

**DIETARY AVAILABILITY AND RETENTION OF SELECTED MINERALS  
ASSOCIATED WITH THE INTENSIVE PRODUCTION OF  
RAINBOW TROUT (*Oncorhynchus mykiss*)**

By

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## ABSTRACT

### **Dietary availability and retention of selected minerals associated with the intensive production of rainbow trout (*Oncorhynchus mykiss*).**

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This research programme aimed to review the nutritional requirements for the main minerals, (namely calcium, phosphorus, magnesium and zinc) formulated in commercial diets that are essential for the health and growth performance for salmonid fish. This was undertaken with the aim of improving our knowledge of their physiology, metabolism and fate in the rainbow trout, *Oncorhynchus mykiss*. Phosphorous (P) featured strongly in this work due to its adverse role in pollution and the environmental impact of intensive fish farming. The first chapter surveyed the gross nutritional requirements of fish and focused on the mineral requirements in particular. Typically the P requirements for trout were found to range from 0.5-0.8% of the diet. The problems of P loading as a consequence of dietary losses was addressed and the physiological and metabolic roles of both calcium and phosphorous were especially noted in relation to fish health and for phosphorous its environmental implications were addressed.

Experimental approaches were evaluated and it was decided to conduct both standard growth trial studies as well as digestibility trials to provide the basis of most investigations with the rainbow trout. Novel approaches and strategies were used in relation to specific experiments such as examining the mineral levels in blood, and various tissues and the testing of different feed ingredients, dietary supplements and mineral sources in successive investigations. Initial investigations appraised commercial diets of varying nutritional profile with respect to mineral retention and availability for rainbow trout under controlled laboratory conditions. The effects of diets containing different fishmeal sources: i.e. brown versus white fishmeals, elevated ash content and also varying in the levels of oil were tested on juvenile rainbow trout in closed recirculated systems. Diet composition caused a significant effect on mineral retention and distribution profile in fish tissues and organs. Typically, both P and Ca were of highest concentration in vertebrae of trout (60mg/g-dry weight), compared with P concentrations for all other major organs/tissues, which were fairly even between 11-20mg/g. A small increase in dietary P level (1.08% vs. 1.22%) did not affect any growth parameters for trout for the first two commercial feeds tested but there were interesting observations with respect to the amount of P excreted in the bile with a 25% increase from 0.8mM to 1.2mM. The P levels in plasma of these fish did not reflect any dietary changes. However, there was a noticeable reduction in the digestibility of P in the diet containing the white fishmeal source (26%) compared with 49% for the higher grade fishmeal diet. High ash content feeds resulted in a marked reduction in the net mineral retention of this element (16% compared to 27% for the lower ash diet). The same was also true for Ca (12% compared with 26%). The effect of oil levels in diets on mineral utilization was investigated under farmed conditions and was of particular interest given the demand for nutrient dense feeds in the industry. There was a strong tendency for improved P and Ca digestibility coefficients at each incremental increase in oil level for juvenile production sized fish (50-100g). This ranged from 55% to over 70% when oil levels were over 26%. However this was not observed for larger fish of over 200g in weight.

Experimental investigations followed are described in (Chapter 4) where fishmeal based diet was supplemented with varying levels of inorganic phosphorous. Phosphorous, calcium and other mineral absorption characteristics in addition to retention were measured in a series of growth and digestibility trials. Interestingly, there was no apparent change in the distribution

of P with increasing dietary levels ranging from 1.39-2.16%. These were above known requirements for these fish with minerals being in excess. Similar results were noted for all other minerals measured in rainbow trout. There was a significant rise in the P concentration of plasma of rainbow trout fed a diet containing over 2% P and this may infer that the homeostatic regulation of P is unable to function at this level. Other haemato-logical parameters were not affected. Although not significant, there appeared to be slight trend in elevated bile P with increasing dietary P supplementation. The faecal concentrations for each of the minerals showed that elevated P in fishmeal diets led to increased faecal output from 25mg/g to over 40mg/g for the highest P diet. Overall digestibility coefficients were lower as dietary P increased above that in the fishmeal control diet. These ranged from 50% to 39% for P, Ca and Mg were not greatly affected. The net retention of P was calculated and this fell from 30% to just below 20% for the range of dietary P used in the investigation.

A preliminary study, reported in chapter 5A, was useful in providing information about the relative absorption profiles for differential mineral absorption from the various regions of the gastro intestinal tract of rainbow trout. A standard commercial diet was fed to large trout (>200g) and subsequently, digesta was removed from fish and analysed. For all minerals and protein, the pyloric and mid intestinal region was the main site for digestion, release and absorption of the macro elements concerned. The protein and mineral digestibility of suitable feedstuffs commonly employed in the formulation of complete diets for trout resulting from a sequence of experimental trials are presented in chapter 5B. These included a selection of marine, animal and plant by-products which were substituted into a reference basal diet designed for salmonids. This involved the inert marker - yttrium oxide and calculations based on nutrient digestibility from diet and faecal concentrations. Mineral digestibility coefficients were found to vary considerably and a number of anomalies such as negative values were obtained for Ca and Zn in certain feedstuffs. Combined diets (reference and test ingredient) gave values that were more consistent and P digestibility ranged from 47-59% in marine and animal protein concentrates compared with plant sources (24-37%). Negative values for Ca and Zn were thought to be attributable to complex interactions with other feed components. Additionally, a group of inorganic mineral supplements were tested by inclusion into a series of diets. These included mono calcium phosphate, di-calcium phosphate (DCP), mono di-calcium phosphate and magnesium phosphate. DCP produced lower Ca and P digestibility values, 31 and 50% respectively, compared with the other sources (44-62%), indicating the importance of choice of mineral supplement in aquafeeds.

A critical appraisal of this work is provided in Chapter 6 and formed a retrospective review of the results generated and integrated these findings into a foundation for further research and development. Nutritional investigations using the rainbow trout as the model salmonid species raised many more questions and possibilities and broadened the scope of the topic. The subject of mineral requirements for fish is very complex and numerous factors are involved at several physiological and biochemical levels in fish. Although the research on rainbow trout involved whole animal studies under both laboratory and commercial farm conditions, the need to explore alternative *in vitro* methods and to utilize larger scale farm and sea cage trials for salmon were suggested. The advent of more advanced diet formulations and feeding strategies were mentioned and the scope for more scientific investigations to improve the utilization and reduce phosphorous discharge into the environment proposed.

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*For my mum and dad...*

## **CHAPTER 1**

### **GENERAL REVIEW AND INTRODUCTION**



## CHAPTER 1

### GENERAL REVIEW AND INTRODUCTION

#### 1.1 Aquaculture and fish production

Fish farming is thought to have been initially practiced in the Far East as long ago as 2000 B.C., and written accounts dating from the first century AD have described the construction of fishponds by Romans, which spread throughout Europe during the Middle Ages (Lovell *et al.*, 1978). However, it was not until the 19<sup>th</sup> century that modern style fish culture operations expanded from the United States to England, and was concerned mainly with the propagation of salmonids for restocking purposes. By the mid 20<sup>th</sup> century the worldwide culture of carp, milkfish, tilapia and various indigenous finfish and crustaceans was generally well established and fairly extensive, with the exception of salmonid culture located mainly in North America and Western Europe (Lovell, 1989).

Aquaculture is now recognised as a practical and profitable worldwide enterprise, due to the great advances made in the late 20<sup>th</sup> century, improvements in technology, interest in research and commercial support have all aided in the growth of the industry. Aquaculture as a commercial enterprise has shown a particularly rapid expansion in the past fifteen years. During this period, worldwide production figures have increased from 10.2 to 39.4 million metric tons, and currently the industry is worth an estimated 50 billion US dollars (FAO, 2000). The coastal mariculture of molluscs, crustaceans and algae constitute approximately half of the production figures, with the remainder being supplied from fish, eighty eight percent of which are freshwater species. Although in relative terms European aquaculture is responsible for a fairly small proportion of the total global production figures, the importance of this industry is paramount with great emphasis placed on the production of freshwater salmonids, with 230,000 metric tons being produced in 1998 (FAO, 2000).

It has been predicted that the fish farming industry will continue expanding to meet the demand for fishery products, as supply can be controlled more effectively as a result of efficient management, predictable yields and high quality products when compared to marine fisheries. Hardy, (1996) stated that widespread and growing concern about the environmental impact of this industry upon its surrounding aquatic ecosystems has accompanied this industrial expansion on a global level.

## **1.2 The environmental impact of aquaculture**

Industrial aquaculture is practiced throughout the world in various aquatic environments, from open coastal reaches to landlocked lakes and ponds. In addition to the increased competition with other water users, the impact of this industry on its surrounding environment has given rise to a great deal of concern (Phillips *et al.*, 1990). These effects are particularly wide ranging: for example it has been shown that the cultivation of aquaculture species can cause profound changes in aquatic ecosystems (Beveridge and Phillips, 1993; Gowen *et al.*, 1990). The extensive and intensive farming of shellfish and finfish can result in various fluctuations in external environmental conditions including; enrichment of receiving water leading to eutrophication, changes in water quality, disruption of food web structure, chemical usage and various interactions between cultured and wild stock populations (Beveridge *et al.*, 1989). It is those changes, caused by the practice of intensive finfish farming that will be focused upon in this introductory section.

In recent years, recognition of problems created from pollution by fish farm and hatchery discharges have caused widespread concern to develop, particularly the release of phosphorus [P] and nitrogen [N] (Enell, 1987; Meade, 1985). The primary consequence of intensive aquaculture on the surrounding aquatic environment is enrichment and potential to alter the trophic status of the water body by the release of metabolic waste products and uneaten food into the water column. In general, organic waste such as uneaten feed and

faecal matter settles into the sediment, with the water column being the recipient for the soluble waste, urinary and leached feed products. Figure 1, demonstrates this process and also indicates that there may be a proportion of material recycling between the water and sediment fractions.

Any measurable increase in the concentration of a dissolved nutrient is defined as hypereutrophication, and any increase in primary production resulting from hypereutrophication is classed as eutrophication (ICES, 1984). These processes can have a significant impact on bodies of water in which farming operations are situated, unless the system is land based; in these circumstances problems can occur if (for example) the discharge is into a nearby river system. Several physical and chemical changes have been identified as a result of land-based fish farms discharging into such systems. Markmann, (1982) recognised these changes as increased levels of suspended solids, increased concentrations of ammonia, nitrate and phosphate, reduced concentrations of dissolved oxygen and the potential of ammonia toxicity.

In marine waters it is nitrogen in the form of nitrate ( $\text{NO}_3^-$ ) that is the primary limiting nutrient for planktonic growth. In contrast, it is dissolved phosphorus in the form of orthophosphate that is the nutrient responsible for restricting primary growth in freshwater ecosystems (Dugdale, 1967; Wetzel, 1983). Ryther and Dunstan, (1971) stated that consequently these elements, in unnaturally high levels, have been held responsible for the eutrophication of various water systems.

Particular attention has been paid to phosphorus due to its role in the growth of phytoplankton and hence the trophic status of the water body (Schindler *et al.*, 1978). Hypereutrophication can result in an increase in phytoplankton growth and can even change the species composition, so that domination by a particular species could be an indication

of increased nutrients that would normally be limited. This can also lead to increased secondary production, which eventually leads to the disruption of energy through the food chain. Additionally, aquaculture releases dissolved inorganic carbon and nitrogen, which can stimulate the growth of bacterial and heterotrophic organisms (Nishimura, 1982). During extreme algal blooms, particular algae and bacteria produce toxins which can have a devastating effect on other aquatic life. In addition to reduced water clarity, oxygen levels can be reduced (Barlow *et al.*, 1963) which can lead to mass fish mortality (Gowen and Bradbury, 1987; Phillips *et al.*, 1985).

Although the levels of phosphorus and nitrogen released from aquaculture are comparatively small relative to other human activities, discharge from aquaculture is often localised from a point source, into surrounding unpolluted waters resulting in significant impact. In 1982 Penczak *et al.*, (1982) undertook a study to ascertain the waste production from floating cage fish farms in freshwater, and estimated that 0.023kg phosphorus and 0.10kg nitrogen waste was produced for each kg of rainbow trout (*Oncorhynchus mykiss*) biomass. These findings are in agreement with various other studies both in the marine and freshwater environment, although variation can be found in the quantities of waste discharged between various operations. Values of waste production on a small scale i.e. per kg of fish can be scaled-up to predict values for the whole farming operation, which may amount to hundreds of tonnes. Loadings of total phosphorus (P) and ammonium (NH<sub>4</sub>-N) have been calculated as being between 11-16 kg ton fish<sup>-1</sup>annum<sup>-1</sup> for P and between 45-55 kg ton fish<sup>-1</sup>annum<sup>-1</sup> for NH<sub>4</sub>-N (Solbe, 1982; Warrer-Hansen, 1982). Quantifying the amount of waste from a farm is an essential preliminary step in assessing the environmental impact of a specific site. Research studies and the use of nutrient modelling into this area are proving to be valuable tools for the prediction of waste production without the need for individual site assessments.

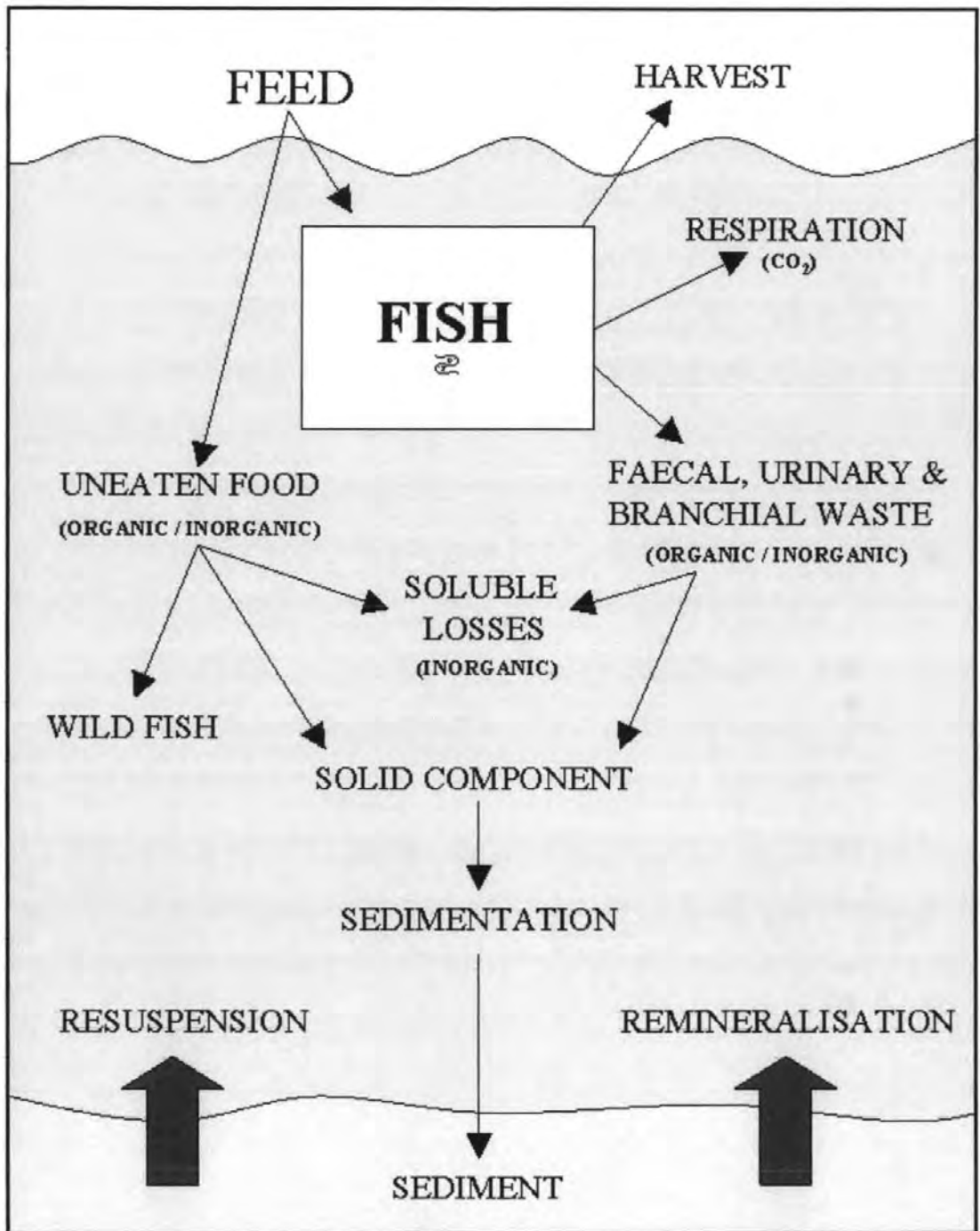


Figure 1.1  
 An illustration of organic and inorganic waste released into aquatic systems from intensive aquaculture (adapted from Gowan 1990).

In order to discharge water from a fish farm, consent has to be provided by the relevant environmental governing body such as the Scottish Environment Protection Agency (SEPA). Such agencies are responsible for the regulation of discharges into controlled waters under the Control of Pollution Act 1974. Discharge consent is given according to various guidelines and is dependent on various factors including the size of the fish farm, location, volume and sensitivity of the receiving water, and type of water body i.e. coastal, inland, still or flowing water. SEPA adheres to a set of environmental quality standards which will determine if the fish farm can freely discharge farm effluent or will be restricted in the levels of nutrients (mainly phosphorus and nitrogen) it can release. As a general rule less than 10µg of phosphorus/L of water is classed as oligotrophic, 10-25µg/L as mesotrophic and more than 25µg/L as eutrophic; it is these high levels that need to be avoided to prevent eutrophication occurring, particularly in freshwater. When water is discharged into a receiving water body that is flowing (i.e. a river), phosphorus levels are restricted to less than 100µg per litre of water. Predictions for nitrogen are less feasible, as it is often difficult to distinguish the source of nitrogen release due to agricultural and sewage outlets, although the release of excess nitrogen into marine waters is more of a problem compared to freshwater areas (SEPA pers. Comm.).

In addition to SEPA the Environment Agency (EA) is the governing body responsible for national discharge regulations nationally in England and Wales. On a wider scale European regulations, particularly those in Nordic countries are considerably more stringent and are seen to lead the way in regulative legislation. In Denmark, freshwater trout farmers have become involved in an environmental battle with bodies such as the Association for the Conservation of Nature, resulting in the implementation of various strict environmental legislations imposed by the Minister of the Environment (Mathiesen, 1991). Danish legislation imposes maximum values for environmental load such as, BOD, suspended solids, total P, ammonium and total N, additionally feed contents are regulated

i.e. gross energy content, level of digestible energy, P and N levels (Iversen, 1995). It has been feared that these regulations may spread and confront the international fish farming industry as a whole (Mathiesen, 1991).

In order to comply with any restrictions imposed on the release of phosphorus from fish farm discharges, considerable attention has been paid to the development of more efficient husbandry practices and careful management strategies that reduce the output of waste materials from intensive aquaculture (Beveridge, 1996; Cripps and Bergheim, 2000; Gowen *et al.*, 1990). In particular, emphasis on efficient farming practices has been placed on trout and salmon production, as a consequence of these fish being the predominant species farmed in Europe. Consequently, fish feed manufacturers with the aid of scientific research are trying to ascertain the optimal levels of dietary phosphorus that sustain high growth rates and health of the fish whilst minimising phosphorus excretion. Therefore, nutritionists are currently investigating the optimum formulation of aquafeeds and selection of feed ingredients that supply a high phosphorus bioavailability in order to supplement the diets with low levels of total phosphorus (Lall, 1991). This is particularly well developed for salmonid aquaculture operations where more detailed knowledge of their nutrition is fully established.

### **1.3 An overview of the nutritional requirements of intensively reared Salmonids**

As a consequence of the demand for aquaculture products the evolution of fish farming has moved towards the need for higher yields and faster growth with maximum profitability. In order to support these trends, fish have become less dependant on natural food sources, and more dependant on prepared feeds. Modern intensive aquaculture is therefore totally dependant on quality formulated feeds. In 1991, Lovell declared that it is necessary to develop balanced diets for most fish species based on optimum protein, energy content and ratio with an adequate supplement of vitamins and minerals.

The nutritional requirements of fish do not vary greatly between species and can usually be applied to the following broad categories; i.e:- marine or freshwater species found in either warm or coldwater conditions. Fish, like terrestrial farm animals require specified levels of nutrients for general good health, growth, reproduction, and to maintain all other physiological functions, although there are notable nutritional differences between fish and warm blooded land animals (De Silva and Anderson, 1995).

This introductory section will focus primarily on the nutrition of salmonids mainly relating to Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*). As a consequence of their poikilothermic metabolism, fish possess relatively low energy requirements, and as a result demand a relatively higher protein to energy ratio in their diet. Additionally, salmonid fish require particular lipid components in their diets, and these are mainly polyunsaturated or highly unsaturated types. The n-6 to n-3 series of fatty acids is essential for various physiological functions and structures, principally the lipid bilayer of plasma membranes, and as fish are unable to synthesise these fatty acids *de novo* they must be supplied in the diet. Essential fatty acid (EFA) requirements have been estimated for rainbow trout with the omega n-3 series fatty acids being required at a level between 0.8-1.7% of a diet that contains 20% lipid (Castell *et al.*, 1972; Watanabe *et al.*, 1974). However a general dietary inclusion level of 1 to 2% is used as a standard in order to prevent essential fatty acid deficiency signs (Watanabe, 1982). The requirement for (n-6) EFA has not been fully identified in rainbow trout. Consequently Cowey, (1992) stated that the need for this EFA cannot be discounted. Practical trout diets used to contain approximately 10-15% crude fats to supply adequate energy and concentrations of (n-3) and (n-6) fatty acids (Hilton and Slinger, 1981), a 4 to 5% dietary inclusion of marine fish oil will provide adequate EFA levels although cod liver oil is often used for experimental diets. In recent years, a trend has developed to include higher lipid levels and very high energy, some diets containing up to 30% oil, but common levels are lower at



approximately 15% to 20%. In 1994, Kaushik and Medale concluded that such increases in dietary oil were thought to be beneficial to salmonid fish by providing a higher level of metabolisable energy (ME), as lipid based energy is less expensive than protein.

