor greater than five, whilst some of the experimental fish had not doubled in weight. The poor growth was not a consequence of a reduction in intake, (fish fed treatment BCaFe had a higher intake than those on the commercial diet). The casein/gelatin diets were found to be less palatable than the Pruteen diets. The poor palatability of casein/gelatin diets has been observed by others (Atack et al. 1979). Factorial analysis (Table 4.5.1.1.) showed that Pruteen had increased intake ($P <= 0.01$). Growth however, was not affected, suggesting that the FCR's of the fish given Pruteen were greater than those of fish given the casein/gelatin. Scrutiny of Table 4.5.1.2. reveals that this is the case but statistical confirmation was not possible. This result was anticipated, as the protein levels in the Pruteen diets were lower than those in the casein/gelatin diets, and the protein level in the Pruteen was probably over-estimated (sect 5.1.). When the final fish weights were analysed factorially, iron was shown to have improved growth ($P <= 0.05$) whilst an interaction between Pruteen X Ca had induced a marked reduction ($P <= 0.01$). This result contradicts those of Arai et al (1975). It is possible that Arai's results reflected the effect of a lack of alpha-cellulose in the diets rather than an increase in calcium, because alpha-cellulose was used to replace calcium in the diets. It is possible that alpha-cellulose affected the availability of other minerals in the diet and induced spurious effects because it is a fibre and may bind minerals (see sect 5.3.). The growth improvement caused by iron confirms the results of several authors, who have shown that iron

is an essential dietary mineral for both freshwater and marine species of fish (Kawatsu 1972, Ikeda et al. 1973, Arai et al. 1974). Several authors have described a requirement for calcium in freshwater fish, (Arai et al. 1975), and marine fish (Sakamoto and Yone 1973), but here no effect was detected on growth when Ca was added to diets (Table 4.5.1.1.). The suppression of growth by the interaction of Pruteen X Ca is an interesting result, given that Pruteen contains low levels of calcium, and suggests that the calcium induced an imbalance of Ca:Mg leading to calcinosis and reduced growth. This hypothesis is supported by the observation (Table 4.5.2.9.1.) that the Ca:Mg ratio of fish carcasses was increased by Pruteen. In the carcasses of fish given Pruteen, Mg levels were higher than in fish given casein/gelatin, but the greater levels of Ca induced by the Pruteen reveal a potential imbalance.

5.5.2. Carcass Minerals:

When the mineral levels in the carcasses were determined and the results subjected to a factorial analysis of variance, a number of significant effects were seen to have been induced by the dietary factors. With the exception of one effect (Pruteen on Mn content), all factors had affected the mineral levels in the same way, either increasing them or decreasing them, where an effect was found to be significant. Thus calcium had reduced the levels of Mg and P in the carcass, and iron had reduced the levels of Mg, Zn, Mn and P. Ogino and Yang (1978) observed that the iron content of fish carcasses was inversely related to the zinc content. Dietary iron had no effect on the carcass iron

levels. The interaction Ca X Fe had increased the Ca and Mn content. Pruteen increased Ca, Mg and P and reduced Mn. The interaction Pruteen X Ca increased Mn and P . The interaction Pruteen X Fe increased Mg, Mn and P . It must be remembered that the factorial analysis is not merely additive. An effect produced by Pruteen X Ca and found to be significant is significant by an amount above the effect produced by the sum of both factors. Phosphorus levels were increased by Pruteen X Ca and Pruteen X Fe, indicating that both Ca and Fe interacted with Pruteen separately to give an increase in phosphorus content relative to diets not containing these factors (See Table 4.5.2.1.1.). However, when both Fe and Ca interacted with Pruteen at the same time, (Pruteen X Ca X Fe), the phosphorus content was increased by less than the amount expected, and thus the factorial effect was significantly reduced. If each factor, Ca or Fe, caused an increase in P by itself, then together they should, in the absence of any interaction, produce still higher levels. Thus the observation that the levels of phosphorus in fish given both Ca and Fe are not significantly greater than in those given either mineral indicate that these minerals interact in the diets. This can be confirmed by scrutiny of Table 4.5.2.1.2.

There is an association between the effects on magnesium and phosphorus. Table 4.5.2.1.1. shows that every effect on Mg is in the same direction and coincides with an effect on P. This suggests that the effects on one are a consequence of effects on the other, probably effects on P affecting Mg, since the effects on the P are generally greater than on the Mg. This hypothesis

explains neither the cause of the effects on P levels nor the mechanism by which they could affect Mg levels. O'Dell (1960) showed that high dietary phosphorus and calcium in rats increased magnesium deficiency.

Ca X Fe interacted to produce a significant increase in the Ca content and the Mn content, possibly by the formation of a complex of the two minerals in the gut which had a reduced availability to the fish. This is tentatively supported by the observation that Ca had no effect on Mn, that Fe suppressed Mn, and that the interaction Ca X Fe increased Mn levels. If Fe reduced manganese availability then the reduction of Fe availability should increase manganese content . The observation that Pruteen X Fe increased Mn content whilst Pruteen X Ca X Fe decreased Mn, casts doubt upon this hypothesis. To clarify the observations made on carcass minerals, and gain insight into the causes of the different levels observed, factorial analysis is invaluable. However this did not show the actual levels of minerals, nor whether they were higher or lower than at the start of the experiment. The data were therefore submitted to a one-way analysis of variance (Table 4.5.2.1.2.). The diet I represented the carcass of the fish immediately prior to the start of the experiment (Initial sample) which had been maintained on a commercial feed and were growing rapidly. These fish thus represent 'normal' values for mineral contents and were compared with the experimental fish. The only mineral present in the fish carcasses at the start of the experiment at a level significantly higher than at the end was zinc. This suggested that the fish

were somewhat zinc deficient at the end of the experiment. In all cases of all minerals except zinc, the fish fed diet 'C' (Control) had not changed significantly in mineral content during the experiment, which suggested that the changes observed at the end of the experiment were induced by the diets, not simply because the fish had grown. To investigate the causes of the different mineral levels observed, it is necessary to analyse different tissues, rather than the total carcass composition, since some tissues will respond more quickly than others to differences in dietary minerals. The mineral content of different tissues varied in response to the levels of different dietary minerals.

5.5.2.1. Bone

Bone was found to 'indicate' (vary in the levels of) Ca, Mg, Fe, Mn and P. Ca was found to cause a reduction in the Ca, Mg and P content of bone. This was surprising, for it indicates a suppression of bone mineralisation in response to dietary calcium. It is possible that the cause was calcium complexing magnesium in the gut and preventing the absorption of Mg. Casein/gelatin has a lower Mg content than Pruteen, and thus one may hypothesise that as Pruteen X Ca caused an increase in bone Ca, Mg and P, the Pruteen supplied sufficient Mg for the extra calcium to be utilised and incorporated into the skeleton. Bone deformity is a symptom of Mg deficiency (Ogino et al. 1978), and it has been shown that calcium will exacerbate this (O'Dell 1960). No evidence of skeletal deformity was seen in any fish during this experiment which suggests that magnesium was not

deficient. The dietary iron increased the iron in the bone. Although bone is not generally regarded as an indicator tissue for iron, this result suggested that bone is sensitive to dietary iron deficiency. The interaction Ca X Fe did not appear to have any effect on the mineral content of the bone. Pruteen induced an increase in bone calcium, magnesium and phosphorus. Tacon and Cooke (1980) demonstrated that the nucleic acids from SCP increase the phosphate content of the fish carcass, because the organophosphorus compounds are easily assimilated. This is supported by the present results. Mineralisation is improved by Pruteen. Bone deformity is a symptom of phosphorus deficiency (Murakami 1970, Ogino and Takeda 1978). Pruteen X Ca induced still higher bone mineralisation (as expressed by the increase in Ca, Mg and P produced by this interaction), indicating a beneficial effect of extra calcium with Pruteen. This may be a consequence of the higher Mg content of Pruteen, which prevents an imbalance of Ca:Mg occurring when extra calcium is given. The increase in bone Mn induced by Pruteen X Ca further suggests that Fe is antagonised by Ca and that Mn is antagonised by Fe. Thus the Fe present in Pruteen was antagonised by the Ca and the Mn availability was improved. The coincident increase in manganese, calcium and phosphorus in the bone supports the work of Knox et al. (1980), who found bone calcium and phosphorus to be reduced in manganese deficient fish.