Therefore, protein deposition is increased and the overall dietary protein requirement reduced. This theory was previously demonstrated during research conducted using turbot (*Scophthalmus maximus*) (Bromley, 1980). Carbohydrate is fairly cost effective as an energy source in aquafeeds, however, energy from this source is inadequate for salmonid fish due to problems associated with digestibility of starch and glucose assimilation.

In addition, all fish require a complete range of vitamins in the diet which are essential for maintaining health and promoting growth. These include the water-soluble B vitamin groups and ascorbic acid together with vitamins A, D, E, and K to meet the health criteria and optimum efficiency for growth and production.

The formulation of salmonid diets depends primarily on the size of the fish, stage of production and target growth rates. The most abundant component of a practical fish feed is protein, the minimum dietary protein level for optimal growth is approximately 45% to 50% for newly hatched fry, 40% to 45% for larger fry, and 35% to 40% for fingerlings up to harvesting size (Hilton and Slinger, 1981). Salmonids are able to synthesise the majority of amino acids needed for protein turnover, but approximately 10 amino acids are deemed essential and must be present in the diet. The requirements for protein and amino acids in fish have been extensively reviewed by Wilson, (1994). Diets that are formulated using high quality fish meal, generally contain sufficient levels of essential minerals to satisfy salmonid growth, but many alternative dietary constituents such as plant based protein sources and high-ash fish meals possess anti-nutritional factors (ANF's) that can reduce the availability of certain cations, particularly zinc. As a consequence, practical feeds are always supplemented with vitamin and mineral premixes, that can be varied

depending on the predominant protein source of the diet and the predicted requirement of the fish species.

Most salmonid feeds are produced in a dry form ranging from granulated diets for juvenile fish to pellets of different sizes suited to each phase of production. Feeds are either manufactured by compression steam pelleting (cold pressed) or as slow sinking expanded diets by steam pressure (extrusion) (Hilton *et al.*, 1981). Feed formulations for fish are high in protein and oil, which generally precludes the use of many feed ingredients such as corn and wheat used in terrestrial farming situations. Diets would be nutrient poor and have a high bulk density if formulated with cereal based materials. Salmonid feeds are therefore formulated using a selected range of raw materials; these include fishmeals, oilseed meals and grain milling by-products, but some of these ingredients have restricted inclusion levels. Soyabean meal may be unpalatable to salmonids especially salmon at high inclusion levels (>20%), due to a bitter or lack of flavour particularly if the soyabean meal is not extracted by alcohol. Similarly, Hilton and Slinger, (1986) observed that high dietary levels of canola meal (15%) are related to thyroid dysfunction in rainbow trout (*O. mykiss*). Traditionally, other protein supplements such as meat and poultry by-product meal, blood meal and feather meal were utilised in fish feeds (Bureau *et al.*, 2000; Bureau *et al.*, 1999; Burel *et al.*, 2000). In recent years, the possible link of inter species disease risks such as BSE and related consumer concerns have negated the use of such protein sources. With the rapidly increasing demand for aquaculture feeds and the production of fishmeal and oils becoming a limited resource, there is pressure for cost effective, renewable alternative protein sources to fulfil the nutritional requirements of most farmed fish (Tacon, 1998). The inclusion of trace element and mineral supplements is also an important cost factor (constituting 3-5% of the overall cost approximately £30/metric ton) and relevant to modern feed production for salmonids. Consequently, scientific research is now turning to these issues.

#### **1.4 Protein metabolism and additional dietary supplements**

Protein forms the largest portion of any standard fish feed, amino acids being the structural component of protein are therefore abundant in the diets of fish (Murai, 1992). Amino acid availability varies with protein source, for example certain plant protein sources have poor apparent digestibility coefficients, resulting in low amino acid uptake levels. It is usually lysine and methionine that are the most likely to have reduced digestibilities when plant protein sources such as soya bean meal are used as the protein base for the diet. Therefore, additional amino acid supplements may be necessary depending on diet formulation and source. Amino acids are divided into essential and nonessential classes, essential amino acids being those that cannot be synthesised at all or in high enough quantities by the animal, whereas nonessential amino acids are those that can be naturally synthesised by the animal. Ten essential amino acids (EAA's) are deemed to be essential in the diets of all fish species i.e:- arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. A critical appraisal for the amino acid requirements of fish was presented by Cowey, (1994).

Research relating amino acid metabolism to mineral nutrition is very limited, but information has been published linking the metabolism of phosphorus to the amino acid phenylalanine. The L-isomer of phenylalanine has been known specifically to inhibit the activity of intestinal alkaline phosphatase, and has consequently been linked to cholecalciferol (Vitamin D<sub>3</sub>). However, both the L and D-isomers were found to increase tissue uptake and storage of phosphate in the intestinal tract of chicks which had been fed on rachitogenic diets and treated with cholecalciferol (Taylor, 1974). Additionally, Holdsworth, (1970) found that phenylalanine increased the uptake of calcium when included in the incubation buffer for everted chick ileums. This amino acid can function as a calcium chelator and therefore may play an important role in calcium transportation in avian and other species.

An important investigation relating dietary supplements to mineral availability was recently undertaken by Sugiura *et al.* (1998), in which a preliminary trial was conducted to establish if a range of feed supplements had the potential to improve dietary mineral availability. The range of supplements included; citric acid, sodium citrate, potassium chloride, sodium chloride, histamine dihydrochloride, EDTA disodium salt, sodium bicarbonate, ascorbic acid, inositol and choline, and cholecalciferol. The apparent availability of a whole range of minerals was measured including phosphorus, calcium and magnesium. It was determined that citric acid increased the apparent availability of the aforementioned three minerals, especially in the monogastric rainbow trout, with phosphorus faecal levels being greatly reduced. Additionally, Vielma *et al.* (1999) demonstrated that additional dietary citric acid can result in an increase in whole-body ash levels, suggesting improved utilisation of phosphorus.

### **1.5 The relevance of Vitamins C and D to mineral nutrition**

An important component of a nutritionally balanced feed for fish is the inclusion of specific vitamins. Vitamins are organic compounds that are required in relatively small levels and are involved in various metabolic processes, to some animals a certain vitamin maybe essential but not necessarily to other species. They maybe classified into either water soluble (B complex and C) or fat soluble types (A, D and K) (Halver, 1982; NRC, 1993). Although these micronutrients are required in very small quantities, deficiencies can result in a range of symptoms, such as poor growth and appetite suppression to severe tissue deformities and subclinical pathologies. Salmonid fish require fifteen different vitamins (NRC, 1993), but levels can vary with various biotic and abiotic conditions, in addition to problems relating to the loss of vitamin potency due to methods of feed processing and storage. Although all vitamins described are vital for the health of salmonid fish, two micronutrients (vitamins C and D) in particular were directly relevant to this programme of research and are reviewed in further detail.

Vitamin C or ascorbic acid is a water-soluble vitamin, occasionally referred to a macronutrient, being required at 100mg/Kg of diet (Halver, 1982; Halver, 1989) for salmonid fish. Unlike animals, many fish are unable to produce vitamin C because they lack the enzyme L-gulonolactone oxidase which is responsible for the synthesis of this vitamin from glucose (Hardie *et al.*, 1991). Vitamin C exists in two forms, the reduced form is ascorbic acid and the oxidised form dehydroascorbic acid, both of which are biologically reversible (Figure. 1.2).

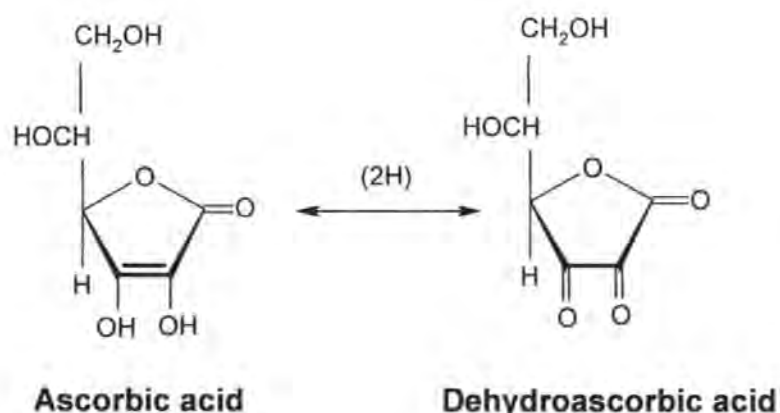
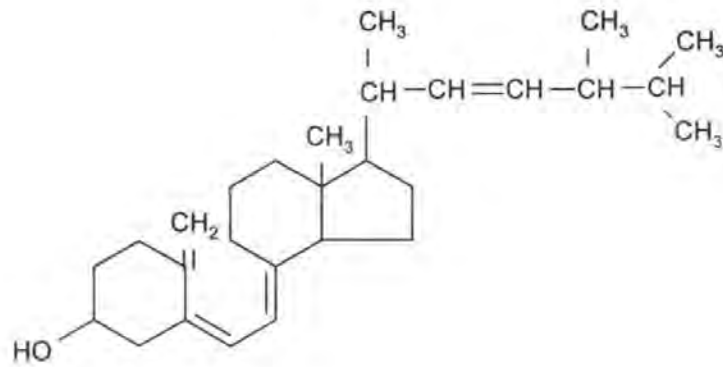


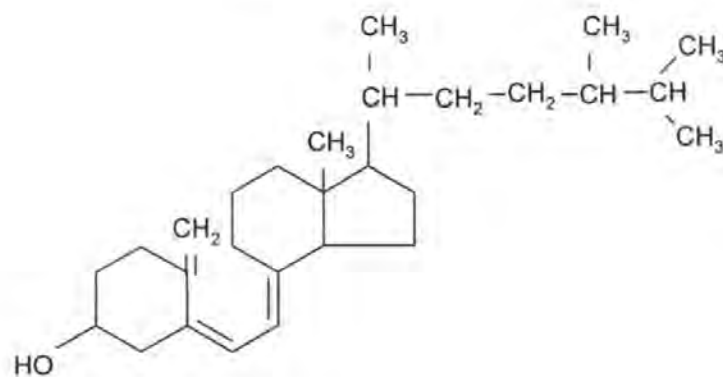
Figure 1.2 The two forms of vitamin C (ascorbic acid).

The predominant physiological role that vitamin C plays is as a strong reducing agent, and is involved in the hydroxylation of proline and lysine to the hydroxy-amino acids that are essential for the conversion of procollagen to collagen. Consequently, any fish deformities that involve a tissue of which collagen is a component, such as bone, cartilage, fins and skin, are usually linked to a deficiency of vitamin C. One of the most noticeable physical deformities resulting from a lack of vitamin C is curvature of the spinal column. Scoliosis is a lateral curvature of the spine and lordosis a vertical curvature. These conditions have been induced in rainbow trout (*Oncorhynchus mykiss*), salmon (*Salmo salar*), tilapia (*Oreochromis niloticus*), catfish (*Ictalurus punctatus*) and carp (*Cyprinus carpio*) by the

feeding of vitamin C-deficient diets (Halver *et al.*, 1969) (Lovell, 1991) (Madsen and Dalsgaard, 1999). In addition to skeletal deformities, Lim and Lovell, (1978) established that a deficiency in vitamin C can also lead to internal haemorrhaging, fin erosion, distorted gill filament cartilage, reduced resistance to bacterial infection and slow wound healing in channel catfish (*Ictalurus punctatus*). In 1969, Halver *et al.* demonstrated that rainbow trout can present deformed gill operculum and abnormal histology of support cartilage in the eye and gill when dietary ascorbic acid levels are low. Therefore, it can be concluded that vitamin C forms an essential dietary component and should be provided at an optimum level to maintain fish health.



Vitamin D<sub>2</sub>



Vitamin D<sub>3</sub>

Figure 1.3 The two biologically active forms of vitamin D.

Vitamin D is naturally found in two forms, ergocalciferol known as D<sub>2</sub> and cholecalciferol – D<sub>3</sub> (Figure 1.3). Fish have the ability to utilise cholecalciferol more readily than the alternative ergocalciferol form. Although research into the metabolic role of Vitamin D in fish is fairly limited, recent papers have indicated that channel catfish (*I. punctatus*) and rainbow trout (*O. mykiss*) have a definitive dietary requirement for vitamin D.

Due to the insufficient level of research involving the role of Vitamin D in fish physiology, it has been assumed that this vitamin plays a similar role to that known in warm-blooded animals. The general function of vitamin D in mammalian physiology is to elevate plasma calcium and phosphorus to a level that will support normal mineralization of bone (DeLuca, 1979). Additionally, vitamin D, in conjunction with the functions of the parathyroid hormone (PTH), is responsible for homeostatic control of plasma calcium and phosphorus. Fish do not have a distinct parathyroid tissue and so are devoid of PTH, so an equivalent hormone thought to be calcitonin and/or stanniocalcin that act as the PTH equivalent. In mammalian physiology PTH stimulates the conversion of vitamin D to its active form (cholecalciferol) during hypocalcemic conditions, which in turn stimulates the specific pump mechanisms in the intestine, bone and kidney to increase plasma calcium and phosphorus levels. Active transport of calcium across the intestinal epithelium is increased (DeLuca and Schones, 1976), calcium and phosphorus are mobilised from the bone fluid compartment to the plasma (Garabedian *et al.*, 1974), and calcium reabsorption is improved in the distal renal tubules (Steele *et al.*, 1975). In 1975, Hughes *et al.* reported that when blood phosphorus levels are low, cholecalciferol production is stimulated and levels of ionic calcium are increased suppressing the parathyroid gland. Lack of parathyroid hormone increases phosphorus retention by the kidney, and as cholecalciferol stimulates both phosphorus metabolism from the bone (Castillo *et al.*, 1975) and increases intestinal absorption (Chen *et al.*, 1974), plasma phosphorus levels become elevated to their normal levels.

The physiological effects of the active form of vitamin D cholecalciferol have led to limited research in fish nutrition, although the existing research has indicated that cholecalciferol has a positive effect on the mineral dynamics in fish. Skonberg *et al.*, (1996) established that the addition of cholecalciferol to rainbow trout diets decreased magnesium concentrations but increased phosphorus and calcium concentrations in whole body, vertebrae and skin. Channel catfish (*I. punctatus*) also require a dietary source of vitamin D for optimal growth and bone mineralisation (Lovell and Li, 1978). Further research has indicated that this vitamin can stimulate intestinal calcium ion uptake in both the American eel (*Anguilla rostrata*) and goldfish (*Carassius auratus*) (Fenwick, (1994) Fenwick *et al.*, 1994). However, this finding could not be repeated by Avila *et al.*, (1999) for rainbow trout, which was attributed to the already high metabolic phosphorus status of the fish. Dietary vitamin D<sub>3</sub> has also been investigated in conjunction with a supplementation of the enzyme phytase to the diet in poultry (Mohammed *et al.*, 1991; Qian *et al.*, 1997), in addition to salmonid fish. However cholecalciferol appeared to have no effect on either phosphorus utilisation or phytase action in rainbow trout (Vielma *et al.*, 1998). The scientific literature to date indicates that the role that cholecalciferol plays in fish nutrition is still equivocal and undefined needing further research for clarification.

## **1.6 Mineral requirements and nutrition**

The requirement for inorganic minerals by fish have been divided into two categories, those needed in large quantities are termed macro minerals. These include calcium, phosphorus, magnesium, sodium, potassium, chlorine, and sulphur (Cowey, 1992; Hilton and Slinger, 1981; Shearer, 1988; Shearer, 1989; Shearer *et al.*, 1994). Trace minerals are required only at low levels and include iron, zinc, iodine, manganese, copper, cobalt, selenium, molybdenum, fluorine, aluminium, nickel, vanadium, silicon, tin and chromium (Lorentzen *et al.*, 1996). The present programme of research addresses the macro element



phosphorus, calcium, magnesium and zinc. These four minerals and particularly phosphorus, are featured in this chapter and are central to future investigations.

Research into mineral metabolism is fairly limited although a number of reviews have been published. Probably the most comprehensive report on phosphorus concerns the digestibility, metabolism and excretion of dietary phosphorus in fish by Lall, (1991). This section will focus on the nutritional and dietary aspects of phosphorus whilst the physiological aspects shall be discussed later. There are several problems encountered during experimental mineral nutrition involving fish that can impede quantification and general outcome of the work. The predominant ones include the formulation of palatable mineral free diets, assessing and overcoming tissue storage of minerals and the absorption of minerals from the water. The latter process can give rise to negative apparent digestibility coefficients.

Dietary minerals are required in quantities that can maintain and sustain optimum growth, tissue formation, feed utilisation and various metabolic processes. As fish are able to absorb minerals from their surrounding aquatic environment, it is only necessary to ensure that dissolved minerals that are in negligible or low levels in natural waters are supplied in a dietary form. Phosphorus is the most important mineral that needs to be added to feeds, due to the lack of phosphorus ions in freshwater (Hilton, 1989) and the high metabolic requirement for this mineral. Salmonid fish have a typical dietary available phosphorus requirement ranging from 0.5 to 0.8% of the diet. In 1978, Ogino and Takeda reported levels of 0.7–0.8% for rainbow trout, 0.61% was reported by Ketola and Richmond in 1994, with 0.5–0.6% for chum salmon (*Onchorynchus keta*) by Watanabe *et al.*, in 1980. It has been suggested that phosphorus requirements should be assessed in relation to energy requirement, as different sizes of fish will need varying levels of minerals depending on varying growth phase. A dietary concentration of 0.25g available phosphorus per MJ

digestible energy was recommended for rainbow trout by Rodehutsord in 1995. Other fish species comply with these estimates and are shown in Table 1.1.

Table 1.1.  
Recommended phosphorus dietary inclusion levels for non-salmonid fish species.

Fish Species	% Phosphorus	Source
Sunshine bass ( <i>Morone chrysops</i> X <i>M.saxatilis</i> )	0.3-0.4	Brown <i>et al.</i> , (1993)
Channel catfish ( <i>Ictalurus punctatus</i> )	0.3-0.4	Eya and Lovell, (1997)
	0.8	Wilson <i>et al.</i> , (1982) Lovell, (1978)
American cichlid ( <i>Cichlasoma urophthalmus</i> )	0.15	Chavez-Sanchez <i>et al.</i> , (2000)
Common carp ( <i>Cyprinus carpio</i> )	0.6-0.7	Ogino and Takeda, (1976)
Blue tilapia ( <i>Oreochromis aureus</i> )	0.5	Robinson <i>et al.</i> , (1987)
Striped bass ( <i>Morone saxatilis</i> )	0.58	Dougall <i>et al.</i> , (1996)

Calcium requirements have been determined for fish to a lesser extent (NRC, 1993), although a range between 0.3 to 1.00% similar to that of phosphorus has been reported for several species of fish (Arai *et al.*, 1975; Hossain and Furuichi, 1998; Robinson *et al.*, 1987; Robinson *et al.*, 1986; Robinson *et al.*, 1984). Magnesium is generally required at levels of 4g/kg and zinc at some 20-50mg/kg, although much higher levels are usually supplemented due to the antagonistic effects of other dietary components (Lovell, 1989; Maage *et al.*, 2001).

Typical signs of phosphorus deficiency in most fish include poor growth, feed efficiency (FCR), specific growth rates (SGR) and inadequate bone mineralisation and mortality. In carp (*Cyprinus carpio*) low phosphorus levels can also lead to increased carcass fat content, skeletal deformities, reduced blood phosphate, increased gluconeogenic enzyme activity according to Ogino and Takeda, (1976). In 1980, Sakamoto and Yone established that low phosphorus intake in sea bream (*Pagrus major*) leads to malformed enlarged vertebrae, reduced liver glycogen content, increased serum alkaline phosphatase and high lipid deposition in the muscle, liver and vertebrae. Lethargy, reduced growth, anorexia and dark colouration were noted in rainbow trout after 5 weeks on a phosphorus deficient diet in 1991 by Hardy *et al.* More recently, Baeverfjord *et al.*, (1998) fed Atlantic salmon diets that were quite deficient in phosphorus (0.35% P), which resulted in inducing a pathological condition of abnormally soft malformed bones including spinal scoliosis. Calcium deficiency leads to poor growth and reduced ash content of bones, whereas lack of magnesium results in poor growth, lethargy, flaccid muscles, cell degeneration, vertebral curvature and mortality in channel catfish (Lovell, 1989). Gatlin and Wilson, (1983) showed that zinc deficiency in channel catfish induces poor growth, lack of appetite, reduced levels of bone zinc and calcium. In the rainbow trout (*O. mykiss*) a zinc deficiency characteristically produces lens cataracts in addition to a number of pathologies, which was demonstrated by research conducted by Ketola in 1979.

Phosphorus is found in many feed ingredients, but levels differ between feedstuffs and also with respect to source and degree of processing. Additionally, the bioavailability of this mineral to fish varies, resulting in the need for inorganic supplementation in sources that have low phosphorus levels and/or availability via trace element premixes. Feedstuffs of animal origin generally contain the highest concentration of phosphorus, particularly meat and bone meal, but these products are now excluded from European fish feeds due to recent legislation. Fishmeal typically contains between 1.5-4.5% phosphorus, for example

high quality herring fishmeal made from whole fish contains a fairly low 1.82% phosphorus, whereas fishmeal comprised from filleted remains and offal contains a higher 4.17% of phosphorus due to the large bone component of the meal. These fish meals contain high levels of ash, and have low phosphorus digestibility especially if the meal has not been finely ground (Pike *et al.*, 1990; Rodehutsord *et al.*, 2000a). Sugiura *et al.*, (2000) suggested this low digestibility may be due to the inorganic mineral content of the bone fraction of the meal. In fishmeals the majority of minerals are found in an inorganic form chiefly as phosphorus complexes of protein, lipid and carbohydrate (Lall, 1991), which are fairly available to fish from the gastro intestinal tract. On the other hand, phosphorus in plant protein sources is present in lower concentrations ranging from 0.3-1.5%, but this phosphorus is stored as phytate which is largely unavailable to fish and all monogastric animals as they do not possess the enzyme phytase responsible for hydrolysing this compound.

Table 1.2  
The biological availability of phosphorus from selected feed ingredients for salmonid fish. Adapted from (Lall, 1991; Ogino *et al.*, 1979).