5.5.2.2. Eye

The eyes of the fish from different treatments differed in levels of Ca, Zn, Fe, Mn and P. Calcium induced an increase in the calcium content of the eye,

and reduced the iron content. This is the first direct evidence of an interference of calcium on iron. In this tissue, the metabolism of P, Ca and Mg appear to be separate, or related in a different manner from that in bone. Pruteen induced the usual increase in P, but suppressed the Ca, and the Pruteen X Ca interaction also did likewise. Ca X Fe interacted to produce an increase in zinc content. The eyes are known to be sensitive to zinc deficiency. Ogino and Yang (1978) showed that 75% of zinc deficient trout developed bilateral cataracts, but cataracts were not seen in in this experiment. It is possible that the increased zinc content induced by Pruteen X Ca was caused by an interaction of the dietary Ca with a substance in Pruteen which bound zinc. Thus the calcium would have acted as a zinc-releasing agent. Ketola (1978) also found that cataracts occurred in zinc deficient trout. Comparison of the data reveals that the zinc levels in the eyes of the experimental fish tended to be higher than in the fish fed EM. This suggests that EM fish may have been suffering from a deficiency of zinc.

Pruteen caused Mn to be reduced.

5.5.2.3. Kidney

In the kidney, only one significant effect was observed: Pruteen caused the Ca content to decrease. Low kidney Ca concentrations indicate a healthy fish, whilst high Ca suggests potential renocalcinosis. As Pruteen has a higher Mg content than casein, this could be the mechanism by which the renal calcium is reduced, i.e. by the absence of a Ca:Mg imbalance. This is supported by the observation that the Ca:Mg ratio in the kidney

was reduced (P<=0.01) by Pruteen (Table 2.5.2.9.1.). Kloppel and Post (1975) found that the Ca content of trout was increased by a deficiency of tryptophan. This amino acid is less abundant in casein/gelatin than in Pruteen.

5.5.2.4. Muscle

The muscle tissue showed differences in concentrations of Mg, Zn and P. Here, Ca reduced the Mg and P content, both as calcium alone and interacting with Pruteen. Iron increased the Zn content, but Ca X Fe suppressed it, providing indirect evidence that Ca interacts with Fe to prevent its absorption. Pruteen reduced the zinc content of the muscle.

5.5.2.5.. Liver

The livers were found to differ in levels of Ca, Mg, Fe and Cu. Calcium was found to increase the calcium content of the liver, and iron was found to increase the iron content. This may be due to the position of the liver as the first tissue to receive nutrients absorbed from the gut. Pruteen decreased the calcium content and increased the magnesium and copper content; Pruteen contains considerably more copper than casein/gelatin mixture. The interaction Pruteen X Ca induced increased Mg, Fe and Cu, suggesting that the fish were less likely to become anaemic when given Pruteen with Ca. Pruteen X Ca X Fe , however, suppressed the copper content. The liver copper levels suggested one reason why the fish fed the experimental diet grew far more slowly than the fish fed on diet EM. This was the only mineral and the only tissue which differed significantly between EM and all the other diets (Table 4.5.2.6.2.), indicating that all the experimental

fish were copper deficient, despite the fact that the carcass copper content had not changed significantly during the experiment (Table 4.5.2.1.2.). The Cu content of the livers of the fish given EM were similar to the levels of liver Cu found in wild trout from unpolluted water (Wilson et al. 1980).

5.5.2.6. Skin

The skin is an important organ in the fish, as it not only acts as a storage organ for minerals, both in the scales and the epidermis, but also actively takes up minerals from the water. (Van Oosten 1957, Mashiko and Jozuka 1961). The skin of the fish differed in Ca, Mg, Fe and P contents, some of the effects appearing similar to those observed on the bone mineral contents. No major differences could detected in the scale morphologies, examined by scanning electron microscope (Plates 1-9). The scale rings of fish fed EM appear to be less well defined than the scale rings of the fish given the experimental diets, which may be a consequence of the faster growth of the former. Calcium reduced the concentration of Ca, Mg, Fe and P in the skin, suggesting once again that the effect of calcium was to cause an imbalance with resultant secondary effects, rather than direct effects, at least in the 'hard' tissues (skin and bone). In the 'soft' tissues (liver and eye) calcium in the diet increased the Ca content. It is possible that in calcium deficient fish, the skin has a high calcium content due to active uptake from the water, whilst in fish receiving sufficient calcium in the diet, the active transport is less. This is rather a simplistic arguement. Mashiko et al. (1961) showed that a mineral present in

a tissue did not necessarily enter the fish via that tissue. The suppression of Fe levels by dietary Ca supports the hypothesis that Ca decreases iron absorption, but the decrease in Fe content induced by Fe in the diet does not. The iron levels of fish fed on diet C were high compared to those in other treatments, which was not a consequence of a single fish having a high Fe content, as all the fish in treatment C had high Fe concentrations. The presence of Fe in the diets may have reduced the active absorption of Fe from the water into the skin and hence reduced the skin Fe. Pruteen caused an increase in the Ca, Mg and P content of the skin, further supporting the hypothesis that the superior phosphorus availability in Pruteen caused increased scale mineralisation irrespective of the source of the Ca and Mg also incorporated into the scales. Whereas an effect was seen in the bone, Pruteen X Ca had no effect on the skin Ca. An hypothesis therefore suggested is that bone 'competes' with scale tissue for available calcium. If calcium is absorbed from the gut, with sufficient P to allow apatite formation and enough Mg for enzyme activity then the bone will 'mop up' most of the calcium, and there will be no effect on skin calcium. If the fish is absorbing most of its Ca from water, and the blood has sufficient P and Mg, then scale formation will also occur. If calcium is added to the diet, then active transport in the skin is switched off, and scale formation decreases, and if this calcium is given without sufficient Mg and P, then the bone formation is also affected, leading to a decrease in bone mineralisation. If no calcium is added to the diet, the skin

becomes the only source of calcium from the water, unless the fish drinks, which is unlikely in freshwater fish. Unlike bone, scales can be resorbed by the fish (Yamada 1956, Wallin 1957). If the calcium in the diet caused an increase in phosphorus requirement, it is possible that the scales might be resorbed to provide the additional phosphorus. This would also reduce the calcium content and result in dietary calcium reducing the scale calcium. With Pruteen, abundant phosphorus would be available and thus the scales would not need to be resorbed to provide more phosphorus if calcium were added to the diet. Thus the interaction Pruteen X Ca would not be expected to produce an effect on scale mineralisation.