Feed Source	% Bioavailability (apparent digestibility)
Casein	91
Blood meal	81
Feather meal	77
Poultry by-product meal	81
Herring meal	52
Whitefish meal	79
Wheat	58
Soybean meal	36

It is evident that the bioavailability of phosphorus is variable between feed ingredients and inorganic mineral supplements, that can be influenced by chemical form, particle size, feed processing, digestibility and interactions with other dietary components (Lall, 1991; Nordrum *et al.*, 1997). Table 1.2 shows bioavailability of phosphorus from selected feed ingredients used in aquafeed formulations. Different fish species can utilise phosphorus from fish meals to varying extents, for example, rainbow trout and salmon have a higher utilisation capacity of phosphorus when compared to carp (*C. carpio*) (Yone and Toshima, 1979) and tilapia (*O. niloticus*) (Watanabe, 1980). This may be linked to the limited gastric secretion by warm water species as suggested by Ogino *et al.*, (1979).

Where inorganic supplements are concerned, the more soluble the salt in neutral ammonium citrate the more available the phosphorus to the fish, a method that was utilised by Li *et al.*, (1996) to evaluate the efficacy of defluorinated phosphates as dietary phosphorus sources for channel catfish (*I. punctatus*). Generally tri-, di- then monobasic phosphates are the sequence in which the phosphorus moiety is better utilised in fish (Ogino *et al.*, 1979) with respective availabilities of 56%, 72% and 90% (Lall, 1991). These findings were also confirmed for rainbow trout by Rodehutschord *et al.*, (2000a). It was also established that dicalcium phosphate and defluorinated phosphates are equally good at providing an adequate phosphorus source for channel catfish (*I. Punctatus*) by Li *et al.*, (1996) and Robinson *et al.*, (1996). In 1993, Satoh *et al.* discovered that excessive dietary phosphorus induces poor growth in rainbow trout, and that the phosphorus portion of tricalcium phosphate may inhibit zinc availability in addition to the calcium to phosphorus ratio. A later study by Porn-Ngam *et al.*, (1993) using rainbow trout suggests that the optimum dietary Ca:P ratio needed to avoid any negative effects of excess phosphorus is 1:1. Generally, diets that contain plant proteins have to be supplemented with higher inorganic mineral supplement levels, due to the fact that the plant phosphorus is chiefly unavailable for the reasons described previously.

It is necessary to provide an adequate mineral level in the diet from a combination of raw ingredients and/or inorganic supplements that will maintain optimal growth and health, whilst at the same time overcoming any anti-nutritional factors, ingredient interactions, and attempting to minimise excretory mineral losses.

In addition to the mineral component of the feed, there are various other dietary factors that influence mineral metabolism, those that can be related to the minerals concentrated on in this thesis are discussed in the following sections.

### **1.7 The physiological role of minerals in salmonids**

Phosphorus is an element essential for life, and is the second most abundant mineral after calcium in the bodies of bony fish. Phosphorus is found incorporated into various compounds, molecules and ions as the free form is not found in the body. Approximately 86-88% of the total body phosphorus is found in fish bones where it exists as tricalcium phosphate and hydroxyapatite (Lall, 1991), and along with calcium develops and maintains the skeletal system. The remaining phosphorus pool functions in a variety of important physiological processes, usually in the form of an organic phosphate and is found in cells and extracellular fluids. Phosphorus is a major constituent of adenosine triphosphate (ATP) and is therefore an essential factor in all energy-producing cellular reactions. Phosphorus is also an important component of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), various co-enzymes, and phospholipids, which are involved in the transportation of molecules across cell and subcellular membranes. Phosphate is also responsible for buffering body fluids to maintain normal pH as a function of homeostatic processes.

Studies involving the absorption and transport of phosphorus in fish are very limited, it has been speculated that intestinal absorption is similar to sodium transport, where a gradient

induces the movement of phosphate into cells of the gut brush border (Murer and Hildmann, 1981). Although it should be noted that only data from carp and eels (*Anguilla anguilla*) established from research conducted by Nakamura, (1985a) lend support to this theory. In higher vertebrates, cholecalciferol (vitamin D<sub>3</sub>) is known to play a role in stimulating intestinal Ca<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup> absorption (Murer and Hildmann, 1981). In 1961, Harrison and Harrison, ascertained that in rats, phosphate transport across the intestinal wall is activated by calcium and potassium and that vitamin D<sub>3</sub> can enhance the cells permeability to calcium, hence increasing phosphate transport. Exposure to cholecalciferol rapidly translocated <sup>32</sup>P across all segments of the small intestine (duodenum, jejunum, ileum) in chicks (Wasserman and Taylor, 1973), suggesting a direct enhancement of phosphorus uptake.

The most abundant mineral found in the bodies of bony fish is calcium, with over 98% of it being found in skeletal tissues and scales, these structures are thought to provide a calcium reservoir from which this ion can be reabsorbed when needed for other physiological functions (Simkiss, 1974). Mineral reabsorption, particularly from scales has been reported during starvation (Ichikawa, 1953), sexual maturation (Mugiya and Watabe, 1977; Ouchi *et al.*, 1972), and by the feeding of rachitogenic diets (Wallin, 1957). Carragher and Sumpter, (1991) established that it is the scales and not the bone of rainbow trout that act as the main reservoir from which calcium is drawn in times of high demand. It is thought that bone may act as a reservoir for phosphorus alone and not calcium, because levels of blood calcium are similar in both bony and cartilaginous fish, whereas blood phosphate levels are much higher in bony fish as cited by Lall, (1991). Calcium also plays an essential role in muscle functioning, blood clotting, nerve impulse transmission, as a cofactor for various enzymes and is a fundamental ion involved in osmoregulatory control (Lovell, 1989).

The regulation of ionic calcium is mandatory for all vertebrates, as only small changes in calcium levels can have pronounced effects on many physiological processes. For instance, hypocalcemia can result in tetany and seizures, whereas hypercalcemia induces myocardial dysfunction and lethargy. There are two systems by which fish regulate their plasma calcium levels; an 'open' system where calcium mainly enters and leaves the body via the fins and gills, some calcium is absorbed across the intestine, and the kidney has some control over calcium loss. In a 'closed' system, calcium is stored in the skeletal structures and is continually recycled to maintain a steady state, bony fish are thought to operate both systems (Dacke, 1979). Control of these homeostatic calcium systems is hormonal and is quite different from terrestrial vertebrates, as fish do not possess parathyroid glands, which in higher vertebrates dominates the control of extracellular calcium (Wendelaar Bonga, 1991). The pituitary gland is thought to be the source of the hypercalcemic hormone prolactin, calcitonin is involved in calcium regulation along with a uniquely produced hypocalcemic hormone called stanniocalcin (Wendelaar Bonga, 1991), the role and function of these hormones will be discussed in a later section.

Magnesium is predominantly found in the hard tissues of bony fish, but also plays an important role as an enzyme activator in carbohydrate metabolism and protein synthesis. Additionally, magnesium is necessary in body fluids to maintain the integrity of smooth muscle. The predominant role of zinc in fish physiology is in various enzyme systems acting as a cofactor to carbonic anhydrase in red blood cells, enzymes involved in protein digestion, and enzymes in carbohydrate catabolism. Furthermore, zinc is a key component of insulin and helps to prevent keratinisation of epithelial tissues.

### **1.8 Skeletal structure, composition and phosphorus.**

Phosphorus is a macro-mineral essential for general health and growth of fish, the above section (1.7) introduces the importance of this element and its role in maintaining the functioning of various physiological systems. Phosphorus in association with calcium



form fundamental components of skeletal material. The endoskeleton of higher animals serves two major purposes; (1) to provide structural support and protection to soft tissue organs and (2) to serve as an ion reservoir for calcium and phosphorus homeostasis (Zerwekh, 1992).

Comparisons between fish and other vertebrates have shown that fishes have more types of supportive tissue and more intermediate tissue between cartilage and bone, including cartilage, bone, notochord and muscle (Harder, 1975 as cited in Yasutake and Wales, 1983). In 1961, Moss defined two types of fish bone, acellular and cellular, the difference being the lack of osteocytes in acellular bone.

Mineralised bone in teleost fish is composed of an organic matrix (25%) and inorganic salts (75%), and is typically made of three components: 1). Bone cells, which are osteoblasts, osteocytes and osteoclasts; 2). The organic matrix, which is composed primarily of collagenous fibres, and an amorphous ground substance of phospho- and glyco- proteins into which the collagen fibres calcify when embedded; 3). The inorganic mineral matrix of calcium phosphates or hydroxyapatite (HAP)  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ . HAP has an extremely complicated structure (Hamada *et al.*, 1995), and other additional minerals can be found in its complex for example carbonate, citrate, sodium and magnesium are found in substantial amounts, with iron, zinc, copper, lead, manganese, tin, aluminium, strontium, boron and silicon being found in trace levels (Zerwekh, 1992). Hamada *et al.*, (1995) examined the hydroxyapatite bone ash from fifteen species of fish by both X-ray diffraction and elemental analysis, the only freshwater teleost examined in detail was carp. Findings showed that carp had a HAP crystalline structure and contained 35.6% Ca, 16.9% P, 0.61% Mg and 280ppm Zn. The molar ratio of Ca to P was 1.63. This is slightly lower than the theoretical value of 1.67, indicating that a small proportion of the Ca was replaced by another element, probably magnesium.

Literature on fish bone tissue particularly the histological definition is limited, with the majority of published work being on mammalian vertebrates, this leads to many fundamental problems when examining the skeleton of lower vertebrates. Higher vertebrates, including humans possess the Haversian organisation of bone, which is very specialised and not encountered in the lower vertebrates, whereas many living fish have bone without osteocytes.

Osteocytes are the most common bone cells and are characteristically star shaped. They are entrapped in the bone matrix as osteoblasts during the synthesis of new bone tissue. Osteocytes show varying features depending on fish species, for example in carp they are found frequently and are regularly distributed, but in Thunnidae they are scarce and found irregularly (Meunier and Huysseuene, 1992), but in many fish the bone lacks osteocytes and has been termed acellular bone. In teleost fish, cellular bone is found in the majority of lower fish groups, such as salmonids whereas acellular bone has developed in advanced species. All non-teleost fish have cellular bone. This oversimplified scheme relates to the endoskeleton, with the exoskeleton or the scales and fin rays being the exception, acellularisation is a more general feature in scales than in normal bone, for example Salmonidae and Cyprinidae have cellular bone and acellular scales (Meunier and Huysseuene, 1992).

As discussed in section 1.7 endo- and exoskeletal structure are thought to act as a reservoir for calcium and phosphorus in times of high metabolic demand. Bone resorption has been demonstrated in tilapia (*O. niloticus*) acellular bone by Witten in 1997, using the enzymes tartrate-resistant acid phosphatase and alkaline phosphatase as markers for osteoclasts (bone resorption cells) and osteoblasts (bone forming cells). In the rainbow trout, oestradiol-17 $\beta$ -induced calcium mobilisation from scales has been displayed due to increased osteoclast activity (Persson *et al.*, 1994). Both mono- and multinucleated

osteoclasts are known to resorb the calcified layer and the fibrillary plate of scales (Bigler, 1989; Ouchi *et al.*, 1972), and they appear similar to terrestrial vertebrate osteoclasts. It has been reported by Yates *et al.*, (1991) that in higher vertebrates and humans, high inorganic concentrations of phosphorus inhibit both the production of new osteoclasts but also inhibit bone resorption by mature osteoclasts. Conversely, Ten-lin *et al.*, (1974) stated that high dietary phosphorus stimulates bone resorption in adult animals. Excess phosphorus entering the bloodstream causes a depression in plasma calcium, which stimulates parathyroid hormone secretion. This hormone acts to restore plasma calcium levels by stimulating bone resorption and the cessation of alkaline phosphatase activity (a marker of bone formation), although the effect of excess phosphorus is not known in relation to fish bone turnover. In addition to hormones, Vitamin D (previously mentioned in section 1.5) is known to play an important role on the skeleton when activated to cholecalciferol.

Vertebral skeletal deformities such as scoliosis and lordosis in adult fish may occur under natural conditions, but over the last decade there has been an increased number of such deformities in cultured fish species (Franceson *et al.*, 1988; Santamaria *et al.*, 1994). To date there are several explanations as to why these spinal malformations occur, it has been shown that heavy metal pollutants (Bengtsson *et al.*, 1988; Hodson *et al.*, 1980), industrial waste (Neilson *et al.*, 1984), pesticides (Haya, 1989) and hereditary factors such as inbreeding (McCrimmon and Bidgood, 1965; Poynton, 1987) can all be responsible for spinal deformities in teleost fish. The bacteria *Flavobacterium psychrophilum* causes a disease known as rainbow trout fry syndrome which when treated with oxytetracycline is linked to spinal fractures (Lorenzen *et al.*, 1997). Additionally, previously infected fry show ataxia, spiral swimming and spinal deformities in later life as reported by Kent *et al.*, (1989) for salmonid fish. However, Madsen and Dalsgaard, (1999) established that feeding diets supplemented with glucans have been found to stimulate the immune response and hence increase the disease resistance of fish.

Diet deficiencies that lead to connective tissue alterations are thought to be associated with spinal malformations in fish (Weis and Weis, 1989). There are two dietary components that when fed to fish in insufficient levels have led to the development of connective tissue deformities, they are ascorbic acid and tryptophan (Hodson *et al.*, 1980; Lovell, 1989; Walton *et al.*, 1984). Tryptophan is utilised in the formation of collagen, hence low levels of this amino acid are thought to limit the formation of tropocollagen molecules.

Additionally, low levels of ascorbic acid lead to a decreased level of hydroxyproline which impairs collagen synthesis for connective tissue production. Therefore, vertebral columns that lack collagen between vertebrae are less flexible and become rigid, leading to a curvature (scoliosis and lordosis) or a shortened, 'stunted,' appearance to the spine. Such physical deformities resulting from in ascorbic acid deficiency have also been noted in coho salmon (*Oncorhynchus kisutch*), rainbow trout (Halver *et al.*, 1969; Madsen and Dalsgaard, 1999) and channel catfish (Lovell, 1991; Lovell, 1989). As most species of fish do not have the ability to synthesise ascorbic acid, it is essential that diets be supplied with sufficient levels to maintain connective tissue integrity and healthy bone growth.

### **1.9 Hormonal regulation of calcium and phosphorus.**

As stated in section 1.6, there is a strong synergistic relationship between calcium and phosphorus under many physiological conditions. In teleost fish there are two predominant hormones involved to a certain extent with the homeostatic control of calcium and phosphorus. The principal hormone is Stanniocalcin (STC), which has previously been referred to as hypocalcin by Pang *et al.*, (1974), or teleocalcin by Ma and Copp, (1978). In 1993, Wagner *et al.* described STC as, "a homodimeric glycoprotein hormone with a unique primary structure," that was first discovered in fish and more recently identified in mammals and is likely to be present in all vertebrates (Wagner *et al.*, 1997a) (Wagner *et al.*, 1998). This hormone is synthesised and secreted by unique endocrine glands located on the kidneys of bony fish known as the corpuscles of Stannius (Stannius,

1839). Early documentation of this find was by Milet *et al.*, (1979), with more recent literature by Wendelaar Bonga and Pang, (1986) and Flik *et al.*, (1989).

In salmonids, such as salmon and trout, STC is known to play an integral part in calcium and phosphorus homeostasis, functioning as an anti-hypercalcemic hormone.

Physiologically high levels of calcium ions [ $\text{Ca}^{2+}$ ] in blood plasma have a positive stimulatory effect on the pathways governing hormone synthesis and secretion (Cohen *et al.*, 1975; Wagner, 1994; Wagner *et al.*, 1989; Wagner *et al.*, 1991). Control of plasma ionic calcium by this hypocalcemic hormone has additionally been recorded by Hansen *et al.*, (1989) for the European eel (*A. anguilla*). Once released into the bloodstream stanniocalcin targets the major organs involved in the regulation of calcium and phosphorus, to aid in the restoration of normocalcaemia. This process has been demonstrated both *in vivo* (Wagner *et al.*, 1991) and *in vitro* (Wagner *et al.*, 1989), the more reliable findings being obtained using cultured cells obtained from the corpuscles of Stannius (CS) in rainbow trout. Wagner, (1989) demonstrated that an increase in extracellular calcium within a realistic physiological range (0.3 to 2.7 mM  $\text{Ca}^{2+}$ ) produced an accompanying elevation in the release of STC from cultured CS cells, thus emphasising the time and concentration-dependent effects of the calcium cation on hormone release.

Stanniocalcin accomplishes normocalcemia by reducing the levels of calcium entering the bloodstream from the aquatic environment, as a consequence of inhibiting the  $\text{Ca}^{2+}$  transport system across the gills (Fenwick and So, 1974; Lafeber *et al.*, 1988; Wagner *et al.*, 1988a). Additionally, STC is able to reduce the levels of gut  $\text{Ca}^{2+}$  transport into the extracellular compartment (Sundell *et al.*, 1992; Tagaki *et al.*, 1985; Wagner *et al.*, 1986), and increase the rate of renal phosphate reabsorption (Lu *et al.*, 1994). Therefore, it could be suggested that stanniocalcin, the gills and gut are responsible for the short-term regulation of calcium and phosphorus homeostasis in fish.

The actions of stanniocalcin in fish are strikingly similar to the actions of the hormone calcitonin in mammals, both in respect to stimulus-secretion and function, although the principle target organs affected by each hormone are different. In mammals, the polypeptide hormone calcitonin (CT) is secreted by the parafollicular "c" cells of the thyroid gland. These cells were found to be homologous with the ultimobranchial bodies by Pearse and Carvalheira in 1967, and are situated between the heart and the oesophagus in fish (Srivastav *et al.*, 1998). Although calcitonin is particularly effective as a powerful hypocalcemic and hypophosphatemic hormone in mammals (Thomas and Keenan, 1986), reports on its effect in fish have been inconsistent and inconclusive.

Hypocalcemic effects induced by calcitonin in teleosts have been reported by Chan *et al.*, (1968), Bradshaw and Sutton, (1970), Lopez *et al.*, (1976), Pang *et al.*, (1971) and Wales and Barrett, (1983). By contrast, many researchers have found that the plasma calcium concentration of teleost fish was not effected by calcitonin (Pang, 1973) (Orimo *et al.*, 1972) (Copp and Ma, 1978) and (Hirano *et al.*, 1981). Due to the doubt and conflicting opinions on the role of CT in fish, some authors such as Hirano *et al.*, (1981) have concluded that this hormone plays no important role of homeostatic control of calcium in fish.

A more probable alternative suggests that the definitive role of CT in fish is not yet known (Pang and Pang, 1986), with evidence supporting this theory being offered by many authors. In several fish species, activity of the ultimobranchial body and plasma calcitonin concentrations both increase as a result of an increase in external calcium levels, (Orimo *et al.*, 1972) (Watts *et al.*, 1975) (Fouchereau-Peron *et al.*, 1986). This suggests a hypocalcemic mechanism of functioning, and implies an involvement in ionic regulation, and possible associations with water balance and osmoregulation (Milet *et al.*, 1979) (Milhaud *et al.*, 1980), especially as Fouchereau-Peron *et al.*, (1986) established that

rainbow trout possess specific high affinity calcitonin receptors in their gills. Additionally, calcitonin receptors have been located in bone (Arlot-Bonnemains *et al.*, 1983), suggesting a possible role in bone resorption and formation (Pechet *et al.*, 1967; Rasmussen and Tenenhouse, 1967). More recently, Wendelaar Bonga and Pang, (1991), argued that the function of CT is essentially to protect the skeleton in times of calcium demand, e.g., growth, vitellogenesis and reproduction.

## **1.10 Fish excretion and phosphorus losses**

### ***1.10.1 Nitrogen excretion***

There are two main sources of nitrogenous waste that must be excreted by fish. The first is as endogenous waste resulting from the transamination and deamination of the amino acids which arise as the result of the metabolic turnover and break down of tissue proteins. Most amino acids released by protein breakdown are re-utilised hence values of endogenous excretion are fairly low, between 30 and 300 mg N kg/day, depending on fish size, species and water temperature (Jobling, 1993). The second source of waste is exogenous and comprises of the direct deamination of amino acids that have been either directly ingested or absorbed from the diet and in a catabolised form. Therefore the exogenous component of nitrogen excretion is influenced by factors such as feeding rate, dietary protein content and amino acid composition of the diet (Jobling, 1993). Most teleost fish are ammonotelic with the primary nitrogenous waste products being ammonia nitrogen, either in the form of ammonia or ammonium ions. This ammonia/ammonium is produced mainly in the liver, with the gills, kidney and muscles producing smaller amounts and is transported via the bloodstream to the gills for excretion across the surface of the gill lamellae into the surrounding water (Wood, 1993). Wood, (1958) also established that smaller quantities of urea, uric acid, and other minor constituents such as creatine may be excreted in the urine, or through the skin, or via the gills in most teleost fish. Estimated figures for total nitrogen excretion for the rainbow trout *Oncorhynchus mykiss* are presented in table 1.3.

Table 1.3.

Rates ( $\mu\text{mol-N/kg/h}$ ) and relative percentages of nitrogen (N) excretion and ammonia-N and urea-N via the gills and kidney for the freshwater rainbow trout *Oncorhynchus mykiss* (from Wood, 1993).

Excretory organ	Ammonia - N	Urea - N
Gills	270 (86%)	34 (11%)
Kidney	4 (1%)	6 (2%)

*Note:* the percentages assume that the total of ammonia-N plus urea-N is 100%. Kidney excretion measured by urinary catheter. Excretion through skin and gastrointestinal tract is therefore included in the gill component.

Freshwater teleosts excrete a greater proportion (over 80%) of their nitrogenous waste as total ammonia, but the proportion of nitrogen excreted as urea and other products may reach up to 40% in some marine species (Boyce, 1999; Jobling, 1993; Walsh, 1997). Concentrations of ammonia-N and urea-N in the blood plasma of the rainbow trout have been evaluated in several studies by Wood. It was established that ammonia-N concentrations were between 0.10-0.68 mmol-N/l and urea-N concentrations were between 4.24-5.00 mmol-N/l (samples were taken by either caudal or cardiac puncture or by indwelling catheters) (Wilkie and Wood, 1991; Wood *et al.*, 1989b). Two factors have a large influence over endogenous excretion they are fish size, which can be described by an allometric function and water temperature which causes an exponential increase in the rate of nitrogenous excretion with increasing temperature (Jobling, 1993; Ogino *et al.*, 1973). The effects of temperature on nitrogenous excretion appear to be similar to those observed for other physiological processes such as oxygen consumption for the plaice *Pleuronectes platessa* by Jobling, (1981). Although the nitrogenous excretion rates of some species such as the bluegill sunfish *Lepomis macrochirus* were established to be independent of temperature over part of the range by Savitz in 1969. The ingestion of food leads to an increase in the rate of nitrogenous excretion, although the excretion peak, which is mainly ammonia/ammonium, occurs several hours after the meal, after which excretion levels fall



slowly. Cho and Kaushik, (1985) concluded that diet composition will also have an effect on exogenous excretion, which can be markedly high when fish are fed diets rich in protein, or when the balance of amino acids is unsuitable for protein synthesis.