5.5.2.7. Viscera

The viscera showed differences in Ca and Zn content. The Pruteen increased the Ca and decreased the Zn content of the viscera, whilst Pruteen X Ca interacted to increase calcium still further.

5.5.2.8. Faeces

Faecal mineral levels were assayed to determine the effects of dietary factors on the excretion of minerals. As the fish had to be killed to remove the faeces, and as the fish were so small, only one sample of faeces was available per treatment (Table 4.5.2.9.1.). Some effects can be discerned from comparing this table with that of dietary mineral levels (Table 3.1.5.11.). Dietary calcium reduced the availability of phosphorus resulting in increased excretion of phosphorus in the faeces. This effect was very pronounced in diets with a casein/gelatin mixture as the protein source. The faeces of all the fish in the experiment had

similar magnesium levels, although diet EM had a higher magnesium concentration. The magnesium in fishmeal is therefore likely to be highly available to fish. In contrast, although the phosphorus level in EM was slightly higher than in the Pruteen diets, the phosphorus in the faeces was far higher. This is possibly due to the higher calcium levels because, as described above, the dietary calcium appears to reduce the availability of the dietary phosphorus. Given that dietary calcium is not necessarily required by fish, and that phosphorus in the effluent from fish farms constitutes a significant pollution load (Rundquist et al. 1977), formulation of fish diets with less calcium may be beneficial to both fish and environment. The iron in Pruteen diets was slightly less than in diet EM, but the levels in the faeces of fish given diets B and BCa were less still. This suggests that the iron in Pruteen is highly available. In contrast, the manganese in Pruteen appears to be rather less available than the manganese in fishmeal. The reduction in manganese induced by Pruteen appears to support this.

5.5.3. Mineral Balance

Having considered the levels of minerals and their differences in the fish, the effects observed can be reconsidered. Dietary calcium suppressed Mg and P, which could have been a consequence of an imbalance of Ca:Mg induced by the calcium. However this is not supported by factorial analysis of the Ca:Mg ratio in the carcasses; this showed that calcium had no significant effect on the Ca:Mg ratio. Alternatively, storage of calcium in fish carcasses may be inversely related to the supply of dietary

calcium. If the diet supplies adequate calcium, then calcium accumulation may be suppressed, or calcium uptake inhibited. A different hypothesis suggested by the results proposes that the apparent differences in the calcium content of the carcasses and the tissues are due to differences in the weights of the fish. It is possible that if fish are given a diet with adequate nutrients other than minerals, they will grow rapidly, but will be unable to absorb sufficient ions from the water to allow carcass mineralisation to keep up with carcass growth. Thus a gradual dilution of the minerals in the carcass occurs, with potentially harmful effects to the fish, especially if rapid growth is maintained for a considerable time. The fish in this experiment did not grow rapidly, if 'rapid' is taken to mean growth rates seen in commercially reared trout. However, differences did occur in the growth, and the relation between growth and calcium content is uncertain. When the calcium content of the diets (Table 3.1.5.11), the calcium contents of the carcasses (Table 4.5.2.1.2.) and the final weights of the fish (Table 4.5.1.2.) are compared, the fish fed on the diet containing one of the lowest levels of calcium (diet C) can be seen to have had a low final weight (only one treatment had a lower final weight). These fish had the fourth highest calcium content. However, the fish fed diet CCa which grew the fastest, were given far greater amounts of calcium in the diet and yet had the lowest calcium level in the carcass, suggesting that dietary calcium may not be availabe for bone mineralisation, but may affect growth. This is obviously a complex problem, which probably involves a number of

interrelated factors. These may include the Ca Mg and P levels in the diet and in the environmental water. The effect may be due to an effect of calcium in the diet on another dietary ingredient, which in turn limits the absorption of calcium. For example, Mahajan and Agrawal (1982) have shown that ascorbic acid is necessary for adequate Ca uptake in fish. If dietary calcium reacted with ascorbic acid or another dietary ingredient to reduce the availability of ascorbate, then this would affect the absorption of calcium. However, no ascorbate deficiency symptoms (Hodson et al. 1980) were seen. Thus the addition of calcium would appear to suppress the calcium in the carcass. Casein/gelatin mixtures and Pruteen contain a large number of different nutrients including ascorbic acid, which will vary both quantitatively and qualitatively, and interact with each other and with dietary minerals. The different responses of fish to diets containing the two dietary materials are not unexpected. It is possible that the concentration of ascorbic acid required for growth is less than that required for adequate uptake of calcium. In which case the casein/gelatin mixture would induce a greater deficiency of calcium than would Pruteen, which is an 'unpurified' dietary material and may well have more ascorbic acid, whilst differences in growth would be less. Kloppel and Post (1975) showed that fish given tryptophan deficient diets had an increased Ca content. Lall and Bishop (1976) noted that moist diets such as were used in Experiment VII may be susceptible to tryptophan loss by acid reaction.

Comparison of the factorial analyses carried out for each

tissue shows that the effect on Ca, Mg and P are generally associated, reflecting the combination of these elements into the hard tissues as calcium and magnesium apatites (Van Oosten 1957). The fact that Pruteen will cause an increase in calcium and magnesium in skin without supplementary Ca, whilst Ca causes a reduction of Ca, Mg and P in bone and skin suggests that the increase in Ca and Mg are a consequence of the improved phosphorus availability in Pruteen. Thus the absorption of Ca and Mg from water (the only route via which significant amounts may be absorbed if the fish are given deficient diets) can be seen to be related to the phosphorus status of the fish. Ca may reduce the availability of phosphorus to the extent that carcass mineralisation is depressed (see sect 5.5.2.8.).

These results raise doubts about the relationship between growth and mineral status. In this experiment, fish with apparently adequate carcass mineral concentrations grew significantly less fast than fish which appeared to be deficient. The exact cause of this effect may lie with a dietary factor of non-mineral nature (e.g. ascorbic acid), in which case this study would not identify it. In addition to the Ca, Mg, P association discussed above, there also seems to be a relation between Fe, Zn and Mn balance. If the effects of Mn in the carcass are examined, the effects of Pruteen and the interaction Pruteen X Ca, Pruteen X Fe or Ca X Fe X Pruteen exerted a series of significant effects in different directions (Table 4.5.1.2.1.). The Pruteen, Pruteen X Ca X Fe and Fe all reduced Mn, Pruteen X Ca, Pruteen X Fe and Ca X Fe increased it. This is evidence that Fe suppresses Mn

absorption, and that Ca interferes with the behaviour of Fe. Waddel and Sell (1964) showed that calcium carbonate or ammonium phosphate in poultry diets increased the Fe requirement. The reduction of Mn levels by Pruteen suggests that muco-polysaccharides (MPS's), which form a large proportion of the cell-coat of M. methylotrophus may adsorb Mn in the gut and render it less available. If MPS preferentially absorbed Ca and Fe rather than Mn then this would explain the increase in Mn content caused by Pruteen X Ca, and Pruteen X Fe. There is some evidence from the faecal minerals (Table 4.5.2.9.1.) that both calcium and iron complex manganese and render it less easily absorbed. If the MPS from Pruteen adsorbed Fe, or prevented its uptake from the gut, then the fish fed casein/gelatin would show a greater increase in Fe content than those fed Pruteen, given the same supplementation. Table 4.5.2.1.2. appears to show this. If the 'iron indicator' tissues (bone, eye, liver and skin) are compared, the effects of Fe on each are not the same. Dietary Fe increased the iron content of bone and liver, whilst the iron content of eye, liver and skin were increased by the interaction Pruteen X Ca. These results support the hypothesis that MPS adsorbs iron, and that dietary Ca reduces the effect of this adsorption. If the interference of Fe uptake from the gut by Ca is accepted, then these results also suggest that calcium may induce an increase in Fe uptake by binding to MPS in preference to iron. The observation that the Pruteen factor did not significantly reduce Fe in fish as it did Mn may be a consequence of the variation in iron content among fish given the same diet,

which renders the identification of significant effects more difficult. Alternatively, the (hypothetical) adsorption of Fe by MPS may be less than that of Mn. In conclusion, there is evidence to support the results of other authors, that dietary Ca may adsorb other dietary minerals reducing their availability. As this process may be enhanced by dietary lipids, with which the Ca may form soaps in the gut (Cuthbertson 1979), the frequent interaction of Ca with Pruteen may be partially explained. The diets containing Pruteen all had a higher fat content than those formulated with casein/gelatin. However, diets containing casein/gelatin became very compact and hard when dried before soxhlet extraction, which may have prevented the solvent from efficiently removing all lipids. This would cause an under-estimation of the fat, and over-estimation of NFE. In addition to Ca, MPS may also adsorb minerals in the gut, and these results indicate that this may be important in limiting the availability of Mn. The adsorption of Mn by MPS may be prevented if there are significant amounts of Ca or Fe in the diet. MPS may bind Zn, or the Zn in Pruteen may be less available than that of casein/gelatin. It is clear from these results that the concentrations of minerals present in a feedstuff do not necessarily reflect their availability to fish, and that the behaviour of dietary mineral supplements will vary depending on the nature of the other dietary ingredients. Adding minerals to a diet which is 'on paper' deficient may not achieve an improvement in the value of the diet. Indeed, the supplemented diet may prove to be inferior to the unsupplemented diet (i.e. diets B, BCa).

Interpretation of the results of the experiment was rendered difficult by the absence of deficiency symptoms observed in the fish. No incidence of spinal curvature, cranial deformity, cataract or fin erosion was seen, despite the deficient diets. The only symptoms of dietary differences were differences of growth and mineral content. Pruteen reduced the concentrations of zinc in the eyes, suggesting that the zinc in Pruteen is less available than that in casein/gelatin, as both materials have approximately the same zinc content. The experimental fish did not appear to be zinc deficient, despite the lack of zinc in the diets. Fig.4.6.2.9. revealed that the total zinc in the water was very often at concentrations believed to be toxic to rainbow trout (Finlayson and Verrue 1980). Ball (1967) found that the 48hr. LD_{50} level of dissolved zinc for rainbow trout was 4.6ppm, and Sprague (1971) believed that the 'no-effect' level was possibly two orders of magnitude lower than this, suggesting that the zinc availability in the water was less than the total levels indicated, because the fish did not die (very often). On average, 70% of the zinc could be absorbed by ion exchange resin, but when extreme levels of total zinc were detected in the water (Fig 4.6.2.9.) the proportion which could be absorbed by resin was reduced. This indicated that much of the variation in water zinc levels was due to complexed zinc which was probably unavailable to the fish either as an essential element for growth or as a toxin.

5.6. Water Analysis

The results of the study of the minerals in the water supplied to the Fish Culture Unit are shown in Figs. 4.6.2.1. to 4.6.2.20. and Table 4.6.1. Minerals were shown to vary widely from day to day, with a basal level above which the concentrations rose and then fell back This pattern was similar in all the metals, although only Mn and Fe were strongly correlated. This correlation accords with the observation of the WRC (ANN. REP. 1976) that mains water contains ferro-manganous precipitates. The extreme fluctuations in Fe and Mn levels are explained by the random presence or absence of solid matter in the sample. This would produce a basal level with random elevations whenever solids chanced to be present. This seems to be the case in this study. The high levels of zinc present in the water explained the random mortality which occurred during experiments (and at other times) in the Fish Culture Unit. The sub-lethal exposure of fish to dissolved zinc probably renders them more susceptible to disease, which destroyed experiments I and IV.

It is possible that the fluctuations in water mineral levels were due to the time of year at which the samples were taken. The samples were drawn before and during Experiment VII, and thus Fig. 4.6.1.4. shows the rising water temperature over this time. It is possible that as the temperature of the water rose, so did its solvent activity, and the minerals which had accumulated in the mains over the previous cold months were leached out and flushed into the system. Nonetheless, the levels of zinc are so high as to remain a constant threat to the well-being of fish held in

this water.

6. CONCLUSIONS

The results of the experiments lead to a number of conclusions regarding the mineral metabolism of trout, the potential use of Pruteen in fish diets and the possible effects of the latter on the former.

In all experiments, mineral supplements in trout diets were found to be of benefit. Of the minerals present in the bulk mineral premix, magnesium and iron are probably those of greatest value. Calcium was not shown to be required in fish diets.

The addition of trace element supplements to Pruteen diets was not found to improve growth, and often reduced it. Growth of fish given fishmeal diets was improved by the addition of dietary trace elements or of dietary minerals. When both minerals and trace elements were added to fishmeal diets, the growth rate of the fish was not higher than that of fish given either supplement. In Experiment II the growth rate of fish given both minerals and trace elements in fishmeal diets was significantly decreased; this interaction was less marked when a different batch of fishmeal was used in Experiment III. This suggested that the benefit of dietary mineral or trace element supplements may vary with the mineral spectrum of the other dietary ingredients, and that detrimental interactions may occur between them.

The palatability of Pruteen to trout was not found to be significantly less than that of fishmeal, even where Pruteen was included in the diets as the sole protein source, except in Experiment III. Here, the reduced palatability of Pruteen diets was believed to have been caused by heat damage; Pruteen may be

more sensitive to thermal degradation than fishmeal. Pruteen diets were found to be well bound and homogeneous, which may not be beneficial in dry diets, because they would be hard and unpalatable to fish. The inclusion of Pruteen in diets may allow a reduction in the amount of synthetic binders required to produce stable pellets.

In all the experiments in which fish were analysed over a period of time, the most rapid change in carcase mineral composition was seen in the first few weeks. The rapid change in mineral content was found in some cases to be temporary, as significant changes in mineral content later disappeared. Temporary changes in mineral content were also induced by a change in water hardness , after which the fish took several weeks to acclimatise. The water hardness was found to affect the response of fish to diets with different mineral levels. Although water hardness is often related to pH, increasing the hardness of the water by the addition of calcium carbonate (oyster shell) did not increase the pH.

Recirculating the water was found to affect the response of fish to mineral-deficient diets, the recirculation reducing the availability of dissolved minerals . Fish in through-flowing water are presented with a continuous (but variable) supply of dissolved minerals. The levels of zinc in the fish were notably lower in recirculated water than in through-flowing water. Concentrations of dissolved zinc (and other metals) were found to fluctuate widely in Birmingham mains water and zinc was often at a potentially dangerous level for trout, which may explain the

sudden deaths of fish during these experiments (and throughout the Fish Culture Unit).