As mentioned in section 1.2 the discharge of nitrogen (N) and phosphorus (P) from intensively run fish farms into the natural surrounding waters has the potential to lead to environmental pollution. N and P are usually the limiting nutrients in aquatic eco-systems and the release of them in large levels from aquaculture operations can lead to eutrophication. N and P are usually released in the form of suspended solids from undigested, or uneaten food and the soluble excretory products resulting from metabolism. Therefore, it is essential to limit the release of such nutrients by utilising highly digestible dietary components to minimise feed waste. Nitrogen excretion rates are closely linked to nitrogen intake (protein level) in different fish species such as the largemouth bass (*Micropterus salmoides*) (Savitz *et al.*, 1977), common carp (*Cyrimus carpio*), rainbow trout (*Salmo gairdneri*) (Kaushik, 1980; Medale *et al.*, 1995), brook trout (*Salvelinus fontinalis*) (Paulson, 1980), plaice (*Pleuronectes platessa*) (Jobling, 1981) and the sturgeon (*Acipenser ruthenus*) (Gershanovich and Pototskij, 1992).

Research has shown that an increase in the dietary level of digestible non-protein energy increases N retention by decreasing N losses (Jayaram and Beamish, 1992; Kaushik and Oliva Teles, 1985; Watanabe *et al.*, 1987). Further research by Robert *et al.*, (1993) indicated that lowering the ratio of digestible protein (DP) to digestible energy (DE) from 22.6mg/kJ (NRC, 1993) to 19 mg/kJ leads to an improvement in feed efficiency and protein retention. Confirmation of these results was provided by Kim and Kaushik, (1992) and Medale *et al.*, (1995) for rainbow trout and by Tibbets *et al.*, (2001) for American eel (*Anguilla rostrata*). Although an investigation by Einen and Roem (1997), found the optimal ratio of DP to DE to decrease with increasing fish weight for Atlantic salmon (*S.*

*salar*). More recently, Ruohonen *et al.*, (1999) fed rainbow trout diets containing low-fat Baltic herring and supplemented with low-protein, these fish had improved protein retention with reduced levels of nitrogen and phosphorus waste compared to a control diet comprising of a standard dry commercial feed. However, fish being fed the treatment diet had 15-25% reductions in growth when compared to the control fish. Lipid and carbohydrate have been used as ‘protein sparing’ feed ingredients to fish diets fed to turbot (*S. maximus*) by Bromley, (1980a) to increase protein efficiency. Conversely, too-high dietary protein can suppress the retention efficiency of protein (Adron *et al.*, 1976; Lee and Putnam, 1973; Ruohonen *et al.*, 1998), which subsequently leads to increased levels of nutrient excretion.

The management of aquaculture wastes can be approached via several different routes, such as; integrated aquaculture (Chopin *et al.*, 2001; Martinez-Aragon *et al.*, 2002) and the use of constricted wetlands that both rely on natural processes primarily of aquatic plants to treat wastewater (Negroni, 2000). More mechanical approaches include a water treatment structures that aid in concentrating and collecting particulate waste material from fish farm effluent (Wong and Piedrahita, 2003), and sedimentation basins and rotating screens/columns that also to remove nutrient particulate waste from aquaculture systems (Cripps and Bergheim, 2000). Another approach is the use geographic information systems (GIS), which aids in modelling the distribution of organic waste from aquaculture, enabling prediction of possible impacts on the environment (Pérez *et al.*, 2002). Additionally, diet formulation, including pellet size (Chen *et al.*, 2003), improvement of feed utilization and producing feeding regimes for specific farm sites (Cho *et al.*, 1994), all play integral roles in the management of aquaculture. As Cho (1994) stated “fish do not pollute – feeds and feeding pollute!”

### 1.10.2 Phosphorus excretion

Phosphorus excretion can be segregated into three divisions namely;

- Endogenous losses, as a result of phosphorus loss from physiological processes.
- Regulated excretion of phosphorus in excess of the demands from the fish.
- Phosphorus that is unavailable to the fish presented in a dietary form.

A significant amount of soluble phosphorus is believed to be excreted via the urine in teleost fish, but the examination of phosphorus excretion through the kidney has received little scientific attention (Bureau and Cho, 1999; Grafflin, 1936; Kaune and Hentschel, 1987; Renfro, 1997). In mammalian renal physiology Bijvoet, (1980) demonstrated that inorganic phosphate (Pi) excretion is controlled by the plasma Pi concentration. Urinary output of Pi by the animal appears to be directly relative to this plasma Pi concentration, below a threshold level Pi excretion is minimal and above which increases comparatively to the increase in plasma Pi (Bureau and Cho, 1999). Mammalian research has indicated that Pi reabsorption in the kidney has been shown to take place predominantly in the proximal tubule and is highly dependent on the sodium ion concentration in the tubular fluid. Dennis *et al.*, (1979) reported that the Pi is actively transported against an electrochemical gradient through brush border membranes of the tubule wall. There is little published information of Pi excretion from fish, although research by Chester Jones *et al.*, (1969) has indicated that Pi may undergo active tubular secretion in the freshwater adapted European eel (*A. anguilla*). Additionally, Elger *et al.*, (1998) postulated that tubular secretion of Pi occurs in the second proximal tubule of the winter flounder (*Pleuronectes americanus*) kidney, with regulation of the urinary Pi content in the subsequent collecting tubule and duct.

A study by Kessler and Fanestil, (1981) suggested a further mechanism of Pi transport in the form of a carrier protein. The proteolipid fraction from rabbit kidney brush borders was investigated for their potential involvement in epithelial transport. It was established

that organic solvent extracts (in which proteolipids are soluble) of renal brush border had the ability to bind to Pi with high affinity and selectivity, they were also inhibited by arsenate which is known to be a competitive inhibitor of Pi transport. Together these factors imply a role for this proteolipid in the transport of phosphate across the renal brush border membrane (Kessler and Fanestil, 1981). As glomerular fish and mammals possess similar renal physiology (Dantzler, 1989), it has been postulated that a similar relationship in the regulation of renal Pi excretion is likely to be present between fish and mammals.

Regulation of renal Pi reabsorption is mediated by various physiological-pathological factors; one of them being acid-balance. Sacktor and Cheng, (1981) established that any process that results in intracellular acidification tends to increase the uptake of Pi from the filtrate of the proximal tubular lumen, implicating a further mechanism involved in regulating renal Pi transport even in the presence of a sodium gradient. Confirmation of acid-base balance playing a role in the regulation of Pi was recently validated by Wood *et al.*, (1999) for fish. Chronic respiratory (RA) or metabolic acidosis (MA) or metabolic alkalosis was induced in rainbow trout by exposure to hyperoxia, low pH or sodium bicarbonate infusion. During RA net H<sup>+</sup> excretion increased as a result of an elevation in efflux of NH<sub>4</sub><sup>+</sup> and HCO<sub>3</sub><sup>-</sup> and was accompanied by a large increase in inorganic Pi excretion, indicating that these responses to RA may be linked to the activation of Pi secretion.

Stanniocalcin (STC) is a homodimeric glycoprotein hormone first discovered in fish (Wagner *et al.*, 1993), where it is produced by unique endocrine glands called the corpuscles of Stannius, as previously mentioned in section 1.9. In freshwater salmonid fish STC is involved in maintaining calcium and phosphate homeostasis, high levels of extracellular Ca<sup>2+</sup> enhance the synthesis and release of STC into the bloodstream. The STC reduces the level of Ca<sup>2+</sup> transport at the gill and gut and stimulates renal Pi

reabsorption hence reduces excretion to restore normocalcemia, as concluded by Wagner *et al.*, (1998) following extensive studies involving seawater salmon. STC has recently been established in other vertebrates and is thought to participate in the renal regulation of Pi homeostasis in mammals in addition to fish (Conlon, 2000; Wagner *et al.*, 1997b).

As stated above, phosphorus (P) excreted by fish may have a direct influence on the enrichment of aquatic ecosystems and eutrophication, therefore the form in which P is excreted is of high importance. Generally P excreted as soluble orthophosphate affects water quality directly and is readily available for algal growth. Alternatively, organic P from the indigestible portion of the diet is faecally excreted in particulate form and settles in the sediment or pond/raceway. Such organic P and inorganic forms of P covalently bound to metal ions are released over time into the surrounding water from the sediment as a result of anaerobic and other biological and bacterial processes (Enell, 1987; Pettersson, 1988). Other inorganic forms of excreted P are usually as calcium phosphates, which are relatively insoluble and are deemed permanently bound and will settle to the bottom of the lake/sea. Persson, (1988) stated that 30% of total P from feed readily dissolves in water, and that P bound to organic compounds was approximately 55% of the feed and 33% of the faeces, with the remainder being considered to be bound and unavailable. Conversely, Cowey, (1995) reported that under farming conditions only 30% of P is in a particulate form with 60% being soluble P and available to plants.

Recently, Rodehutsord *et al.*, (2000), conducted an investigation into the effect of P intake on faecal and non-faecal P excretion in rainbow trout and established that with increasing P intake, faecal P excretion increased non-linearly. Basal non-faecal P excretion was estimated to be 3.7 mg/kg BW/day, which was similar to a figure of 7 mg/kg BW estimated by Bureau and Cho, (1999). Basal faecal excretion was calculated to be 2.9 mg/kg BW. The non-faecal P excretion was unaffected by P intake until the estimated P

requirement was met. When dietary levels of P reached 5 g/kg, DM non-faecal P excretion increased and serum Pi remained unchanged, suggesting that when the blood level is at its maximum (or close to) P retention, the surplus P is excreted primarily via the urine. Similarly, results were established by Vielma and Lall in 1998 for the Atlantic salmon, when the dietary P concentration was increased three fold the urinary P levels had increased by a factor of 20. It has been concluded by Bureau and Cho, (1999) that blood plasma Pi is the main factor in determining the soluble excretory output of fish. Above a threshold plasma Pi concentration of 86 mg Pi/l, urinary excretion was directly related to plasma Pi, and these results are in agreement with those presented by Rodehutsord *et al.*, (2000).

It can be concluded that dietary phosphorus source and availability will affect the amount of soluble and particulate phosphorus excreted by the fish, in addition to the level of phosphorus that could be subsequently biologically degraded and released from phosphorus forms settled in the sediment. Therefore, dietary phosphorus form warrants careful consideration when formulating aquafeeds. Additionally, at a physiological level plasma Pi measurements could aid in the estimation Pi urinary output and of the P adequacy of the diet.

### **1.11 Alternative protein sources and mineral utilisation**

As previously stated, protein constitutes the largest component of fish feed at 40-45% (Hilton and Slinger, 1981) and with the growing demand for limited fishmeal resources, the need has arisen for alternative protein sources to be found. Such alternative protein sources are predominantly from plant origin or animal by-products, but due to the stringent regulations on the re-use of animal products, only plant based protein sources can be utilised in the UK and the EU. As highlighted in section 1.3, soyabean and various grains such as barley and corn are currently the preferred alternative to fishmeal.

Table 1.4.

Phytate content of various feedstuffs of plant origin, adapted from (Ravindran *et al.*, 1995; Ravindran *et al.*, 1994)

Feed Ingredient	Phytate P – as % of total P
<b>Cereals</b>	
Maize ( <i>Zea mays</i> )	84.6
Rice ( <i>Oryza sativa</i> ), brown unpolished	73.7
Wheat ( <i>Triticum aestivum</i> )	69.0
Barley ( <i>Hordeum vulgare</i> )	64.0
Oats ( <i>Avena sativa</i> )	67.0
Sorghum ( <i>Sorghum vulgare</i> ), dark seeds	65.9
Maize gluten meal	59.0
<b>Oilseed meals</b>	
Soya bean ( <i>Glycine max</i> )	61.7
Soya bean meal, solvent extracted	60.3
Cottonseed meal ( <i>Gossypium sp</i> )	70.0
Peanut meal ( <i>Arachis hypogaea</i> )	80.0
Rapeseed meal ( <i>Brassica napus</i> )	59.0
Sunflower meal ( <i>Helianthus annus</i> )	77.0
Sesame meal ( <i>Sesamum indicum</i> )	81.0
<b>Grain legumes</b>	
Chick peas ( <i>Cicer arietinum</i> )	51.2
Peas ( <i>Pisum sativum</i> )	50.0

The primary storage form of phosphorus in all seeds and plant tissues is phytate (sometimes known as phytic acid). Phytate is myoinositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) and in mature seeds the phosphorus is predominantly in the form of phytic acid (Ravindran *et al.*, 1995). It is found as a complex salt of calcium, magnesium and potassium, sometimes combined with protein (Makower, 1969) and over 99% of this phytate phosphorus is present in a water-soluble form (Lolas and Markakis, 1975). Phytate contributes approximately two-thirds of phosphorus in various grains, examples of which are shown in table 1.4. Other feedstuffs such as roots and tubers contain less phytate (21-25% of total phosphorus).

Fish and other monogastric species such as poultry and pigs utilise phytate poorly due to the fact that the intestinal mucosa of these animals does not secrete the enzyme phytase. Phytase is needed to hydrolyse phytate into more digestible components (Lall, 1991; Ravindran *et al.*, 1995; Sugiura *et al.*, 1999). Subsequently, this phosphorus source passes through the animal virtually undigested. As a consequence, it is necessary to supplement animal diets containing a high proportion of plant protein with additional inorganic phosphorus. A recent study by Sugiura *et al.*, (1999) demonstrated that phosphorus discharge from fish could be reduced by incorporating a low-phytate mutant strains of corn and barley as a dietary protein source. Additionally, the enzyme phytase has been successfully used as a feed additive for poultry and pigs (Engelen *et al.*, 1994) to improve digestibility and reduce phosphorus excretion. Recently, fish nutritionists have advocated the addition of this enzyme to fish feeds (Cain and Garling, 1995; Jackson *et al.*, 1996; Vielma *et al.*, 2000) with the aim of improving P availability.

The phytate ion forms complexes with mono, di- and trivalent ions such as  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Fe^{2+}$ ,  $Fe^{3+}$ , and usually forms insoluble compounds (Reddy *et al.*, 1978, Reinhold *et al.*, 1973). Phytate has a particularly high affinity for zinc in physiological pH ranges, and



the complex formed is very stable (O'Dell and Savage, 1960; Maddaiah *et al.*, 1964). This zinc complex is insoluble, which leads to decreased dietary absorption (Richardson *et al.*, 1985), particularly across the gastrointestinal tracts of monogastric animals (Oberleas *et al.*, 1966; Oberleas and Harland, 1981). Roberts and Yudkin, (1960) have also demonstrated this process in phytate complexes formed with calcium and magnesium. Phytate also has the ability to bind with protein at neutral pH, (Cosgrove, 1980) and with digestive enzymes (Singh and Krikorian, 1982). These phytate-protein complexes may reduce the utilisation of proteins and amino acids as well as decreasing digestive enzymatic activity and leading to poor protein digestibility.

Although it would be easy to attribute all negative effects on the availability of minerals to the presence of phytate and its chelating capabilities, another feed component may be held responsible for reducing the availability of minerals. Fibre, which is an important fraction of most foods, particularly those of plant origin, is also known to have a high affinity for minerals (Reinhold *et al.*, 1975). Therefore, Torre *et al.*, (1991) suggested it may be difficult to ascertain the primary cause of reduced mineral availabilities if phytate and fibre cannot be assessed on an individual basis.

Interest in phytate and phytases has arisen due to the poor natural ability of monogastric animals to digest phytate and the strong chelating action of phytate for mono-, di-, and trivalent metal ions, resulting in the reduced bioavailability of minerals. As stated in the above section, the enzyme phytase has been used as a dietary supplement in animal nutrition, to improve phosphorus digestibility and reduce excretion. Recently, aquaculturists have been trying to achieve the same results with fish nutrition and feed formulation.

Phytase is an acid phosphatase that catalyses the sequential hydrolysis of inorganic orthophosphate from phytic acid. As this enzyme is non-specific it can remove phosphate from a number of natural and synthetic phosphorylated substrates (Gibson and Ullah, 1990). There are two recognised classes of natural phytase; 3-phytases are characteristically found in micro-organisms and filamentous fungi (particularly the *Aspergilli*) and the seeds of higher plants contain 6-phytases (Gibson and Ullah, 1990). Microbially produced phytases are commercially produced, usually from *Aspergillus ficuum* and *Aspergillus niger*, which are used to dephytinise plant feedstuffs (Storebakken *et al.*, 1998). As phytases are thermolabile, they have to be added to the feeds after the high temperatures of extrusion, or by incubating the individual feed ingredients in an aqueous suspension prior to drying and dietary formulation (Cain and Garling, 1995).

#### **1.12 Primary objectives and rationale for the programme of investigations.**

The role of macroelements and phosphorus in particular in the nutrition of salmon and trout was reviewed in the context of aquaculture. There were a number of issues pertaining to those factors governing the bioavailability of phosphorus and other minerals from the diet. These mainly reflected their relative net absorption, distribution and retention into various tissues and organs during growth and development at different stages.

It was previously stated that defining phosphorus retention is of central importance, with respect to quantifying losses to the aqueous environment with the possibility of minimising adverse impact in aquaculture practice. Various dietary interactions may markedly influence the absorption and assimilation of phosphorus in fish. Since commercial diets differ considerably in specification and vary in P levels and source, it is necessary to examine these implications in detail.

Indeed, the digestibility of feed components and release of minerals and elements including phosphorus is subject to a number of biotic and abiotic factors. The gastrointestinal physiology associated with P absorption and post-prandial systemic levels requires elucidation. Various commercial considerations warrant further investigation, with respect to the availability of phosphorus from selected raw materials that may be used in balanced feed formulations destined for salmon and trout culture.

For those reasons, a series of experiments were implemented to further understanding of primarily phosphorus nutrition and that of related macroelements critical for growth performance, production and health of salmonid fish.

The rainbow trout served as the model experimental fish representing salmonid fish in general. Studies embraced the main points previously stated with the main objectives being:-

1. To evaluate commercial diets differing in their phosphorus and mineral profile with respect to overall performance and retention of these nutrient components in the fish.
2. Establish the interactions of nutrient/calorific density (varying oil levels) on phosphorus and selected mineral retention and assimilation during growth.
3. Determine and refine the basis of phosphorus requirements in relation to dietary P level on growth performance, feed utilisation, and health in juvenile trout.
4. Assess the apparent digestibility of P and other related minerals such as Ca in selected raw materials, ingredients etc, and to compare the digestibility of these minerals from different commercial inorganic sources, commonly employed in animal nutrition.

5. Finally to further knowledge of the physiological constraints and processes involved in P absorption from the gastro-intestinal tract. To determine the differential uptake and availability of P and Ca from selected regions associated with the digestive system in the rainbow trout.

The investigations aimed to further understanding of the macroelemental requirements of the rainbow trout and concurrent losses to the aquatic medium. These experiments provide an investigative approach and were based on *in vivo* trials mainly under controlled laboratory conditions or under managed farm systems appropriate to the species. At all times, the welfare and husbandry conformed to standard aquaculture practice such that the result of these studies would have relevance to practical operations in the field.

## **CHAPTER 2**

### **GENERAL MATERIALS AND METHODS**

## CHAPTER 2

### GENERAL MATERIALS AND METHODS

#### 2.1 Nutrition Trials

##### 2.1.1 Stock Animals

The salmonid fish rainbow trout *Onchorynchus mykiss* (Walbaum) was selected as the experimental species for the following studies, due to its popularity as a farmed fish in the United Kingdom and accessibility from local sources (Fig 2.1). The fish utilised in the first trial (chapter 3.1) were supplied from Mill Leat Trout farm, Ermington, Ivybridge, Devon at a mean weight of approximately 30g.

During grading and transportation all fish were held in oxygenated water at an optimum temperature of 15°C. These fish were reared on a stock diet until reaching a weight of 50g, suitable for feeding trials. Furthermore this period acted as an acclimation period to the experimental facilities. During the first 10 days of this feeding regime, the diet was spray coated with the antibiotic oxalinic acid (2mg/Kg dry weight), as a precautionary measure against bacterial and fungal outbreaks due to transportation stress. The health and feeding response of fish were carefully monitored on a daily basis.

For the subsequent growth and nutritional studies that were conducted at the University of Plymouth, the experimental fish were obtained from Hatchland Fisheries, Greysshoot Lane, Rattery, Devon. Fish were brought in at the appropriate size relating to the type of experimental trial being conducted. All fish were held on stock diets, for a two week acclimation period. As mortality levels were less than 10% for all sets of 'new' stock fish, they were deemed to be in good health before the commencement of each trial.