Although Pruteen is less variable in mineral content than fishmeal, it is by no means of uniform mineral composition. Different batches were found to have different concentrations of Zn, Fe and Mn. The Fe in Pruteen was found to be more available to fish than the Fe of fishmeal, but the Zn and Mn were less easily absorbed. The absorption of minerals may have been affected by the addition of sodium phytate to fish diets. The inclusion of phytate in diets affected the carcase mineral composition, with both long and short term effects.

Pruteen was found to be more palatable to trout than a casein/gelatin mixture, but the feed conversion of fish given Pruteen was generally poor. This may be due to the relatively high concentrations of nitrogen-free extract present in Pruteen, and the high amounts included in the diet. Pruteen was found to be a very good source of dietary phosphorus, and may even supply excessive amounts if given in large quantities. Calcium in the diets of fish given casein/gelatin was seen to reduce the absorption of phosphorus from the gut, but in Pruteen diets, Ca had little effect. Pruteen increased carcase mineralisation in fish, probably as a consequence of the increased phosphorus concentrations.

Dietary iron was shown to improve the growth of trout.

ADDENDUM

The following were omitted from the list of references:

Cuthbertson, D.P. (1979) "Metabolism of the major mineral elements (Ca, P, Mg, S and also Fe) in relation to requirements." In: Reichigl, M. Jr. (Ed.) "Comparative animal nutrition volume II - Nutrient elements and toxicants." (Karger Basel, Munich)

Halver, J.E. (1957) "Nutrition of Salmonoid fishes III. Water soluble vitamin requirements of chinook salmon (Onchorynchus gorbuscha)." J. Nutrition 62: 225-243

Holm, L.W., and Svensson, P. (1954) "Wet combustion of ion exchange resins." Acta Chem Scand. 8: 527-9

Kraus, K.A. and Moore, G.E. (1953) "Anion Exchange Studies-VI. The bivalent transition elements manganese to zinc in hydrochloric acid." J. Am. Chem. Soc. 75: 1460

Lundgren, D.P. (1960) "Phosphate Analysis with the Technicon Autoanalyser. Selective Orthophosphate and Total Inorganic Phosphate Determinations." Analytical Chemistry 32: 824-828

Mahajan, C.L. amd Agrawal, N.K. (1980) "The role of vitamin C in calcium uptake in fish." Aquaculture 19: 287-294

Waddell, D.G. and Sell, J.L. (1963) "Interrelation between dietary calcium, phosphorus and iron for the chick." In 52nd. Annual Meeting of the Poultry Science Association. Poultry Science 42: 1315

Waiwood, K.G. and Beamish, F.W.H. (1978) "The effect of copper, hardness and pH on the growth of rainbow trout, Salmo gairdneri." J. Fish. Biol. 13: 591-599

Wilson, D., Finlayson, B. and Morgan, N. (1981) "Copper, zinc, and cadmium concentrations of resident trout related to acid-mine wastes." Calif. Fish and Game 67: 176-186

Wood, E.M. Griffin, P.J. and Snieszko, S.F. (1954) "Synthetic binding of trout diets." Prog. Fish Culturist 16: 19-24

7. REFERENCES

Andrews, J.W., Murai, T. and Campbell, C. (1973) "Effects of dietary calcium and phosphorous on growth, food conversion, bone ash and haematocrit levels of catfish." J. Nutr. 103: 766-771

Anon. (1976) "I.C.I. trial report 76/f/1: The gradual replacement of fish and herring meal by up to 30% 'Pruteen' in the diet of trout fry. Brixham, U.K." I.C.I. Agric. Div. Billingham, England.

Arai, S., Muller, R., Shimma, Y. and Nose, T. (1975) "Effects of calcium supplementation to yeast grown on hydrocarbons as feedstuff for rainbow trout." Bull. Fresh. Fish. Res. Lab. Tokyo. 25: 33-40

Arai, S., Nose, T.and Kawatsu, H. (1974) "Effects of minerals supplemented to the fish meal diet on growth of eel, (Anguilla japonica)." Bull. Fresh. Fish. Res. Lab. Tokyo. 24: 95-99

Association of Official Analytical Chemists. (1970) "Official methods of analysis." Horowitz, W. (Ed.) 11th edition. A.O.A.C., Washington D.C.

Atack, T.H., Jauncey, K. and Matty, A.J. (1979) "The utilization of some single-cell proteins by fingerling mirror carp, (Cyprinus carpio)." Aquaculture 18: 337-348

Atack, T.H. and Matty, A.J. (1978) "The evalution of single-cell proteins in the diet of rainbow trout II. The determination of net protein utilization, biological

values and true digestibility." Symp. Fin-Fish. Nutr. and Feed. Technol. Hamburg. FRG., June 20 1978. E.I.F.A.C./78/Symp. E/59 FAO, Rome. FAO access No. 41435.

Atz, J.W. (1964) "Some principles and practices of water management for marine aquariums." In: Clark, J.R. and Clark, R.L. (Eds.), "Sea water systems for experimental aquariums." U.S. Dept. of the Interior, Bureau of Sport, Fisheries and Wildlife. Res. Rep. 63: 3-16

Bailey, N.T.J. (1959) "Statistical Methods in Biology." Hodder and Stoughton Ltd. Lond. 2nd edition.

Ball, I.R. (1967) "The relative susceptibilities of some species of fresh-water fish to poisons - II. Zinc." Water Res. 1: 777-783

Barton, B.A. and Peters, R.E. (1982) "Plasma cortisol stress response in fingerling rainbow trout, (Salmo gairdneri) to various transport conditions, anaesthesia and cold shock." J.Fish. Biol. 20: 39-51

Beck, H., Gropp, J., Kroops, H. and Tiews, K. (1978) "Single cell proteins in trout diets." EIFAC '78 Symp. Hamburg, W. Germany.

Beerstecker, E. (1979) "The chemical element needs of animals." In: Reichgl, M., Jr., (Ed.) "Comparative Animal Nutrition, Vol. II, "Nutrient elements and toxicants." 1-27 Karger, Basel, Munich.

Berg, A. (1968) "Studies on the metabolism of calcium and strontium in freshwater fish - I. Relative contribution of direct intestinal absorption." Mem. Inst. Itha. Idrobiol.

23: 161-196

Bhatia, D. (1932) "Factors involved in the prodution of annual zones on the scales of the rainbow trout (S. irideus) II." J. Exp. Biol. 9: 6-14

Blondin, G.A., Kulkarni, B.D. and Kes, W.R. (1967) " A study of the origin of vitamin D from 7-dehydrocholesterol in fish." Comp. Biochem. Physiol. 20: 379-390

Bower, C.E., Turner, D.T. and Spotte, S. (1981) "pH maintenance in closed seawater culture systems: Limitations of calcareous filtrants." Aquaculture 23: 211-217

Bremner, I., Young, B.W. and Mills, C.F. (1976) "Protective effect of zinc supplementation against copper toxicosis in sheep." Br. J. Nut. 36:551-561

Brett, J.R., Shelbourn, J.E. and Shoop, C.T. (1969) "Growth rate and body composition of fingerling sockeye salmon, (O. nerka), in relation to temperature and ration size." J. Fish. Res. Bd. Can. 26: 2363-2394

Brown, M.E. (1946a) " The growth of brown trout (S. trutta) I: factors influencing the growth of trout fry." J. Exp. Biol. 22: 118-129

Brown, M.E. (1946b) "The growth of brown trout (S. trutta) II: The growth of 2 year old trout at a constant temperature of 11.5 C." J. Exp. Biol. 27: 130- 144

Burrows, R.E. (1972) "salmonid husbandry techniques." In: "Fish Nutrition." 375-402 Halver, J.E. (Ed.) Acad. Press.