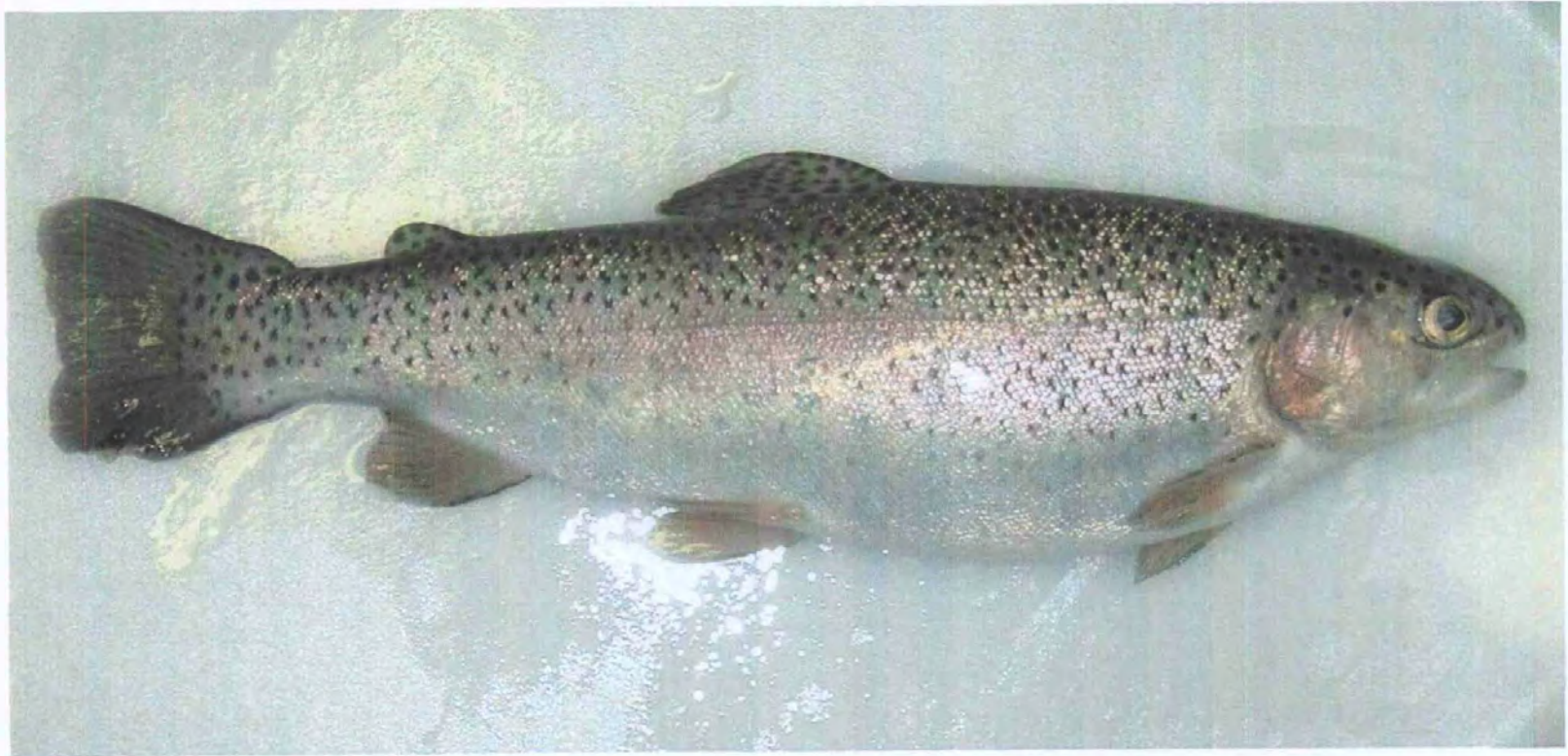


Figure 2.1  
Rainbow trout *Oncorhynchus mykiss* typically used in all experimental investigations.

Two trials were conducted outside of the University's facilities, one at Hatchland Fisheries (as mentioned above) where fish were either held in 1m circular tanks or concrete raceways. The other was based at Sparsholt Agricultural College, Sparsholt, Winchester, Hampshire, where fish were held in 1m circular tanks only. On these occasions fish used were already available at the facility. Water supply to these tanks was sourced from a natural bore-hole at a temperature of approximately 12°C.

During the acclimation period, the feeding regime was to satiation twice daily (am and pm). Fry and small trout (<20g) were initially maintained on a commercially available, high energy starter feed for trout fry (Nutra Aminobalance – TROUW Aquaculture), pellet size was dependent on fish size (either crumb or 1.8mm). Larger stock fish were fed once daily on a commercially produced fully floating feed for trout (standard expanded – TROUW Aquaculture), with a pellet size of 4mm.

During nutrition trials, fish were fed as a percentage of their body weight, which was calculated by following commercial guidelines for weight class and temperature. Actual ration size was determined at fortnightly intervals after fish were bulk weighed.

### ***2.1.2 Aquarium systems***

During the period in which experiments were conducted at the West Aquarium, University of Plymouth, two culture systems were used to hold fish. The initial trial was conducted in a re-circulating system comprising six square 400 L fibreglass tanks suspended over a 1200 L bio-filter (system 3, Fig 2.2). To aid in the filtration process, a foam fractionation basket was fitted to the pre-filter to assist in mechanically removing solid particles from the water before bio-filtration.





Figure 2.2  
Configuration of aquarium system '3' used during the first experimental trial.



Figure 2.3  
Configuration of aquarium system '1' used for all subsequent trials.

Subsequent trials were performed in a twenty tank, re-circulating system (system 1, Fig 2.3), each square fibreglass tank held approximately 160L of water, and had a flow rate of 13L/minute. All tanks were suspended over three linked tanks; a 824L pre-filter containing plastic 'pan-scourers' to assist in mechanical filtration and inorganic magnospheres to aid the waters buffering capacity. The second tank, with a volume of 1565L, contained a porous ceramic material, 'Alpha-grog' (J & K Aquatics), this acted as the main biological filter. Before being pumped into the trial tanks, water passed through a 250L water compartment in which the submersible cooling system was located. Additionally, a Hi-rate sand filter (Lacron) was inserted into the systems circuit to aid in the removal of solid suspended particles from the water. Twice a day, at 8am and 4pm, a backwash cycle was automatically initiated, in which the water flow was reversed through the filter to waste, in replacement clean water entered the system from the city mains supply, which totalled an approximate 10% total water change per week. This water was standard chlorinated tapwater, however, the force at which this water was entering the system 'drove off' a large amount of the chlorine as a gas.

The aquarium systems described above were both thermostatically controlled by a thermostat (Dryden Aquaculture) and kept at a constant temperature of  $15.5 \pm 1^{\circ}\text{C}$ . Photoperiod was fixed to a 12 hour day/night regime, which was held throughout the year.



Figure 2.4  
Production size rainbow trout held in aquarium system '1'.



Figure 2.5  
View of tanks 1-10 in aquarium system '1', sand filter (bottom left) and pre-filter tank (bottom right).

### 2.1.3 Water quality analysis

Throughout each respective trial and acclimation periods, aquarium water was subjected to various water quality tests on a weekly basis. Dissolved oxygen, pH and conductivity were measured by portable waterproof meter and microcomputer pH meter (Hanna Instruments HI 9142 and HI 9023). pH was held constant at  $7 \pm 0.5$ , dissolved oxygen was always above 7 mg/L, conductivity varied between 25-100mv.

A multiparameter bench spectrophotometer (C 100 series - Hanna Instruments), was used to measure the following water borne ions (range indications are also given): Ammonia [ $\text{NH}_3/\text{NH}_4^+$ ], by the Nessler method giving a yellow colouration relative to ammonia level when read at 470nm, range  $0.23 \pm 0.06$  mg/L. Nitrate [ $\text{NO}_3$ ], by an adaptation of the cadmium reduction method giving an amber colouration relative to nitrate level when read at 555nm, range  $15.33 \pm 3.04$  mg/L. Nitrite [ $\text{NO}_2$ ], by an adaptation of the EPA (Environmental Protection Agency) method 354.1 which causes a pink tint in the sample relative to nitrite level when read at 470nm, range between  $0.13 \pm 0.02$  mg/L.

Table 2.1.  
Mineral levels (mMol) in various water sources.

Mineral (mMol)	Mains supply	Hatchlands fish farm	Sparsholt College
Calcium	0.661	1.037	4.211
Magnesium	0.048	0.221	0.106
Phosphorus	Negligible	Negligible	Negligible
Zinc	0.004	Negligible	Negligible

The aforementioned bench spectrophotometer was initially used to analyse water samples for phosphate ions, but in order to improve accuracy and gain better detection limits it was decided to use the inductively coupled plasma – atomic emission spectrophotometer (ICP-AES) for ionic levels of water. The minerals levels for water from the various sources involved in this research are presented in Table 2.1.

#### **2.1.4 Diet Formulation and Materials**

All test diets formulated for use during either the growth or digestibility trials were based on commercially available diets, which allowed for modifications relevant to the need of each trial. Where possible diets were commercially made to precise specifications. Alternatively, diets were made 'in-house' using ingredients commonly used in the manufacture of commercial diets to a practical or semi-purified standard. Test diets typically contained 45% protein and 25% oil, 'in-house' diets generally contained less oil, approximately 15% inclusion level was standard. Due to the diversity of the test diets, the exact formulation, proximate composition and mineral levels are presented for the appropriate investigations in each relevant chapter.

Basic ingredients used to manufacture practical test diets were LT94 Scottish herring fishmeal, hi-pro full fat soya bean meal in addition to a mineral and vitamin premix provided by TROUW Aquaculture. Semi-purified diets were prepared using casein, gelatin, cornstarch, dextrin,  $\alpha$ -cellulose supplied by the Sigma Chemical Company (Poole, Dorset) and cod liver oil was provided by Seven Seas Ltd, Hull. Dietary ingredients deviating from this basic list are stated in the relevant chapter.

### **2.1.5 Diet Preparation**

As previously stated, various diets used were commercially prepared for experimental work. Diets that were manufactured 'in-house' were either practical or of a semi-purified nature, but both were produced in a similar manner. Thus, all dry ingredients were weighed and mixed together in the bowl of a Hobart A101 food processor (Hobart Manufacturing Company Ltd, London), when the mixture was uniformly blended the oil was added. De-ionised water was slowly added during continuous mixing until the mash was sufficiently moist for extrusion. The diet mixture was then extruded through a die with holes of 2-3mm diameter, using the extruder attachment. The resulting moist strands were spread evenly on foil covered trays and dried in a fan assisted drying cabinet at 45°C. These were subsequently broken into uniform size pellets and stored in airtight containers prior to use. The semi-purified diets were not dried but stored in plastic boxes at -20°C, daily requirements were removed as necessary throughout the trial.

## **2.2 Biochemical analysis – Proximate composition**

### **2.2.1 Moisture Determination**

The moisture content of the feed, faecal and whole fish carcasses were determined as described in the A.O.A.C. handbook (1997). Pre-weighed samples of feed, diet ingredients, and whole fish carcasses were evenly distributed in low aluminium foil trays and dried at 100°C to a constant final weight in a fan assisted oven (Pickerstone E 70F). Faecal samples, however were dried at 60°C due to their higher water content.

Tissues, organs and skeletal materials were weighed and dried, from a pre-frozen state in a freeze dryer (Edwards, Super Modulyo – Pirani 1001) for either 24 hours or until a vacuum pressure of  $10^{-1}$  microns of Hg was reached. All materials were stored in sealed plastic bags in a dessicator containing anhydrous silica crystals prior to subsequent analysis.

The percentage moisture in the original sample was calculated as follows:

$$\text{Moisture (\%)} = \frac{\text{Change in weight (g)}}{\text{Initial weight (g)}} \times 100$$

### **2.2.2 Crude protein determination**

The crude protein content (N X 6.25) of fish feed, feed ingredients, faeces and whole fish carcasses were determined by employing the Kjeldahl method. A micro-kjeldahl digestion block was used to enable replication of small samples. Typically, 100mg samples were weighed into a borosilicate digestion tube, 10mL of concentrated sulphuric acid (Sp.Gr. 1.84) and 1 kjeldahl catalyst tablet (3g K<sub>2</sub>SO<sub>4</sub>, 105mg CuSO<sub>4</sub>.5H<sub>2</sub>O and 105mg TiO<sub>2</sub> BDH Ltd U.K.) was also added to each tube. The digestion process was performed on a 40 position Gerhardt micro-kjeldatherm digestion block (C. Gerhardt Laboratory Instruments, Bonn, Germany). Initially, heating was for 30 minutes at 220°C; the temperature was then increased to 380°C for a further 60 minutes. During this digestion period, the acid fumes produced were passed through 15% NaOH held in a Gerhardt Turbosog scrubber unit, which effectively neutralises the acid. After digestion the tubes were removed from the block and allowed to cool at room temperature.

In a Gerhardt Vapodest distillation unit, the sample was diluted with distilled water and then neutralised with 40% NaOH, the resulting inorganic ammonia (NH<sub>3</sub>) in the sample is then collected in a conical flask along with 50ml of saturated orthoboric acid (H<sub>3</sub>BO<sub>3</sub>) containing BDH '4.5' indicator by steam distillation. This distillate was then titrated against 0.1M HCl and the percentage protein in the dry sample calculated as below:

$$\% \text{ Crude protein} = \frac{(\text{Titre sample (ml)} - \text{Titre blank (ml)}) \times 0.25 \times 14 \times 6.25}{\text{Weight of sample (mg)}} \times 100\%$$

Where:

0.25	=	[HCl] in moles
14	=	Relative molecular mass of nitrogen (N)
6.25	=	Constant describing relationship between nitrogen and protein content of sample

### 2.2.3 Total lipid determination

Lipid determination involved the use of a method developed from that of (Folch *et al.*, 1957), this procedure is based on the extraction of lipid from the sample by a suitable organic solvent system. Approximately 0.5g of either feed or fish carcass sample was weighed into a 50mL erlenmeyer flask, and 10mL of chloroform : methanol (2:1) added. All flasks were sealed with parafilm and left at room temperature for 24 hours. The filtrate containing the extracted lipid was then filtered into a test tube, the remaining residue in the erlenmeyer flask 'rinsed' with a further 10ml of the solvent and the filtering process repeated. The residue was then removed and weight recorded, after evaporation of any excess solvent. The filtrate was then distributed between three pre-weighed test tubes which were placed in a rack located in a water bath with a temperature of 55°C, the chloroform and methanol was then allowed to fully evaporate off. Once the tubes were completely dry they were reweighed, and the lipid content of the samples calculated using the following equation.



$$\% \text{ Lipid} = \frac{\text{Weight of collected lipid residue (g)}}{\text{Weight of sample (g)}} \times 100$$

#### **2.2.4 Ash determination**

The ash content of feed, faeces and whole ground fish were determined as outlined in the A.O.A.C handbook (1997). Samples of either 300mg (for faeces) or 1g for all other samples were weighed in triplicate into porcelain crucibles, and placed into a temperature controlled muffle furnace (Carbolite GLM 11/7 – Carbolite Furnaces Ltd, Bamford, Sheffield), which were then subjected to a temperature of 525°C for 8 hours. After cooling, crucibles were re-weighed, the increase in weight was indicative of the sample's ash content and calculated as follows:

$$\% \text{ Ash} = \frac{\text{Weight gained by crucible (g)}}{\text{Weight of sample (g)}} \times 100$$

### **2.3 Determination of minerals**

#### **2.3.1 Microwave digestion**

Microwave digestion involves heating sealed vessels under pressure using microwave radiation. This process of reaction kinetics, acts by accelerating the wet acid digestion. The result is the production of sample material in a soluble acidic format suitable for analytical analysis.

Prior to microwave digestion all samples were dried, as described in section 2.3.1.

Weighed samples of up to 150mg were placed in large teflon bombs (capacity of 120ml), and 5ml of concentrated nitric acid (GPR grade) added. The teflon bombs were then assembled into the rack provided and placed in the CEM microwave, MDS-2000 (CEM Corporation, 3100 Smith Farm Road, Matthews, NC 28105, USA), and an appropriate digestion programme selected. The program used for digestion of all fish related samples followed the following four steps:

10% power 60 PSI – 5 minutes

50% power 120 PSI – 10 minutes

20% power 120 PSI – 1 minute

0% power 20 PSI – 5 minutes

Bombs were allowed to cool in the fume extraction cupboard, and dismantled before using a 25ml volumetric to make the sample up to volume with de-ionised water. All samples were stored in 30ml nalgene bottles before analysis by ICP-AES.

As samples were to be analysed for soluble minerals, all equipment used in the digestion process was washed initially with detergent in warm water, rinsed twice with tap water and twice in de-ionised water before drying. Occasionally an accumulation of lipid would appear on the side of the teflon bombs, which were then left to soak overnight in hot water containing 'Decon' detergent. After sample analysis, the nalgene bottles were decanted, washed with mains water, cleaned with acetone to remove any lipid adhesion followed by soaking and two rinses in de-ionised water before drying.

### 2.3.2 Analysis by ICP-AES

The instrumentation employed for elemental analysis was an inductively coupled plasma atomic emission spectrophotometer (ICP-AES), which was preferred in comparison with an atomic absorption spectrophotometer (AAS) due to its lower detection limits (see table 2.2) and ability to analyse multiple elements in a small sample volume.

Table 2.2.

A comparison of mineral detection limits for ICP and flame AAS (from Varian).

Element	ICP ( $\mu\text{g/L}$ )	Flame AAS ( $\mu\text{g/L}$ )
Calcium	0.03	1
Magnesium	0.1	0.3
Phosphorus	18	40000
Zinc	0.9	1.0

Emission spectroscopy works on the principal of producing emission lines that are the result of excited atomic electrons moving from a higher energy state to a lower energy state. As these electrons shift between these respective states, light is emitted, the wavelength of emitted light being proportional to the spacing of the energy. Since every element has a unique set of energy levels, each element has a unique set of wavelengths at which it will emit energy. To produce an emission, an atomic vapour needs to be generated at high temperature the ICP plasma achieves temperatures between  $6000^{\circ}\text{K}$  and  $10,000^{\circ}\text{K}$  and therefore can excite many elements simultaneously.

The ICP-AES system operates by aspirating a liquid sample through a nebuliser and into a spray chamber as an aerosol mist. The plasma is sustained by a flow of argon, which also transfers the sample into the centre of the plasma. A radiofrequency (RF) generator operates whilst power is supplied to the plasma induction coil. The sample mist undergoes

several reactions; the final ionisation/excitation stage emits light radiation, which is directed to a diffraction grating where dispersion into constituent wavelengths occurs. A photomultiplier tube then converts the radiation discharge to a detectable potentiometric differential. The amplified current is then processed by the appropriate software in a linked computer.

As the experimental samples were in a 1:5 ratio with concentrated nitric acid, all of the standards and blank were formulated in the same 1:5 acid ratio matrix. Two sets of three standards were prepared, one set was for the following minerals; calcium, magnesium, phosphorus and zinc, the other set was for yttrium and is outlined overleaf.

Table 2.3.  
Composition of standards used for ICP analysis.

Compound	Standard concentrations	Matrix used	Detection wavelength (nm)
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.1, 1, 10 mM	1:5 HNO <sub>3</sub>	393.366
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.1, 1, 10 mM	1:5 HNO <sub>3</sub>	279.553
KH <sub>2</sub> PO <sub>4</sub>	0.1, 1, 10 mM	1:5 HNO <sub>3</sub>	213.618
ZnSO <sub>4</sub>	0.01, 0.1, 1 mM	1:5 HNO <sub>3</sub>	213.856
Yt <sub>2</sub> O <sub>3</sub>	10, 20, 40 ppm	1:5 HNO <sub>3</sub>	371.03

These standards were used to calibrate the ICP, prior to each set of sample analysis, calibration curves plotted for each set of standards consistently produced correlation coefficients > 0.98, indicating a good linear relationship between each standard and intensity. During sample runs, standards were routinely analysed in order to establish if the machine had 'drifted.' If an appreciable drift occurred and was more than 8%, the ICP was re-calibrated under the same conditions.

## **2.4 Tissue and organ harvesting from fish**

In order to assess the whole body distribution of various minerals, it was necessary to conduct a full fish dissection to obtain the internal organs and skeletal components required for analysis. Rainbow trout were anaesthetised using 2-Phenoxy-ethanol (0.2ml/L), and culled in accordance with the Schedule 1 Practice of the Home Office Animals (Scientific Procedures) Act 1986. Dissection was conducted by an initial dorso-ventral incision from the anus to vent, thus opening the body cavity. Once exposed, internal organs were removed in the following order, intestine (anus to pyloric region, not including caeca), spleen, liver, heart, kidney (mid-section), gill arch including lamellae (2 middle arches from the basket of four), eye. From the area under the dorsal fin, a 2cm<sup>2</sup> sample of white muscle (epaxial) and corresponding square section of skin was taken, from the mid section of the vertebral column a 5cm section of vertebrae was cut and several pleural ribs. During the initial trial all samples were collected in aforementioned manner.

In further trials, all soft organs (excluding skeletal parts and muscle) were harvested in the above manner. After their removal, individual fish were wrapped in cling film and frozen at -20°C, until further analysis could be performed. When the whole body was needed for analysis, fish were taken, bagged and labelled then stored at -20°C prior to processing.

During dissection all instrumentation was rinsed clean in de-ionised water between individual fish samples, to avoid any cross contamination.

### **2.4.1 Blood, bile and faecal collection**

Initially, fish were lightly anaesthetised using 2-Phenoxy-ethanol (0.2ml/L), to facilitate handling. For blood collection either 1, 2 or 5mL sterile disposable syringes with Luer fitting needles (0.5 x 25mm/25G x 1") were used. To prevent blood clotting syringes were flushed with a heparin solution (1000 units/ml - sodium salt). Once anaesthetised, the fish

was held with its ventral surface uppermost and with the needle held obliquely, a puncture made past the anus and anal fins towards the tail of the fish. The needle was inserted until the vertebrae was reached and caudal vein punctured, the needle was then pulled slightly away, and the plunger gently pulled back until enough blood was collected. After removing the needle (to prevent cell destruction), the syringe was purged into an Eppendorf vial. If plasma was required, the whole blood sample was quickly centrifuged in a micro centrifuge (Micro-Centaur MSB010. CX2.5, Sanyo Gallenkamp PLC, Uxbridge, UK) at 13000xg for 2 minutes, subsequently the plasma was then poured from the supernatant into a clean eppendorf. Plasma samples were then stored at either -20°C or -60°C until further analysis could be conducted.

The total volume of bile was collected from the gall bladder during dissection procedures, before liver removal using 0.5mL sterile insulin syringe (Becton and Dickenson). The bile was then aliquoted into eppendorf tubes and stored at -20°C until analysis could be carried out.

Faecal collection was by the manual stripping method as employed by Austreng, 1978, and commonly applicable to most salmonid species. Prior to collection fish were lightly anaesthetised using 2-Phenoxy-ethanol (0.2ml/L), to facilitate handling. Initially gentle pressure was applied to the abdominal region from the pelvic fins to the anal vent, the faeces were gently expelled and collected in foil dishes, which were subsequently stored at -20°C for further analysis.

#### **2.4.2 *Skeletal cleaning***

To aid cleaning of tissue from skeletal material, fish were wrapped in cling film and microwaved on low power 750 watt (Proline, Microwave) the length of time needed to gently 'cook' the fish was obviously relative to size, with small fish taking seconds and

larger fish minutes. Once the fish was sufficiently cool, samples of skin, muscle, and skeletal structures such as operculum cover, ribs and vertebrae were taken in the manner described above.