Chan, D.K.O., Chester-Jones, I. and Smith, R.N. (1968) "The effects of mammalian calcitonin on the plasma levels of

calcium and inorganic phosphate in the European eel (Anguilla anguilla)." Gen. Comp. Endocr. 11: 243-245

Coffin, C.C., et al. (1949) "Exchange of materials in a lake as studied by the addition of radioactive phosphorous." Can. J. Res. Sect. D 27: 202-222

Copp, D.H., Brooks, C.E., Low, B.S., Newsome, F., O'Dor, R.K., Parkes, C.O., Walker, V. and Watts, E.G. (1970) "Calcitonin and ultimobranchial function in lower vertebrates." In: Tayor, S. (Ed.) "Calcitonin 69" Heinemann, London. 281-294

Cotzias, G.C. (1960) "Metabolic relations of manganese to other minerals." Fed. Proc. 19: 655-658

Cowey, C.B., Adron, J., Blair, A. and Shanks, A.M. (1974) "Studies on the nutrients of marine flatfish. Utilization of various dietary proteins by plaice (Pleuronectes platessa)." Br. J. Nut. 31: 297-306

Cowey, C.B., Pope, J.A., Adron, J.W. and Blair, A. (1971) "Studies on the nutrition of marine flatfish. Growth of the plaice (Pleuronectes platessa) on proteins derived from plant and other sources." Mar. Biol. Berlin. 10: 145-153

Cowey, C.B. and Sargent, J.R. (1972) "Fish nutrition." Adr. Mar. Biol. 10: 383-492

Cowey, C.B. and Sargent, J.R. (1979) "Nutrition." In: Hoare and Randall (Eds.) "Fish Physiology" Vol. 8. Acad. Press. N.Y.

Cowey, C.B. et al. (1977) "The production of renal calcinosis by magnesium deficiency in rainbow trout, (S. gairdneri)."

Brit. J. Nut. 38: 127-135

Crichton, M.I. (1935) "Scale resorption in salmon and sea trout." Salm. Fish. Edinb. 4: 1-8

Cuplin, P. (1969) "Performance evaluation of chelated minerals in Idaho formula diets." Trans. Am. Fish. Soc. 98: 772-776

Duncan, D.B. (1955) "Multiple range and multiple F tests." Biometrics 11: 1-42

Elliott, J.M. (1976) "Body composition of brown trout (Salmo trutta) in relation to temperature and ration size." J. Anim. Ecol. 45(1): 273-289

Finlayson, B.J. and Verrue, K.M. (1980) "Estimated safe zinc and copper levels for chinook salmon (Onchoynchus tsawytscha) in the Upper Sacramento River, California." Calif. Fish and Game 66: 68-82

Fish Comission of Oregon (1972) "Oregon Diet Specifications." Report of the Fish Comission of Oregon.

Flemming, W.R. (1967) "Calcium metabolism in teleosts." Am. Zool. 7: 835-842

Flemming, W.R., Stanley, J.G. and Meier, A.H. (1964) "Seasonal effects of external calcium, oestradiol and ACTH on the serum calcium and sodium levels of Funulus kansae." Gen. Comp. Endoc. 4: 61-67

Garrod, D. and Newell, B.S. (1958) "Ring formation in Tilapia esculentia." Nature. London. 181: 1411-1412

Grayton, B.D. and Beamish, F.W.H. (1977) "Effects of feeding frequency on food intake, growth and body composition of rainbow trout (Salmo gairdneri)." Aquaculture 11: 159-172

Halver, J.E. (1972) "The vitamins" In: "Fish Nutrition" Halver, J.E. (Ed.) Acad. Press. 29-103

Halver, J.E. and Coates, J.A. (1957) "A vitamin test diet for long term feeding studies." Prog. Fish Cult. 19: 112-118

Hastings, W.H. and Dickie, L.M. (1972) "Feed formulation and evaluation." In: "Fish Nutrition." Halver, J.E. (Ed.) Acad. Press. 327-374

Hevesey, G. (1945) "Rate of renewal of fish skeleton." Acta. Physiol. Scan. 9: 234-247

Hevesey, G., Lockner, D. and Sletten, K. (1964) "Iron metabolism and erythrocyte formation in fish." Acta. Physiol. Scand. 60: 256-266

Hickman, C.P., Jr. (1968) "Ingestion, intestinal absorption and elimination of sea water salts in the southern flounder (Paralichthys lethostoma)." Can. J. Zool. 46: 457-466

Hickman, C.P. and Trumps, B.E. (1969) "The kidney." In: Hoar, W.S. and Randall, D.J. (Eds.) "Fish Physiology." Acad. Press. Lond. 1: 91-239

Hodson, P.V., Hilton, J.W., Blunt, B.R. and Slinger, S.J. (1980) "Effects of dietary ascorbic acid on chronic lead toxicity to young rainbow trout (Salmo gairdneri)." Can. J. Fish. Aquat. Sci. 37: 170-176

Holt, S., Heading, R., Carter, D., Prescott, L. and Tothill P. (1979) "Effect of gell fibre on gastric emptying and absorption of glucose and paracatamol." Lancet. 1: 636-639

Ichikawa, R. (1953) "Absorption of fish scales caused by

starvation." Rec. Oceanogr. Wks. Jap. 1: 101-104

Ichikawa, R. (1960) "Strontium/calcium distribution in the rainbow trout." Rec. Oceanogr. Wks. Jap. 5: 120-131

Ichikawa, R. and Oguri, M. (1961) "Metabolism of radionucleides in fish I. Strontium-calcium discrimination in gill absorption." Bull. Jap. Soc. Sci. Fish. 27: 351-356

Ichikawa, R., Oguri, M. and Takada, N. (1962) "Metabolism of radionuclides in fish III. Comparative behaviour of strontium and calcium at gill absorption in environmental water with different amounts of calcium." Bull. Jap. Soc. Sci. Fish. 28: 1160-1163

Ikeda, Y., Ozaki, H and Umatsu, K. (1973) "Effects of enriched diets with iron in the culture of yellow-tail." J. Tokyo Univ. Fish. 59: 91-99

Jozuka, K. et al. (1963) "Studies on the calcium uptake by marine teleosts." Ann. Rep. Noto. Mar. Lab. Univ. Kanazawa. 3: 11-16

Kaushik, S.J. and Luquet, P. (1980) "Influence of bacterial protein incorporation and of sulphur amino acid supplementation to such diets, on growth of rainbow trout, Salmo gairdneri Richardson." Aquaculture 19: 163-175

Kawatsu, H. (1972) "Studies on the anaemia of fish, V. Dietary iron deficient anaemia in brook trout (Salvelinus fontinalis). Bull. Fresh. Fish. Res. Lab. Tokyo. 22: 59-69

Ketola, H.G. (1975a) "Mineral supplementation of diets containing soybean meal as a source of protein for rainbow

trout (S. gairdneri)." Prog. Fish. Cult. 37: 73-75

Ketola, H.G. (1975b) "Requirement of the tlantic salmon (Salmo salar) for dietary phosphorous." Trans. Am. Fish. Soc. 3: 548-551

Ketola, H.G. (1978) "Dietary zinc prevents cataracts in rainbow trout." Fed. Proc. 37: 584 (abs.)