## **2.5 Haematological Indices**

### **2.5.1 Haematocrit**

Haematocrit values were determined directly after blood collection, whole blood was aliquoted into Eppendorf tubes. Samples of blood were then drawn by capillary action into heparinised capillary tubes (Hawksley, Lancing, Sussex), the clear end was heat sealed. The micro haematocrit tubes were then spun in a mini haematocrit centrifuge (Centurion) for 2 minutes at 12500x g, and the packed cell volume was established by using the Hawksley micro-haematocrit reader. The haematocrit percentage was calculated as follows:

$$\text{Haematocrit \%} = \frac{\text{Length of packed red cell volume (mm)}}{\text{Total length of blood volume (mm)}} \times 100\%$$

### **2.5.2 Total Haemoglobin**

In order to ascertain levels of total haemoglobin in whole blood, a Sigma Diagnostic kit (525-A) was used. This procedure involves using a stable Drabkin's reagent (alkaline potassium ferricyanide), which oxidises haemoglobin and its derivatives (except sulphaemoglobin) to methaemoglobin. The methaemoglobin reacts with potassium cyanide to form cyanmethaemoglobin, which is proportional to the total haemoglobin concentration when the colour intensity is optically measured.

A cyanmethaemoglobin standard was prepared by reconstituting a vial of haemoglobin standard (SIGMA Catalogue Number 525-18), with 50mL of drabkin's reagent, this solution was mixed and allowed to stand for 30 minutes. A standard curve was produced relating to total blood haemoglobin levels (0, 6, 12, 18 g/dL). To each of a triplicate series of tubes, 0.5mL of Drabkin's solution was added, to the blank 20  $\mu$ L of double distilled water additionally added, and to all other tubes 20  $\mu$ L of the relevant sample pipetted. Tubes were immediately mixed well and left to stand at room temperature for at least 15 minutes, before reading the absorbance against the blank at 550nm on a UV/VIS scanning spectrophotometer (Philips, PU 8720). The total haemoglobin concentration (g/dL) could be read directly from the calibration curve.

### **2.5.3 Total plasma protein**

The Sigma Diagnostic kit (541-2) was used to ascertain concentrations of blood protein. The total protein reagent determines protein according to the candidate reference method developed by Doumas *et al* (1981). The principle of this reaction is as follows; copper ions in the alkaline biuret reagent, react with peptide bonds of serum proteins to form a coloured complex, which can be optically measured.

A set of serially diluted standards (2, 4, 6, 8, and 10 g/dL) were produced using bovine serum albumin as the protein reference source; the standard curve that was produced was linear to 16 g/dL. A triplicate series of tubes were set up and into each, 0.5 ml of total protein reagent was pipetted. Then into the appropriate tube either de-ionised water (blank), a standard or blood plasma sample was added. Each tube was subsequently mixed gently on a Whirlimixer (Fisons), and allowed to stand at ambient temperature (18 - 26°C) for 10 minutes. After incubation, 300  $\mu$ L aliquots from each tube were pipetted onto a micro plate, each well was read at a wavelength of 540 nm against a water reference, using the endpoint programme on a MRX Dynatech Laboratories visible spectrophotometer.



When samples appeared to be turbid or haemolysed, samples blanks were run. This involved adding the sample to isotonic saline in place of the reagent; the absorbance of this mixture read against the water blank was subtracted from the corresponding absorbance value obtained from the above procedure before calculating the protein concentration.

#### **2.5.4 Plasma Glucose**

Concentrations of blood plasma glucose were calculated by the use of the Sigma Diagnostic kit (Procedure number 16-UV). Serum glucose was determined by an enzymatic method, which principally involved utilising the coupled enzyme reactions catalysed by hexokinase and glucose-6-phosphate dehydrogenase to yield a reduced form of NADH which is directly proportional to the glucose concentration.

A serial dilution of glucose (concentrations) were used to produce a standard curve which was linear in the range in which this assay was run (up to glucose concentration of 120mg/dL). To test for glucose, 0.5mL of glucose (HK) reagent, which had been allowed to warm to assay temperature (ambient), was added to tubes containing 5  $\mu$ L of sample, standard, and blank (de-ionised water) respectively, additionally each tube was triplicated. This mixture was then vortexed on a Whirlimixer (Fisons), after an incubation period of 5 minutes at assay temperature, 300  $\mu$ L from each tube was pipetted onto a micro plate. The absorbance of each well was then read against that of the blank at a wavelength of 340 nm using the endpoint programme on a MRX Dynatech Laboratories visible spectrophotometer. When samples appeared to be turbid or haemolysed, sample blanks were also run. This involved adding the sample to isotonic saline in place of the reagent, the absorbance of this mixture read against the water blank was then subtracted from the corresponding absorbance value obtained from the above procedure before calculating the glucose concentration.

The concentration of glucose in the sample was calculated relative to the values read from the calibration curve as mg/dL, to convert the results into SI units (mmol/L) the glucose value was multiplied by 0.0555.

### **2.5.5 Plasma minerals**

Initial analysis involving plasma mineral determination relied on Sigma Diagnostic kits (Nos. 587 and 360) for calcium and inorganic phosphorus respectively. The determination of calcium relied on the reaction of calcium with o-cresolphthalein to produce a red complex in highly alkaline conditions (pH 10-12), to be optically viewed at an absorbance of 575nm. The resulting colour intensity is directly proportional to the calcium concentration in the sample at the designated wavelength.

A range of calcium standards (5, 10, 20, 30, 40 mg/dL) was used to produce a standard curve, which was linear in the range in which this assay was run. To analyse for calcium, 0.5mL of calcium reagent working solution, which had been allowed to warm to assay temperature (ambient), was added to tubes containing 5  $\mu$ L of sample, standard, and blank (de-ionised water) respectively, additionally each tube was triplicated. This mixture was then vortexed on a Whirlimixer (Fisons), 300  $\mu$ L from each tube was pipetted onto a micro plate within 3 minutes. The absorbance of each well was then read against that of the blank at a wavelength of 340 nm using the endpoint programme on a MRX Dynatech Laboratories visible spectrophotometer.

The concentration of calcium in the sample was calculated relative to the values read from the calibration curve as mg/dL, to convert the results into SI units (mmol/L) the calcium value was multiplied by 0.25.

The test kit for inorganic phosphorus followed the principle involving the reaction of phosphorus with ammonium molybdate in the presence of sulphuric acid, to produce an unreduced phosphomolybdate complex. The absorbance of this complex at 340nm is directly proportional to the inorganic phosphorus concentration. The above methodology was employed for this test kit with the exception of wavelength, which became 340nm. Conversion to mmol/L could be calculated by multiplying by 0.323.

## **2.6 Definitions and related formulae.**

Throughout this programme of research it was necessary to quote various parameters related to growth performance and feed utilisation. These are fundamental to nutritional studies with fish and are defined as follows:-

### **Feed Conversion Ratio (FCR)**

FCR can be used to establish to what extent the feed provides the necessary energy for growth. A lower value indicates an improved outcome, for example an FCR of 1 indicates that 1g of feed produces an increase in 1g of fish weight, therefore the FCR can be expressed as: -

$$\text{FCR} = \frac{\text{Amount of feed consumed (dry/g)}}{\text{Live weight gain of animal (g)}}$$

### Feed Conversion Efficiency (FCE)

FCE may also be used to express feed efficiency, although the FCR is much more commonly used and is widely accepted in animal nutrition trials.

$$\text{FCE (\%)} = \frac{\text{Live weight gain (g)}}{\text{Feed consumed (g)}} \times 100$$

### Protein Efficiency Ratio (PER)

PER gives a measure of how well the diet provides the essential amino acids to the animal, in addition to the balance of the diet with respect to energy and protein.

$$\text{PER} = \frac{\text{Increase in live weight (g)}}{\text{Protein intake in feed (g)}}$$

PER values also measures the deposition of fat in addition to protein, therefore fatty fish can also be associated with high PER values, due to this constraint the **Net Protein**

**Utilisation** (NPU) is a more reliable index of the protein feed quality compared to PER.

$$\text{NPU (\%)} = \frac{\text{Protein gain in fish (g)}}{\text{Protein intake in feed (g)}} \times 100$$

### Specific Growth Rate (SGR)

SGR is defined as the relative instantaneous rate of growth resulting from the linear transformation of growth data, the equation used to calculate SGR is as follows.

$$\text{SGR \% body weight/day} = \frac{\ln W2 - \ln W1}{\text{Number of days}} \times 100$$

Where W1 = Initial weight

W2 = Final weight

### Digestibility coefficients

Digestibility of a diet or specific component of the feed was indirectly determined by the use of an inert marker (yttrium oxide), which was introduced into the feed at a level of 0.1% unless otherwise declared. The marker concentrates relative to the undigestible material constituting the faeces. The faecal marker concentrations relative to those in the diet provide a measure of the extent of digestibility of the diet/dietary component in question. **Apparent Digestibility Coefficient (ADC)** is determined as follows:

$$\text{ADC (\%)} = 100 - 100 \left( \frac{\% \text{ marker in diet}}{\% \text{ marker in faeces}} \times \frac{\% \text{ nutrient in faeces}}{\% \text{ nutrient in feed}} \right)$$

Where the nutrient refers to any component of the diet, such as protein or a particular mineral. During the digestibility trial (chapter 5) it was necessary to establish the individual ingredient digestibility of various protein sources. The procedure followed was first introduced by Cho *et al.* (1974), and involves the production of a reference diet for which the ADC is known. Then to prepare a test diet 30% of which is the test ingredient; the other 70% of this diet comprises of the reference diet. The ADC of any particular nutrient in question can then be established for this test diet using the following equation:

$$\text{ADC (\%)} = \frac{100}{30} \left( \text{nutrient digestibility of TD} - \frac{70}{100} \text{nutrient digestibility of RD} \right)$$

Where TD = Test diet and RD = Reference diet

#### **Apparent Net Nutrient/or Mineral Retention (ANMR)**

This expression is derived for the apparent retention or utilisation efficiency for any given nutrient.

$$\text{Apparent Net Mineral Retention ANMR\%} = \frac{W2 * \text{Min Conc.} - W1 * \text{Min Conc.} * 100}{\text{Total Feed Intake (g)} * \text{Min Conc.}}$$

Where W2 = final mean weight of fish  
W1 = initial mean weight of fish

The final and initial fish weights multiplied by the concentration of any nutrient (Mineral) provide the total amount of the specified nutrient within final and initial fish. The difference is the amount deposited or retained. When expressed over the mean total dietary intake, \*100, the expression provides the percent mineral retained relative to intake.

It is an apparent term due to the fact that endogenous losses of nutrient cannot be accounted. For mineral especially there would be a flux due to turnover of stored elements as these might not originate from the diet directly.

### **Hepato-Somatic Index (HSI)**

HSI is defined as the relative weight of the liver to the whole fish body weight. It is indicative of general hepatic status and health, and can be calculated as follows.

$$\text{HSI} = \frac{\text{Weight of liver (g)}}{\text{Whole body weight (g)}} \times 100$$

### **Statistical Analysis**

Statistical analysis was carried out using the 'GMAV 5' statistical software package.

Initially data was checked for homogeneous variances using a Cochran's test, a log transformation was applied to heterogeneous data before further analysis. Either a single factor ANOVA or 'nested' two-way ANOVA was conducted on the data in question.

Further multiple range testing was conducted using a Student-Newman-Keuls (SNK) test.

A probability level of  $P < 0.05$  was considered statistically significant. Regression lines, curves and their corresponding equations and R-squared values were fitted to data in Excel.

## **CHAPTER 3**

# **APPRAISAL OF COMMERCIAL DIETS OF VARYING NUTRITIONAL PROFILE WITH RESPECT TO MINERAL RETENTION AND AVAILABILITY**



## CHAPTER 3

### APPRAISAL OF COMMERCIAL DIETS OF VARYING NUTRITIONAL PROFILE WITH RESPECT TO MINERAL RETENTION AND AVAILABILITY FOR RAINBOW TROUT

#### 3.1 Introduction

##### 3.1.1 *The quality of fishmeal*

Fishmeal originates from many different sources and most high quality fishmeal products are derived from whole fish carcasses, providing a rich source of protein, energy and minerals. These are highly palatable and digestible but as a consequence are an expensive commodity compared to most alternatives e.g. soyabean and other plant protein concentrates. The commercial fish industry produces a substantial quantity of waste that cannot be used for human consumption, comprising mainly the fish carcass and remaining offal once filleted and processed. As disposal of this waste is often limited and costly, many fish processing plants manufacture fishmeal as a form of disposal. This type of fishmeal is usually classed as a high ash source, due to the substantial proportion of minerals contained in the meal which originate from the skeletal component of the filleted scrap. Additionally, they possess lower protein levels but are less costly to produce. These are often termed 'white fishmeals' compared to the higher oil, low ash 'brown fishmeals' that are mainly included in aquafeeds (Pike *et al.*, 1990).

Fishmeal based diets composed of filleted waste have potential use in aquaculture feeds, but the high levels of ash may cause restricted use. Studies by Ketola, (1979) have indicated that the use of high ash fish meal in diets for rainbow trout (*Oncorhynchus mykiss*) negatively affects zinc bioavailability. Additionally Satoh, (1987a) established that high ash fish meals can cause increased mortality, reduced growth and pathologies such as cataracts and skeletal malformations in rainbow trout (*O. mykiss*). Although Shearer and Hardy, (1987) reported that high dietary ash in feedstuffs is not detrimental in

trout diets as long as sufficient supplementary zinc was present. It was also found that a reduction in the bone content of scrap meal resulted in the phosphorus level becoming lower than is nutritionally required. Further research by Spinelli *et al.*, (1983) and Hardy *et al.*, (1984) has implied that zinc bioavailability can be influenced by other mineral levels such as calcium and phosphorus, in addition to dietary phytate (Gatlin and Phillips, 1989; Richardson *et al.*, 1985). Additionally, Shearer *et al.*, (1992) suggested that dietary ash levels of up to 17.5% were not deleterious when fed to juvenile Atlantic salmon (*Salmo salar*) providing that the dietary zinc level was 100ppm after the addition of a commercial mineral supplement.

### **3.1.2 Levels of dietary lipid**

The major source of energy in aquafeeds is lipid, as it contains more energy per unit weight than protein or carbohydrate. Consequently, they have gained considerable attention as key components of the diet (Hilton and Slinger, 1981). The maintenance energy requirements for salmonids are estimated by Kaushik and Medale, (1994) are 75-100 KJ kg<sup>-1</sup>(BW) d<sup>-1</sup>, with a large proportion of this demand being supplied from a lipid source. Fish, in addition to many vertebrate groups are unable to synthesise *n*-3 or *n*-6 fatty acids (essential fatty acids – EFA), which consequently have to be presented in the diet in adequate levels. Dietary lipids are supplied in various forms usually from a marine or vegetable source, the most commonly used source of supplemental oil being from fish extracted during the production of fishmeal, which is also rich in essential fatty acids. However, as resources from marine fisheries are declining, fish oil is becoming a very limited and expensive commodity. Consequently, nutritionists' are presently searching for alternative lipid sources, such as sunflower, rapeseed, and various grain related oils for inclusion in aquafeeds.

The level of lipid in practical trout diets was previously between 6 and 14% in the 1970's, but recent research has indicated that increased dietary levels of lipid may be beneficial to trout and salmon. This is mainly because fish preferentially utilise protein to lipid as an energy source, making it important to improve protein utilisation for tissue synthesis rather than energy release (Peres and Oliva-Teles, 1999). Kaushik, (1994) suggested that the dietary digestible protein to digestible energy ratio (DP/DE) plays an important role in protein and energy utilisation. By increasing the digestible energy content of diets by supplementation with more oil, a protein sparing effect has been demonstrated in salmonid fish by Cho and Kaushik, (1990), which leads to a reduction in the dietary protein level. In Atlantic salmon, feed and protein utilisation were improved whilst waste levels of nitrogen and phosphorus were reduced when fish were fed diets containing up to 30% lipid in an investigation conducted by Johnsen and Wandsvik, (1990). Although these findings have been reported for salmonid fish, data obtained for other fish species is either scarce or contradictory in nature (Lanari *et al.*, 1998; Peres and Oliva-Teles, 1999).

Although the increase of dietary lipid may be beneficial both economically and nutritionally careful consideration needs to be associated with this dietary change. It has been established that elevated dietary lipid may alter the body composition of the fish by increasing the lipid deposition. This has been noted in Atlantic salmon (*Salmo salar*) by Hillestrad and Johnsen, (1994) and Refstie *et al.*, (2001), and for Gilthead seabream (*Sparus aurata*) by Vergara *et al.*, (1999). Further research by Vergara *et al.*, (1999) has demonstrated that excesses of lipid have been found to cause hepatocyte abnormalities. The location and composition of fat deposits may also influence nutritional value, organoleptic properties and carcass storage time (Peres and Oliva-Teles, 1999).

### 3.1.3 *Experimental aims*

This first experimental study aims to assess the outcome of feeding juvenile rainbow trout (*Oncorhynchus mykiss*) various commercially manufactured diets. These diets varied with respect to fishmeal source and oil content. Three trials were conducted separately.

1. To compare two proprietary diets with feed formulations differing with respect to fishmeal source and mineral supplementation.
2. To determine if a relatively higher ash content in a diet affected growth, feed conversion, digestibility and mineral tissue and whole body retention levels.
3. To ascertain any effect of oil level on mineral and whole diet digestibility in fish of four different size classes (50, 100, 200 and 400g respectively).

Various parameters including FCR, SGR, proximate and elemental composition of whole body and tissues were used to evaluate the dietary treatments.

### 3.2a **Methods – Part A, the comparison of two commercial diets.**

#### 3.2.1a *Experimental facilities and diets*

Forty-five female rainbow trout (*Oncorhynchus mykiss*) with an initial weight of 50g were randomly allocated to each of six 400L fibreglass tanks. These tanks were suspended over a 4350L bio-filter that was an integral component of this closed recirculation system. Prior to grading, during an acclimation period fish were treated with an anti-parasite agent (0.05ppm malachite green) and fed a medicated antibiotic (2mg/kg dry wt oxalinic acid) diet. Freshwater flowed into each tank at 12L/min, water temperature was maintained at  $15\pm 1^{\circ}\text{C}$  by the use of a submerged coolant system. Various water parameters were monitored including pH, dissolved oxygen (DO), phosphorus, nitrite, nitrate and ammonia/ammonium. These were held within ranges tolerated by this species of fish as defined by Klontz *et al.*, (1983). The pH levels were maintained within the range of 6.5-

7.5 by the use of calcium carbonate and 'magnospheres' to aid in the buffering capacity of the water. Photoperiod was maintained at a 12hr light: 12hr dark regime, by a timed lighting regime.

Two proprietary commercially available diets, Trouw Royale Crystal (A) and Trouw Hi-performance (B) both with a 4.0mm pellet were the test diets evaluated for this trial being fed in triplication. These diets were produced on an industrial scale and are typical of those currently used in modern aquaculture practice. The diets varied in fishmeal source, one being formulated with high quality Scottish herring and the other a mixture of this fishmeal and industrial white fishmeal. Additionally diet A was supplemented with an industrially chosen mineral supplement, di-calcium phosphate, whilst diet B had no inorganic supplement of these elements. The test diet formulations are presented in table 3.1. with proximate compositions in table 3.2.

Table 3.1.  
Percentage composition of experimental diets.

Dietary Component	Diet A - % inclusion	Diet B - % inclusion
Industrial white fishmeal	-	25
Scottish herring fishmeal	40	22
Maize gluten	10	11
Soyabean meal –solvent extracted	15.5	15.5
Wheat	12.5	12.5
Fish Oil	20.5	13
Di-calcium phosphate	0.06	-
Vitamin/mineral premix* <sup>1</sup>	1	0.9
Vitamin E	0.56	0.34

\* Vitamin/ mineral premixes supplied in excess of NRC (1993) salmonid requirements

<sup>1</sup> Specification according to TROUW Aquaculture UK Ltd

Table 3.2.  
Proximate and elemental composition of experimental diets

Component (%)	Diet A	Diet B
Protein	41.33	42.33
Lipid	26.90	21.40
Moisture	8.38	9.34
Ash	7.30	7.58
Calcium	1.41	1.79
Phosphorus	1.08	1.22
Magnesium	0.18	0.19
Zinc	0.021	0.026

### 3.2.2a Feeding, sampling and analysis

After fish had been allocated to tanks, surplus fish were used for whole body composition (n=10), blood and organ collection (n=15). The following organs/structures were harvested; skin, eye, vertebrae, muscle, liver, kidney, spleen, intestine, gill arches including lamellae and heart (refer to chapter 2.5 for methodology). Due to the small size of these fish some of the organs were pooled together for analysis, therefore some n values are less than 15. This procedure was repeated at the termination of the trial with a sample of 6 fish per tank.

Total tank biomass was calculated, with this value and the figures produced in the manufacturer's guidelines, it was estimated that fish should be fed 2% and 1.82% of their body weight per day of Royale Crystal and Hi-Performance diets respectively. Differences in feed rations between diets were due to the unequal levels of digestible energy (20.39 MJ/Kg versus 21.46 MJ/Kg). It was decided to feed the fish equal energy rations in order to obtain comparable results on the basis of energy intake.

Fish were fed twice daily at 9.30am and 4.00pm for a period of 12 weeks. At biweekly intervals fish were re-weighed and feed rations adjusted to compensate for growth. Every fourth week, three fish were randomly taken from each tank and stored for whole body analysis. Throughout the growth trial; weight gain, specific growth rate (SGR), feed intake, feed conversion ratio (FCR), any mortalities and water quality were monitored. At the termination of the growth trial and following a 24-hour starvation period three fish from each tank were desanguinated from the caudal vein via heparinised syringes, and bile was removed from the gall bladder. Proximate composition and elemental analysis was conducted on appropriate samples following the procedures outlined in chapter 2.

### **3.2b Methods – Part B. High ash vs. standard ash diets.**

#### ***3.2.1b Experimental site, fish husbandry and feed***

This trial was conducted at Sparsholt Agricultural College, Winchester, Hampshire, during the spring of 1999. Circular fibre-glass tanks with a 1m diameter were held undercover and were subjected to ambient photoperiod, water was supplied from a natural bore hole at a temperature of 10°C. Prior to the start of the experiment female single strain (Hooke Springs) rainbow trout (*Oncorhynchus mykiss*), were fed a commercial feed during an acclimation period of two weeks. Two experimental feeds (diets C and D) were assessed during this trial therefore two sets of tanks in triplicate were utilised. Fish were size graded and randomly allocated to the six tanks, 22 fish per tank with mean weights of 178.3g and 181.6g for respective diets.

Table 3.3.  
Proximate and elemental composition of experimental diets

Component (%)	Diet C	Diet D
Protein	45.43	41.25
Lipid	24.96	26.31
Moisture	6.60	8.60
Ash	6.70	11.0
Calcium	1.68	3.19
Phosphorus	1.33	1.98
Magnesium	0.17	0.16
Zinc	0.023	0.022
Yttrium Oxide	0.124	0.130

Two experimental diets were produced industrially (Table 3.3), based on a commercial formulation for salmonid fish, i.e: a standard diet formulated with high quality fishmeal and the other with a high ash content fishmeal. Feeding was based on manufacturer's guidelines with an extra allowance of 20%, with two rations per day. Feed intake was adjusted on a weekly basis and fish were bulk weighed at biweekly intervals. The growth trial was conducted for a 9-week time period and growth performance and feed utilisation parameters recorded.

### ***3.2.2b Digestibility, sampling and analysis***

After completion of the growth phase of the trial at nine weeks, feeding continued using the same diets but with a coating of yttrium oxide  $Yt_2O_3$  (0.124% for diet C; 0.130% for diet D) as an inert marker. The marker (at a 0.1% level) was dissolved in water and sprayed evenly onto the pelleted feed and mixed for 3 minutes, the diet was then top-dressed with fish oil (0.5% of the total feed weight). Fish were fed the yttrium oxide diets for a further 3 days after which faeces were collected from the fish in each tank by manual stripping (Austreng, 1978). This procedure was repeated with another faecal collection



being carried out 8 days later with the two sets of faeces being pooled. Faeces were immediately frozen at -17°C, and transported frozen to the University of Plymouth where they were subsequently freeze-dried. Yttrium oxide and mineral content was determined by ICP-AES and apparent digestibility coefficients for the mineral and protein components were calculated following the procedures outlined in chapter 2.

A sample of 20 fish were taken from the common pool at day one, 10 were used for assessment of whole body mineral and proximate composition and the other 10 individuals for mineral composition of selected tissues, namely, the vertebrae, skin, operculum and muscle. At the end of the growth trial the aforementioned procedure was repeated with 20 fish being randomly selected from each dietary treatment for analysis. All fish selected for analysis were frozen at Sparsholt College and transported to the University of Plymouth where all analysis was carried out. Mineral determinations were by ICP-AES, with ash, protein, lipid and moisture being calculated following the procedures outlined in chapter 2.

### **3.2.c Methods – Part C. Diets with varying oil content.**

#### ***3.2.1c Experimental site, fish husbandry and feed***

This trial was conducted at Hatchland Fisheries, Greysshoot Lane, Rattery, Devon. Circular fibre-glass tanks (1m diameter) were located outside and were subjected to an ambient photoperiod. Water was supplied from a natural borehole at a temperature of 10°C to the whole farm. Additionally, two 8 metre concrete raceways were utilised. Female, single strain, rainbow trout (*Oncorhynchus mykiss*) were used for the trial. Prior to the experiment, fish were fed a standard commercial feed during an acclimation period of 10 days, in which the fish were placed in either tanks or raceways. As the tanks were limited to 12 in total, the four diets were tested in triplicate for each fish size. The process was then repeated with the next size of fish in a consecutive fashion. Fish were size graded, to an average of either: 50, 100 or 200g, and randomly allocated into the twelve tanks,

approximately 2kg of fish biomass was allocated to each tank. The largest 400g sized fish were located in raceways, initially they were placed in tanks, but the fish became stressed and mortalities became frequent making utilisation of the raceways necessary.

Four different 'off the shelf' commercial diets were being assessed as the experimental feeds, each diet varied in primarily in oil levels (8%, 21%, 26%, 30% respectively).

However, as these diets were formulated commercially the source of fishmeal between diets varied, although the overall protein contents were similar. Table 3.4 presents their mineral and proximate composition, and table 3.5 presents their formulations. As fish were being fed purely for the purpose of faecal collection, feeding was to satiation twice a day (early morning and late evening).

Table 3.4.  
Proximate and elemental composition of experimental diets evaluated in rainbow trout.

Component %	Amino Balance	Royale Crystal	High Performance	Standard Expanded
Protein	43.80	43.60	44.70	40.90
Lipid	27.30	26.30	21.70	7.70
Moisture	6.22	5.78	5.88	6.26
Ash	7.74	7.34	7.62	10.40
Calcium	1.21	1.13	1.06	0.78
Phosphorus	0.94	0.90	0.83	0.74
Magnesium	0.13	0.14	0.15	0.18
Zinc	0.089	0.018	0.020	0.022
(Yttrium oxide)	0.061	0.059	0.054	0.055

Table 3.5.  
Feed formulation of commercial diets evaluated in rainbow trout

Ingredient %	Amino Balance	Royale Crystal	High Performance	Standard Expanded
Fishmeal, Peruvian	9.80	5.75	38.42	17.36
Fishmeal, Scottish	9.80	5.75	-	-
Fishmeal, Icelandic	19.60	23.73	-	-
Fish Protein Concentrate	3.75	5.00	-	-
Fish Oil	24.12	18.45	13.01	-
Soya meal, (full fat)	-	15.07	15.00	21.40
Soya Protein Concentrate	5.00	-	-	-
Maize gluten meal	17.01	15.00	20.00	20.00
Whole wheat	10.00	10.46	12.88	30.61
Amino acids, vit & min premixes	0.92	0.84	0.69	0.62
Remix*	-	-	-	10.00

\*Remix:- This is the added fraction obtained from industrial sweepings of previous diet production in the feed mill.

### *3.2.2c Digestibility, sampling and analysis*

Diets utilised during this experimental trial were produced as part of a large scale commercial operation, consequently it was unfeasible to incorporate a dietary marker within the diet. Therefore, all diets were coated with the inert marker yttrium oxide, at an approximate level of 0.05% level. The marker was dissolved in water and sprayed evenly onto the pelleted feed, which was mixed for 5 minutes. The diets were then “top dressed” with gelatine coating and allowed to set. Fish were fed the yttrium oxide coated diets for 5 days before faeces were collected from the fish in each tank by manual stripping (Austreng, 1978). The stripping process was always conducted 14-16 hours after the feeding of the last meal in order to obtain continuity. This process was then repeated using the next size category of fish, for each of the four experimental diets evaluated in the trials. Faeces were immediately transported to the University of Plymouth at frozen at -17°C prior to freeze-drying. Yttrium oxide and mineral content was determined by ICP-AES and apparent digestibility coefficients for the mineral and protein components were calculated following the procedures outlined in chapter 2.

Statistical analysis was carried out using the ‘GMAV 5’ statistical software package to conduct either a single or two-way ANOVA on the appropriate data in question, after an exploratory Cochran’s test. Where paired comparisons were directly made a T-test was used as the statistical analysis. If further multiple range testing was necessary a Student-Newman-Keuls (SNK) test was applied to the data. A probability level of  $P < 0.05$  was considered statistically significant. For the dose response data (Part C) curves were fitted to the data in Excel (exponential, power, logarithmic or linear), with corresponding equations and R-squared values reported on each graph.

### 3.3a Results - Part A

#### 3.3.1a Mortality, growth, and feed efficiency

Feed acceptance was good for all tanks of fish, the rainbow trout fed the Royal Crystal diet grew from 52 to 201g during the course of the 72 day growth trial, with the fish fed Hi-performance growing similar levels from 51 to 204g. No significant differences were noted between diets for growth, weight gain, FCR or SGR; the values of which are summarised below in table 3.6. Total mortality during the experiment was less than 0.5% (9 fish), the majority of which occurred within 4 days of moving 3 tanks of fish due to a lighting strike and power failure. All other mortalities were not treatment related and all fish appeared to be visually healthy at the end and throughout the experiment. In each case the weight of the dead fish was recorded and feed allowances adjusted accordingly.

Table 3.6.

Weight gain, SGR and FCR of experimental fish after 72 days of growth, (n=3,  $\pm$  S.E).

Growth parameter	Royal Crystal diet	Hi-Performance diet
Initial weight, (g)	52.2 $\pm$ 0.7	51.0 $\pm$ 0.7
Final weight, (g)	201.3 $\pm$ 7.1	203.6 $\pm$ 7.2
Cumulative SGR	1.82 $\pm$ 0.03	1.87 $\pm$ 0.03
Weight gain % (of initial weight)	285.63	299.22
Cumulative FCR	0.93 $\pm$ 0.05	0.99 $\pm$ 0.05

### 3.3.2a Mineral status

Dietary treatment had a significant effect on the phosphorus (P) and calcium (Ca) concentrations of the average whole body mineral concentrations (Table 3.7), with values being highest for the initial fish then those fed the Royal Crystal diet (RC) compared with those fed the Hi-performance diet (HP). Although magnesium levels appeared to be higher in the RC dietary category, values were not significant.

Table 3.7.

Mean whole body distribution of minerals ( $\mu\text{g/g-wet weight}$ ) in the rainbow trout fed both experimental diets, ( $n=10, \pm \text{S.E.}$ ).

Mineral ( $\mu\text{g/g-wet weight}$ )	Initial fish	Royal Crystal	Hi-performance
Phosphorus	$4949 \pm 93^a$	$4712 \pm 98^a$	$4378 \pm 109^b$
Calcium	$4189 \pm 94^a$	$3926 \pm 173^a$	$3284 \pm 237^b$
Magnesium	$322 \pm 5.0^a$	$334 \pm 2.2^a$	$295 \pm 7.4^a$
Zinc	$27.0 \pm 1.8^a$	$27.0 \pm 2.0^a$	$27.0 \pm 1.0^a$

Unlike superscripts denote a significant statistical difference ANOVA  $P > 0.05$ .

The initial mean mineral concentrations for various soft organs and skeletal structures is presented in Table 3.8, which are ranked in order of highest to lowest phosphorus concentrations. As expected, values for phosphorus and calcium are the highest in samples containing skeletal structures such as the vertebrae, gill arch and skin (including scales). The calcium to phosphorus ratio in bone was estimated from the vertebrae to be 1.43. Values for phosphorus were similar ranging from 11.59 to 15.92 mg/g-dry weight for all major soft organs: corresponding values for calcium are very low with the exception of the eye at 3.83 mg/g-dry weight.

Table 3.8.  
 Mean body distribution of minerals (mg/g-dry weight) in the rainbow trout (initial fish), (n=10, except for heart and spleen where n=6,  $\pm$  S.E).

Organ	Phosphorus	Calcium	Magnesium	Zinc
Vertebrae	67.51 $\pm$ 2.76	96.40 $\pm$ 3.25	1.73 $\pm$ 0.09	0.093 $\pm$ 0.017
Gill	19.48 $\pm$ 0.66	31.60 $\pm$ 1.70	0.76 $\pm$ 0.03	0.13 $\pm$ 0.01
Skin	18.40 $\pm$ 0.43	10.74 $\pm$ 0.88	1.22 $\pm$ 0.07	0.59 $\pm$ 0.13
Liver	15.92 $\pm$ 0.24	0.17 $\pm$ 0.03	0.87 $\pm$ 0.02	0.09 $\pm$ 0.002
Kidney	15.65 $\pm$ 0.22	0.44 $\pm$ 0.17	0.90 $\pm$ 0.02	0.12 $\pm$ 0.009
Spleen	13.96 $\pm$ 0.56	0.17 $\pm$ 0.029	0.67 $\pm$ 0.06	0.17 $\pm$ 0.04
Muscle	13.39 $\pm$ 0.21	1.26 $\pm$ 0.12	1.73 $\pm$ 0.04	0.01 $\pm$ 0.001
Intestine	11.88 $\pm$ 0.21	0.50 $\pm$ 0.09	0.78 $\pm$ 0.03	0.92 $\pm$ 0.04
Heart	11.59 $\pm$ 0.25	0.22 $\pm$ 0.014	0.83 $\pm$ 0.05	0.081 $\pm$ 0.008
Eye	6.73 $\pm$ 0.15	3.83 $\pm$ 0.164	0.59 $\pm$ 0.02	0.47 $\pm$ 0.02

The vertebrae, muscle and skin contain the highest levels of magnesium, with the intestine, skin and eye containing the highest levels of zinc. Of all the four minerals determined, it is apparent that phosphorus is the most abundant mineral in the fish body relative to the remaining elements.

When the body mineral distribution was compared between the initial and final fish (table 3.9), it was observed that the mineral concentrations in the various organs were similar. With significant differences only being demonstrated in the skeletal structures, although significantly higher levels of phosphorus were distributed in the spleen while lower levels were seen in the liver ( $P < 0.05$ , T-test).

Figure 3.1, illustrates the mineral distributions of phosphorus and calcium in the vertebral column from samples of initial and final fish (both treatments). Both minerals were significantly higher ( $P < 0.05$ , T-test) in the fish fed the Royal Crystal diet when compared with the fish fed the Hi-performance diet. When compared with the initial fish, the calcium levels in both sets of final fish had decreased whereas the phosphorus levels had increased, producing a calcium to phosphorus bone ratio of approximately 1:1.



Table 3.9.

Mean body distribution of minerals (mg/g-dry weight) in rainbow trout fed both Royal Crystal (RC) and Hi-performance (HP) diets, (n=8,  $\pm$  S.E).

Organ	Diet	Phosphorus	Calcium	Magnesium	Zinc
Vertebrae	RC	80.35 $\pm$ 1.23 *	79.24 $\pm$ 3.11 *	2.15 $\pm$ 0.06 *	0.11 $\pm$ 0.003 *
	HP	71.94 $\pm$ 2.08	70.24 $\pm$ 2.32	1.92 $\pm$ 0.06	0.099 $\pm$ 0.004
Gill	RC	26.04 $\pm$ 1.0 *	14.70 $\pm$ 1.06 *	1.63 $\pm$ 0.07 *	0.76 $\pm$ 0.05
	HP	21.20 $\pm$ 0.51	11.75 $\pm$ 0.92	1.34 $\pm$ 0.05	0.77 $\pm$ 0.04
Skin	RC	17.77 $\pm$ 0.28	26.47 $\pm$ 1.52	0.83 $\pm$ 0.03	0.11 $\pm$ 0.13
	HP	19.92 $\pm$ 0.62 *	30.02 $\pm$ 0.98 *	0.93 $\pm$ 0.04 *	0.09 $\pm$ 0.05
Liver	RC	13.69 $\pm$ 0.75 *	0.19 $\pm$ 0.02 *	0.70 $\pm$ 0.02 *	0.09 $\pm$ 0.005
	HP	12.51 $\pm$ 0.05	0.15 $\pm$ 0.01	0.67 $\pm$ 0.01	0.09 $\pm$ 0.002
Kidney	RC	15.18 $\pm$ 0.65	0.33 $\pm$ 0.02	0.79 $\pm$ 0.01	0.11 $\pm$ 0.03
	HP	14.54 $\pm$ 0.50	0.38 $\pm$ 0.04	0.77 $\pm$ 0.02	0.12 $\pm$ 0.008
Spleen	RC	16.29 $\pm$ 0.34 *	0.30 $\pm$ 0.06 *	0.85 $\pm$ 0.01 *	0.13 $\pm$ 0.01
	HP	15.37 $\pm$ 0.27	0.18 $\pm$ 0.04	0.78 $\pm$ 0.03	0.13 $\pm$ 0.01
Muscle	RC	14.17 $\pm$ 0.55 *	0.99 $\pm$ 0.17	1.47 $\pm$ 0.05 *	0.02 $\pm$ 0.005
	HP	12.82 $\pm$ 0.42	0.55 $\pm$ 0.14	1.39 $\pm$ 0.04	0.02 $\pm$ 0.003
Intestine	RC	14.10 $\pm$ 0.33 *	0.34 $\pm$ 0.03 *	0.81 $\pm$ 0.02	2.20 $\pm$ 0.29 *
	HP	14.42 $\pm$ 0.45	0.25 $\pm$ 0.009	0.75 $\pm$ 0.02	1.66 $\pm$ 0.07
Heart	RC	11.88 $\pm$ 0.60	0.25 $\pm$ 0.09	0.83 $\pm$ 0.03 *	0.11 $\pm$ 0.006 *
	HP	10.57 $\pm$ 0.34	0.22 $\pm$ 0.02	0.74 $\pm$ 0.05	0.09 $\pm$ 0.007
Eye	RC	6.85 $\pm$ 0.24	3.78 $\pm$ 0.18	0.49 $\pm$ 0.17	0.35 $\pm$ 0.02
	HP	6.82 $\pm$ 0.71	3.80 $\pm$ 0.37	0.48 $\pm$ 0.05	0.37 $\pm$ 0.04
Ovary	RC	18.50 $\pm$ 1.02 *	0.31 $\pm$ 0.06 *	1.07 $\pm$ 0.05 *	0.28 $\pm$ 0.02 *
	HP	14.52 $\pm$ 0.95	0.27 $\pm$ 0.01	0.82 $\pm$ 0.56	0.22 $\pm$ 0.02

Asterisks denote a significant statistical difference,  $P < 0.05$ , T-test.

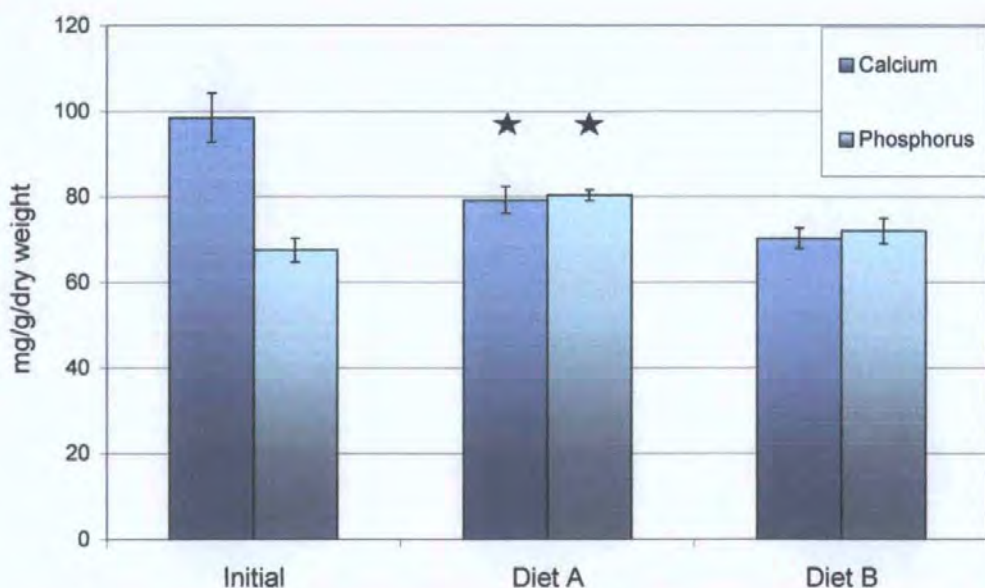


Figure 3.1 Concentrations of vertebral calcium and phosphorus, for initial and final fish fed Royal Crystal (A) and Hi-performance (B) diets, (n=8,  $\pm$  S.E, asterisk denotes statistical difference,  $P < 0.05$ , T-test).

By the end of the trial the fish had grown substantially and had started to mature.

Therefore, it was possible to sample the ovaries (although still small) for both treatments (Table 3.9). When compared with fish fed the Hi-performance diet, fish fed the Royal Crystal diet generally had higher concentrations of phosphorus, calcium, magnesium and zinc distributed through-out their hard and soft organs, with exceptions of the skin, kidney and eye (Table 3.9). Conversely, fish fed the Hi-performance diet had significantly higher levels of phosphorus (19.92 vs. 17.77 mg/g-dry weight), calcium (30.02 vs. 26.47 mg/g-dry weight) and magnesium (0.93 vs. 0.83 mg/g-dry weight) in the skin (Table 3.9,  $P < 0.05$ , T-test).

Blood glucose and phosphorus levels were not significantly different between treatments. However, a statistically significant difference was found for phosphorus levels in the bile between diets, with 0.75 Mmol/l for the Royal Crystal diet compared to a greater level of 1.22 Mmol/l for the Hi-performance fish ( $P < 0.05$ , T-test, table 3.10).

Table 3.10.

Blood glucose, phosphorus and bile phosphorus levels for rainbow trout fed Royal Crystal (RC) and Hi-Performance (HP) diets, (n=15,  $\pm$  S.E).

Mmol/L	Royal Crystal	Hi-performance
Blood glucose	4.50 $\pm$ 0.59	4.01 $\pm$ 0.21
Blood phosphorus	5.03 $\pm$ 0.24	5.05 $\pm$ 0.31
Bile phosphorus	0.75 $\pm$ 0.07	1.20 $\pm$ 0.15*

Asterisk denotes a significant statistical difference  $P < 0.05$ , T-test.

### 3.3.3a Mineral digestibility

Table 3.11 presents data on the apparent digestibility for selected minerals (P, Ca, Mg and Zn) from the experimental fish fed both the Royal Crystal and Hi-Performance diets.

Digestibility values for fish fed the Royal Crystal diet were significantly higher than those of fish fed the Hi-Performance diet. Digestibility values given for calcium and zinc from fish fed the Hi-Performance diet are negative (-9.66 and -2.5% respectively) indicating that the fish were excreting higher levels of this mineral than they are ingesting from the dietary source.

Table 3.11.

Apparent digestibility coefficients (%) for minerals for rainbow trout fed both Royal Crystal and Hi-Performance diets for 72 days, (n=3, ± S.E).

Mineral	Royal Crystal diet	Hi-Performance diet
Phosphorus	49.35 ± 2.34*	26.66 ± 0.33
Calcium	13.28 ± 3.66*	-9.66 ± 2.11
Magnesium	63.76 ± 4.71*	43.64 ± 1.96
Zinc	11.10 ± 3.22*	-2.5 ± 0.75

Asterisks denote a significant statistical difference  $P < 0.05$ , T-test.

For both dietary treatments magnesium digestibility appeared to be the highest, then phosphorus followed by calcium and zinc, the latter two of which gave very poor digestibility figures.