Kloppel, T.M. and Post, G. (1975) "Histological alterations in tryptophan deficient trout." J. Nutr. 105: 861-866

Knox, D., Cowey, C.B. and Adron, J.W. (1981a) "Studies on the nutrition of salmonid fish. The magnesium requirement of rainbow trout (Salmo gairdneri)." Brit. J. Nutr. 45: 137-148

Knox, D., Cowey, C.B. and Adron, J.W. (1981b) "The effect of low dietary manganese intake on rainbow trout (Salmo gairdneri)." Brit. J. Nutr. 46: 495-501

Knox, D., Cowey, C.B. and Adron, J.W. (1982) "Effects of dietary copper and coppper:zinc ratios on rainbow trout (Salmo gairdneri)." Aquaculture 27: 111-119

Lall, S.P. and Bishop, F.J. (1976) "Studies on the nutrient requirement of rainbow trout (Salmo gairdneri) grown in sea water and fresh water." Symp. on "Recent Advances in Aquaculture" FAO. Tech. Conf. on Aquaculture. Kyoto, Japan. May 26 - June 2. Pillay, T.V.R.and Dill, W.A. (Eds.)

Lall, S.P. and Bishop, F.J. (1977) "Studies on mineral and protein utilization by Atlantic salmon (S. salar) grown in sea water." Fish. Mar. Ser. Res. Der. Tech. Rep. 688:

Launer, C.A., Tiemeir, O.W.and Deyoe, C.W. (1978) "Effects of dietary addition of vitamins C and D3 on growth and calcium and phosphorous content of pond cultured channel catfish." Prog. Fish Cult. 40: 16-20

Likuski, H.J.A. and Forbes, R.M. (1965) "Mineral utilization in the rat, IV: effect of calcium and phytate on the utilization of dietary zinc," J. Nutr. 85: 230-234

Mashiko, K. and Jozuka, K. (1961) "Pathway of Ca uptake and excretion by teleost fish." I. Jap. J. Limnol. 22: 217-224

Mashiko,K. and Jozuka, K. (1962) "Studies on the calcium uptake by teleost fishes, I. Ca uptake by crucian carp." Sci. Rep. Kanyawa Univ. 7: 107-126

Mashiko, K. and Jozuka, K. (1964) "Absorption and excretion of calcium by teleost fishes with special reference to routes followed." Annot. Zool. Jap. 27: 41-50

Mashiko, K., Jozuka, K. and Morita, O. (1964) "Turnover of Ca by the scale of The crucian carp." An. Rep. Note. Mar. Lab. Univ. Kanayawa." 4: 53-58

McCay, C.M. et al. (1936) "The calcium and phosphorous content of the body of brook trout in relation to age, growth and food." J. Biol. Chem. 114: 259-263

Mighell, J.L. (1969) "Rapid cold-branding of salmon and trout with liquid nitrogen." J. Fish. Res. Bd. Can. 26: 2765-2769

Ministry of Agriculture Fisheries and Food (1976) "Manual of Nutrition." H.M.S.O. London.

Moss, M.L. (1961a) "Studies of the acellular bone of teleost

fish, I. Morphological and systematic variations." Acta. Anat. 46: 343-426

Moss, M.L. (1961b) "Osteogenesis of acullular fish bone." Am. J. Anat. 108: 99-110

Moss, M.L. (1962) "Studies of the acellular bone of teleost fish, II. Response to fracture under normal and acalcaemic conditions." Acta. Anat. 48: 46-60

Moss, M.L. (1963) "The biology of acellular teleost bone." Ann. N.Y. Acad. Sci. 109: 337-350

Moss, M.L. and Freilich, M. (1963) "Studies of the acellular bone of teleost fish fish, IV. Inorganic contents of calcified tissues." Acta. Anat. 55: 1-8

Mudge, J.E. and Neffe, W.H. (1971) "Sodium and potassium levels in the serum of acid-exposed brook trout (Salvelinus fontinalis)." Proc. Pa. Acad. Sci. 45: 101-103

Murai, Takeshi, Andrews, J.W. and Smith, R.G. Jr. (1981) "Effects of dietary copper on channel catfish." Aquaculture. 22: 353-357

Murakami, Y. (1970) "Supplemental effects of phosphates to a commercial diet for carp fry." Chosa Kenkyu Hokoku, Hiroshimaken Tansuigyo Shidosho. 9: 33-45

Norris, W.P., Chavin, W.and Lombard, L.S. (1963) "Studies of calcification in a marine teleost." Ann. N.Y. Acad. Sci. 109: 312-336

Nose, T. (1974b) "Effects of amino acids supplemented to petroleum yeast on growth of rainbow trout fingerlings, I a preliminary experiment." Bull. Fresh. Fish. Res. Lab.

Tokyo. 24(1): 57-63

Nose, T. (1974c) "Effects of amino acids supplemented to the petroleum yeast on growth of rainbow trout fingerlings, II. Methionine and cystine." Bull. Fresh. Fish. Res. Lab. Tokyo. 24(2):101-109

Nose, T. (1976) "Recent advances in studies on mineral nutrition of fish in Japan." Proc. FAO Technical Conference on Aquaculture, Japan. (Kyoto) May 26 - June 2.

Oberleas, D., Muhrer, M.E. and O'Dell, B.L. (1962) "Effects of phytic acid on zinc availability and parakeratosis in swine." J. Anim. Sci. 27: 57-62

Oberleas, D., Muhrer, M.E. and O'Dell (1966) "Dietary metal complexing agents and zinc availability in the rat." J. Nutrit. 90: 56-62

O'Dell, B.L. (1960) "Magnesium requirement and its relation to other dietary constituents." Fed. Proc. 19: 668-654.

O'Dell, B.L., Burpo, C.G. and Savage, J.E. (1972) "Evaluation of zinc availability in food stuffs of plant and animal origin." J. Nutrit. 102: 653-660

Ogino, C. and Chiou, J.Y. (1976) "Minerla requirement in fish, II. magnesium requirement of carp (Cyrinus carpio L.)." Bull. Jap. Soc. Sci. Fish. 42: 71-75

Ogino, C., Takashima, C. and Chiou, J.Y. (1978) "The requirement of rainbow trout for dietary magnesium." Bull. Jap. Soc. Sci. Fish. 44: 1105-1108

Ogin, C. and Takeda, H. (1976) "Mineral requirements of fish,

III. Calcium and phosphorous requirement in carp." Bull. Jap. Soc. Sci. Fish. 42: 793-799

Ogino, C. and Takeda, H. (1978) "Requirement of rainbow trout for dietary calcium and phosphorous." Bull. Jap. Soc. Sci. Fish. 44(9): 1019-1022

Ogino, C., Takeuchi, L., Takeda, H. and Watanabe, T. (1979) "Availability of dietary phosphorous in carp and rainbow trout." Bull. Jap. Soc. Sci. Fish. 45: 1527-1532

Ogino, C. and Kamizono, M. (1975) "Mineral requirements of fish, I. Effects of dietary salt-mixture levels on growth, mortality and body composition in rainbow trout and carp." Bull. Jap. Soc. Sci. Fish. 41: 429-434

Ogino, C. and Yang, G.Y. (1978) "Requirement of rainbow trout for dietary zinc." 44(9): 1015-1018

Ogino, C. and Yang, G.Y. (1980) "Requirement of carp and rainbow trout for dietary manganese ans copper." Bull. Jap. Soc. Sci. Fish. 46: 455-458

Palaheimo, J.E. and Dickie, L.M. (1966b) "Food and growth of fishes III. Relations among food, body-size and grwth efficiency." J. Fish. Res. Bd. Can. 23(8): 1209-1248

Pagenkopf, G.K., Russo, R.C. and Thurston, R.V. (1974) "Effects of complexation on toxicity of copper to fishes." J. Fish. Res. Bd. Can. 31: 462-465

Pang, P.K.T. and Pickford, G.E. (1967) "Failure of hog thyrocalcitonin to elicit hypocalcaemia in the teleost fish Fundulus heteroditus." Comp. Biochem. Physiol. 21: 573-578

Phillips, A.M. Jr. (1962) "Effects of diet and water temperature on the blood phosphorous of brook trout." Prog. Fish. Cult. 24: 22-25

Potts, W.T.W. and Parry, G. (1964) "Osmotic and ionic regulation in animals." Macmillan Co. N.Y.

Roeder, M. and Roeder, R.H. (1966) "Effects of iron on the growth of fishes." J. Nutrit. 90: 86-90

Rosenthal, H.L. (1960) "Accumulation of Sr and Ca by freshwater fishes." Proc. Soc. Exp. Biol. Med. 104: 88-91

Rosenthal, H.L. (1963) "Uptake, turnover and transport of bone-seeking elements in fishes." Ann. N.Y. Acad. Sci. 109: 278:293

Rundquist, J., Gall, G. and Goldman, C.R. (1977) "Watercress-crayfish polyculture as an economic means of stripping nutrients from enriched waters." Freshwater Crayfish 3: 141-149

Rozin, P. and Mayer, J. (1961) "Regulation of food intake in the golfish." Am. J. Physiol. 201(5): 968-974

Sakamoto, S. and Yone, Y. (1973) "Effects of dietary calcium/phosphorous ratios upon growth, feeding and blood plasma levels in red sea bream." Bull. Jap. Soc. Sci. Fish. 39: 343-348

Sakomoto, S and Yone, Y. (1978a) "Iron deficiency symptoms of carp." Bull. Jap. Soc. Sci. Fish. 44(10): 1157-1160

Sakamoto, S. and Yone, Y. (1978b) "Requirement of red sea bream for dietary trace elements." Bull. Jap. Soc. Sci. Fish. 44(12): 1341-1344

Sakamoto, S. and Yone, Y. (1979a) "Requirement of red sea bream for dietary magnesium." Bull. Jap. Soc. Sci. Fish. 45(1): 57-60

Sakamoto, S. and Yone, Y. (1979b) "Availabilities of three iron compounds as dietary iron sources for red sea bream." Bull. Jap. Soc. Sci. Fish. 45(1): 231-235

Sakamoto, S. and Yone, Y. (1979c) "Mineral mixture in purified diet for red sea bream." Bull. Jap. Soc. Sci. Fish. 45(7): 873-877

Samuelson, O. (1963) "Wet combustion of ion exchange resins." In: "Ion Exchange Separation in Analytical Chemistry." Samuelson, O. (Ed.) Wiley and sons, N.Y. and Lond.

Sanchez Muniz, F.J., De La Higuera, M., Mataix, F.J. and Varela, G. (1978) "Haematological consequences of feeding trout with a single cell protein (Hansenula anomala)." Proc. Nutrit. Soc. 37: 81A

Shaw, H.M. et al. (1975) "Effects of dietary sodium chloride on growth of atlantic salmon (Salmo salar)." J.Fish. Res. Bd. Can. 32: 1813-1819

Shehadeh, Z.H. and Gordon, M.S. (1969) "Role of the intestine in salinity adaptation of the rainbow trout Salmo gairdneri." Comp. Biochem. Physiol. 30: 397-418

Shekhanova, I.A. (1956) "On the possibility of assimilation by fishes of inorganic phosphorous from water." Dokl. Acad. Nauk. USSR. 166: 161-164

Sherwood, M. (1974) "Single-cell protein comes of age." New Scientist. 64: 634-639

Shimma, Y. and Nakada, M. (1974a) "Utilization of petroleum yeast for fish feed, I. Effect of supplemental oil." Bull. Fresh. Fish Lab. Tokyo. 24: 47-56

Simkiss, K. (1972) "The calcium metabolism of fish in relation to ageing." Symp. on the ageing of fish. Reading.

Simmonds, D.J. (1971) "Calcium and skeletal tissue physiology in teleost fishes." Clin. Orthopaedics. 76: 244-280

Simmonds, D.J., Simmonds, N.B. and Marshall, J.M. (1970) "The uptake of Ca in the acellular boned toadfish." Calc. Tiss. Res. 5: 206-221

Smith, H. (1930) "The absorption and excretion of water and salts by marine teleosts." Am. J. Physiol. 93: 480-505

Spotte, S. (1970) "Fish and invertebrate culture: water management in closed systems." Wiley Interscience. N.Y.

Sprague, (1971) "Measurement of pollutant toxicity in fish, III. Sub-lethal effects and safe concentrations." Wat. Res. 6: 217-230

Srivastava, P.N. (1960) "Thyroid control of radiophosphorous metabolism in yearling salmon (S. salar)." Can. J. Zool. 38: 249-256

Tacon, A.G.J. (1978) "The effects of activated sludge -single cell protein (ASCP), derived from the treatment of domestic sewage, in trout diets." Proc. World Symp. on Finfish Nutrition and Fishfeed Technology, Hamburg. 20-23

Tacon, A.G.J. (1979) "Nutritional evaluation of animal and food processing wastes." Symp. "Foodstuffs from Waste" London.

Tacon, A.G.J. and Cooke, D.J. (1980) "Nutritional value of

dietary nucleic acids to trout." Nut. Rep. Int. 22: 631-640

Tacon, A.G.J. and Ferns, P. (1976) "Use of activated sludge from domestic sewage in trout diets." Nutr. Reps. Int. 13(6): 549-562

Tacon, A.G.J. and De Silva, S.S. (In press) "Nutritional composition of commercial fish feed in the U.K. with special reference to minerals.

Tiews, Von K., Koops, H., Gropp, J. and Tiews, J. (1973) "Uber die korperzusammensetzung netzkafiggehaltener forellen (S.gairdneri) im alhangigkeit von futterung und wachstum." Archiv. fur Fischerei Wissenschaft. 24: 261-269

Tomiyama, T., Kobayashi, T.K. and Ishio, S. (1956b) "Absorption of PO4 ion by carp." In: Proc. Conf. on "Research in the effects and influences of the nuclear atom bomb explosion." Tokyo. 1195-1200

Umehara, S. and Oguri, M. (1978) "Effects of calcium content on plasma calcium levels in goldfish." Bull. Jap. Soc. Sci. Fish. 44(8): 827-833

U.S. National Research Council (1973) "Nutrient requirements of domestic animals. No. 11. Nutrient requirements of trout, salmon and catfish." Nat. Acad. Sci. Wash. D.C.

Van Oosten, J. (1957) "The skin and scales." In: Brown, M.E.(Ed.) "The Physiology of Fishes." Acad. Press. Lond. I: 207-244

Van Someren, V. (1939) "A preliminary investigation into the causes of scale absorption in salmon (Salmo salar)." Salm.

Fish. Edinb. No.II: 1-12