Faecal mineral concentrations from fish fed both dietary treatments are illustrated in figure 3.2. Fish fed the Hi-Performance diets excreted significantly ( $P < 0.05$ ) higher levels of phosphorus, calcium and magnesium. Although zinc was not depicted on this graph due to the comparatively low values when compared with the other minerals, levels between diets were statistically higher for the Hi-performance diet with a value of 0.728 mg/g-dry weight compared to 0.675 mg/g-dry weight for the Royal Crystal diet.

The apparent net mineral retention for trout fed both Royal Crystal and Hi-performance diets are given in Table 3.12. Percentage values for all minerals were significantly higher for those fish fed the Royal Crystal diet compared with the Hi-performance diet.

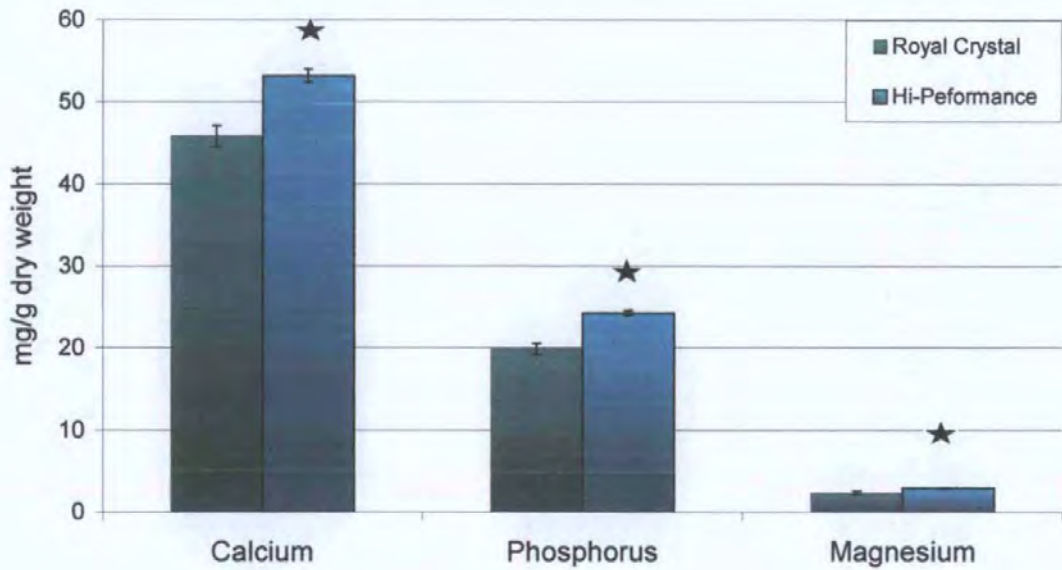


Figure 3.2 Mineral concentrations of faecal material from rainbow trout fed Royal Crystal and Hi-Performance diets (n=3,  $\pm$  S.E, asterisk denotes statistical difference, ANOVA  $P < 0.05$  )

Table 3.12.

Apparent net mineral retention (%) for rainbow trout fed both Royal Crystal and Hi-Performance diets for 72 days (n=3).

Mineral	Royal Crystal diet	Hi-Performance diet
Phosphorus	45.83*	34.67
Calcium	29.18*	16.82
Magnesium	20.20*	15.20
Zinc	13.86*	10.52

Asterisks denote a significant statistical difference,  $P < 0.05$ , T-test.

### 3.3b Results - Part B

#### 3.3.1b Mortality, growth, feed efficiency and digestibility

The feed intake and growth parameters over the nine weeks of the trial are summarised in Table 3.13. Feed conversions ratios for fish fed on both experimental diets were cumulatively below 1, indicating a high feed efficiency and good growth with fish more than doubling their weight during the period of the trial.

Table 3.13.

Weight gain, feed intake, SGR and FCR of experimental fish after 9 weeks, (n=3,  $\pm$  S.E).

Growth parameter	Standard ash diet	High ash diet
Initial weight, (g)	178.33 $\pm$ 1.78	182.5 $\pm$ 0.71
Final weight, (g)	405.67 $\pm$ 9.63	397.5 $\pm$ 0.71
Feed intake, g fish <sup>-1</sup>	191.36 $\pm$ 0.99	191.5 $\pm$ 0.84
Weight gain (% of initial weight)	127.33 $\pm$ 7.56	118.0 $\pm$ 1.41
Cumulative SGR	1.30 $\pm$ 0.05	1.24 $\pm$ 0.01
Cumulative FCR	0.84 $\pm$ 0.04	0.89 $\pm$ 0.0

There was no significant difference between treatments for weight gain, feed intake, SGR or FCR. Fish accepted both diets C (standard ash) and D (high ash) readily, there was no mortality, fish grew well in parallel and no behavioural differences were observed between the treatments.

As the data in table 3.14 indicates, protein digestibility did not differ significantly between treatments these protein ADC's were less than expected being 68.4 and 69.2 for diets C and D respectively. However, it is clear that the mineral digestibility profile was appreciably and significantly different for fish fed the high ash diet when compared with

the fish on the standard ash diet, as the majority of these digestibility figures were negative values.

Table 3.14.

Protein and mineral apparent digestibility coefficients for rainbow trout fed standard and high ash diets for 9 weeks, (n=3).

Apparent digestibility coefficient % - (ADC)	Standard ash diet	High ash diet
Calcium	1.38*	-16.15
Magnesium	70.45*	41.18
Phosphorus	45.48*	-4.71
Zinc	9.15*	-34.66
Protein	68.40	69.20

Asterisks denote a significant statistical difference,  $P < 0.05$ , T-test.

Table 3.15.

Mineral concentrations of faecal material from rainbow trout fed standard and high ash diets for 9 weeks (n=3,  $\pm$  S.E).

Mineral mg/g – dry weight	Standard ash diet	High ash diet
Calcium	63.07 $\pm$ 3.17	126.38 $\pm$ 3.87*
Magnesium	1.93 $\pm$ 0.04	3.19 $\pm$ 0.12*
Phosphorus	27.71 $\pm$ 0.25	70.92 $\pm$ 1.34*
Zinc	0.80 $\pm$ 0.02	1.02 $\pm$ 0.03*

Asterisks denote a significant statistical difference,  $P < 0.05$ , T-test.

### 3.3.2b Mineral status of the fish

Table 3.16 shows the data relating to the mineral levels in the whole-body of rainbow trout. Initial mean whole-body Ca, Mg, P and Zn concentrations were significantly higher, when compared with those for either fish fed diets C or D at the end of the growth trial. Significant differences were also noted between the fish on the two treatments, with the fish fed the high ash diet having an overall lower mineral status by the end of the trial.

Table 3.16.

Whole-body Ca, Mg, P and Zn concentrations for initial fish and rainbow trout fed either a standard or high ash level diet at end of trial, (n=10,  $\pm$  S.E).

Mineral level ( $\mu\text{g/g-wet weight}$ )	Initial fish	Standard ash diet	High ash diet
Calcium	5037 $\pm$ 174 <sup>ab</sup>	5520 $\pm$ 193 <sup>a</sup>	4925 $\pm$ 189 <sup>b</sup>
Magnesium	328 $\pm$ 11 <sup>a</sup>	323 $\pm$ 7 <sup>b</sup>	291 $\pm$ 10 <sup>ac</sup>
Phosphorus	4793 $\pm$ 115 <sup>a</sup>	4714 $\pm$ 125 <sup>b</sup>	4275 $\pm$ 112 <sup>ac</sup>
Zinc	25.0 $\pm$ 1.2 <sup>a</sup>	21.4 $\pm$ 0.6 <sup>b</sup>	18.8 $\pm$ 0.4 <sup>c</sup>

Unlike superscripts denote a significant statistical difference  $P < 0.05$ , ANOVA.

Table 3.17.

Apparent net mineral retention (%) for rainbow trout fed both standard and high ash diets for the growth trial, (n=3).

	Standard ash diet	High ash diet
Phosphorus	27.51*	16.52
Calcium	26.44*	12.22
Magnesium	14.66*	13.96
Zinc	12.63*	5.98

Asterisks denote a significant statistical difference,  $P < 0.05$ , T-test.

Data for the apparent net mineral retention between diets is presented in the table 3.17 above. Retention values for all minerals are statistically higher for the fish fed the standard ash diet with the exception of magnesium. Data relating the mineral concentrations for various organs and skeletal structures from rainbow trout fed either a standard ash level or high ash level diet, is presented in table 3.18. In general, fish fed the standard ash diets had higher levels of minerals in their soft organs (muscle, kidney and liver), with the exception of a higher calcium concentration in the muscle of fish fed the high ash diet. The mineral levels related to the skeletal structures (vertebrae, skin with scales and operculum) were similar between treatments, the only differences being for vertebral phosphorus (76.96 vs. 73.89 mg/g-dry weight), and operculum phosphorus and zinc levels (109.76 and 0.18 mg/g-dry weight respectively).



Table 3.18

The Ca, Mg, P and Zn concentrations for various organs and skeletal structures from rainbow trout fed either a standard (SA) or high (HA) ash level diet (n=12,  $\pm$  S.E).

	Calcium mg/g-dwt	Magnesium mg/g-dwt	Phosphorus mg/g-dwt	Zinc mg/g-dwt
Vertebrae (SA)	110.27 $\pm$ 1.17	2.57 $\pm$ 0.06	73.89 $\pm$ 0.73	0.08 $\pm$ 0.003
Vertebrae (HA)	112.86 $\pm$ 1.21	2.65 $\pm$ 0.17	76.96 $\pm$ 0.1*	0.08 $\pm$ 0.002
Skin (SA)	26.73 $\pm$ 1.19	0.86 $\pm$ 0.04	17.14 $\pm$ 0.72	0.069 $\pm$ 0.003
Skin (HA)	26.13 $\pm$ 1.09	0.96 $\pm$ 0.06	17.64 $\pm$ 0.73	0.09 $\pm$ 0.004
Operculum (SA)	152.17 $\pm$ 2.59	3.70 $\pm$ 0.05	105.52 $\pm$ 1.35	0.14 $\pm$ 0.004
Operculum (HA)	152.53 $\pm$ 1.85	3.82 $\pm$ 0.06	109.76 $\pm$ 1.53*	0.18 $\pm$ 0.007*
Muscle (SA)	0.66 $\pm$ 0.06	1.14 $\pm$ 0.02*	9.75 $\pm$ 0.17*	0.014 $\pm$ 0.004
Muscle (HA)	0.81 $\pm$ 0.04*	1.07 $\pm$ 0.02	9.27 $\pm$ 0.21	0.014 $\pm$ 0.004
Kidney (SA)	0.85 $\pm$ 0.04*	0.64 $\pm$ 0.01*	13.32 $\pm$ 0.16*	0.087 $\pm$ 0.002*
Kidney (HA)	0.74 $\pm$ 0.05	0.57 $\pm$ 0.04	11.59 $\pm$ 0.55	0.079 $\pm$ 0.003
Liver (SA)	0.34 $\pm$ 0.03*	0.44 $\pm$ 0.02*	9.31 $\pm$ 0.3*	0.066 $\pm$ 0.005*
Liver (HA)	0.28 $\pm$ 0.02	0.38 $\pm$ 0.02	8.48 $\pm$ 0.29	0.055 $\pm$ 0.003

Asterisks denote a significant statistical difference between diets,  $P < 0.05$ , ANOVA.

These three parameters were all significantly higher ( $P < 0.05$ , T-test) for fish fed the high ash diet in comparison with the standard ash diet fish. Therefore it could be suggested that no obvious trends in mineral levels were seen in the skeletal structures between treatments

### **3.3c Results - Part C**

#### ***3.3.1c Mineral and protein digestibility***

Feed acceptance during the trial was good, although an extended acclimation period of fifteen days was necessary for the 200g sized fish, as they were unaccustomed to containment in a tank after being in a pond. No mortality occurred for fish sizes 50, 100 and 400g, but three fish from the 200g group of fish died, probably due to hierarchical fighting developing in the tanks.

The apparent digestibility coefficients for protein are presented in table 3.19, the 50g and 400g fish produced a similar set of results throughout all levels of dietary oil, with ADC's ranging from 82 to 88%. Both the 100 and 200g fish exhibited lower ADC's in general for all dietary treatments. Levels were particularly low for the 200g fish, which ranged from 66 to 75%. This may be due to the fact that these fish were unable to adapt to the tank's confinement resulting in high stress and low digestibility values.

Digestibility figures for most of the fish categories follow a general trend with digestibility being significantly higher in the 8 and 30% oil diets, compared with the 20 and 26% diets ( $P < 0.05$ , T-test). This is clearly demonstrated in the 50 g fish size category where digestibility significantly decreases from 86 % for the 8% oil diet to 82% for the 20 and 26% diets and then increases to 88% for the 30% diet. For the 200g fish digestibility coefficients are initially reduced at 66% and remain at this low level for three of the four dietary oil levels, significantly increasing to 75% for the 30% oil diet ( $P < 0.05$ , T-test).

Table 3.19. Protein apparent digestibility coefficients for different sized rainbow trout fed diets with varying oil level, (n=8, except for 400g fish where n=3,  $\pm$  S.E).

Fish size (g)	8% oil	20% oil	26% oil	30%oil
50	86.72 $\pm$ 0.33 <sup>a</sup>	82.62 $\pm$ 0.75 <sup>b</sup>	82.61 $\pm$ 0.43 <sup>b</sup>	88.55 $\pm$ 0.58 <sup>a</sup>
100	82.26 $\pm$ 0.58 <sup>a</sup>	70.14 $\pm$ 0.43 <sup>a</sup>	67.13 $\pm$ 1.68 <sup>c</sup>	71.48 $\pm$ 0.84 <sup>b</sup>
200	66.02 $\pm$ 0.87 <sup>a</sup>	66.13 $\pm$ 1.13 <sup>a</sup>	66.95 $\pm$ 1.10 <sup>a</sup>	75.53 $\pm$ 0.95 <sup>b</sup>
400	84.54 $\pm$ 0.58 <sup>a</sup>	80.79 $\pm$ 0.99 <sup>b</sup>	83.57 $\pm$ 0.20 <sup>a</sup>	84.19 $\pm$ 0.94 <sup>a</sup>

Unlike superscripts denote a significant statistical difference,  $P < 0.05$ , ANOVA.

Apparent digestibility coefficients (ADC's) for the dietary minerals components of the diet, namely the phosphorus, calcium, magnesium and zinc components of the diets are illustrated in a series of four graphs (figures 3.3 to 3.6). For all size categories the apparent mineral digestibility coefficients for magnesium and phosphorus are clearly higher than the corresponding values for calcium and zinc.

The mineral ADC's for the two smaller sizes of fish (50g and 100g, figures 3.3 and 3.4) appear to all follow a similar general trend, an increase in dietary oil level results in a significant increase in mineral digestibility. For the 50g fish, all minerals ADC's were best fitted to an exponential curve. Whereas mineral ADC's for 100g were fitted to a range of mathematical curves, power, exponential, log and linear. Mineral digestibilities for the 50g fish are at least 30% higher for the all minerals at all oil levels, when compared with the other fish size categories, with the exception for the 30% dietary oil treatment where differences are only approximately 10% between mineral sets.

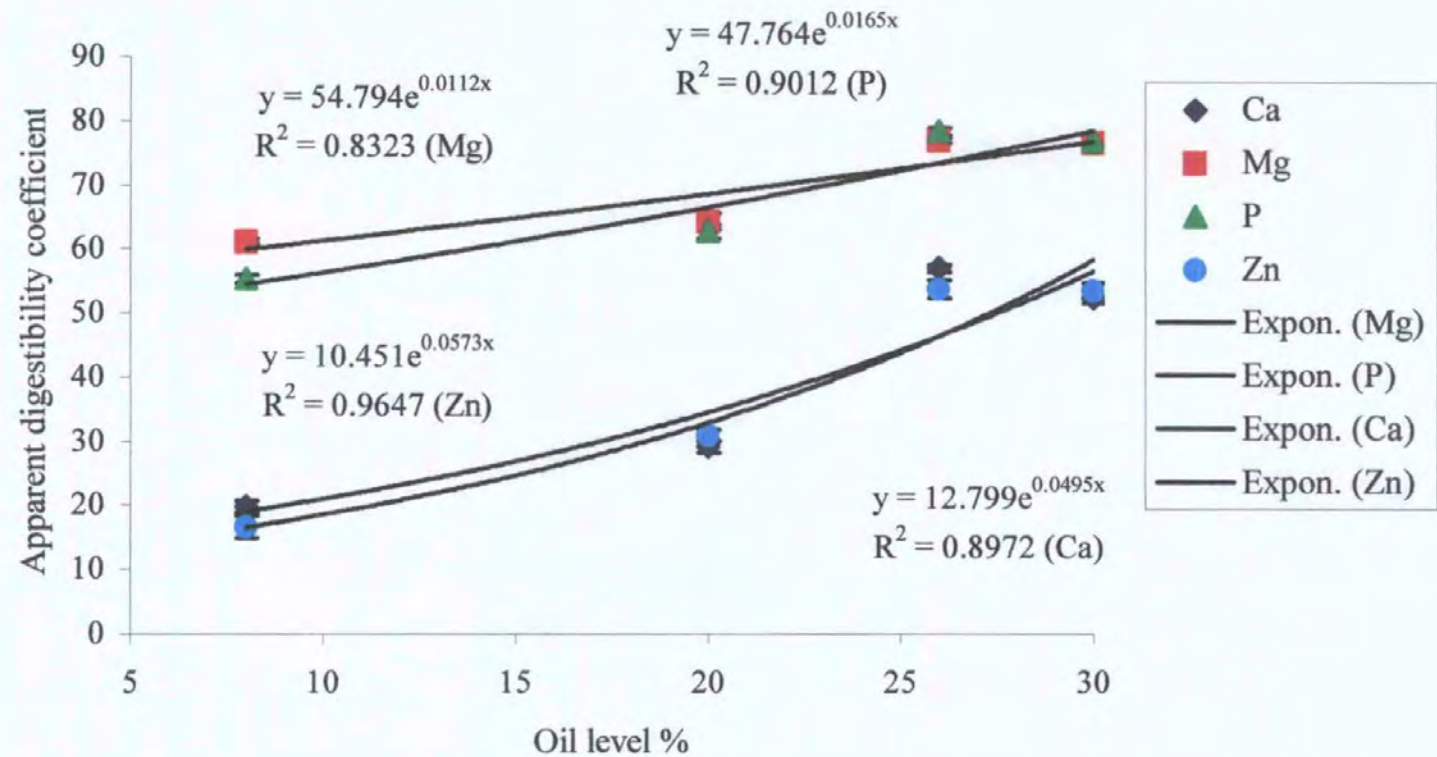


Figure 3.3 Apparent digestibility coefficients of minerals for 50g rainbow trout fed diets with varying oil level (n=9, error bars denote S.E.)

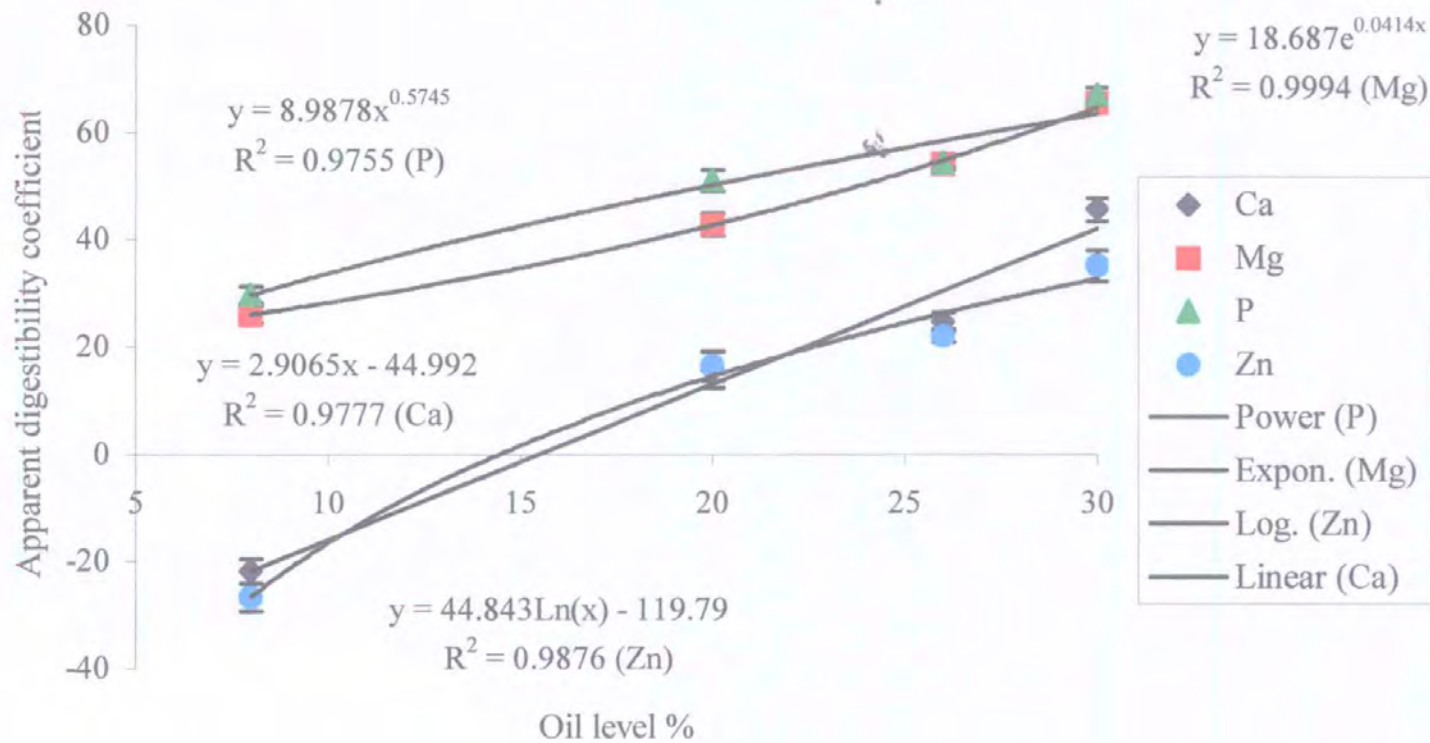


Figure 3.4 Apparent digestibility coefficients of minerals for 100g rainbow trout fed diets with varying oil level (n=9, error bars denote S.E.)

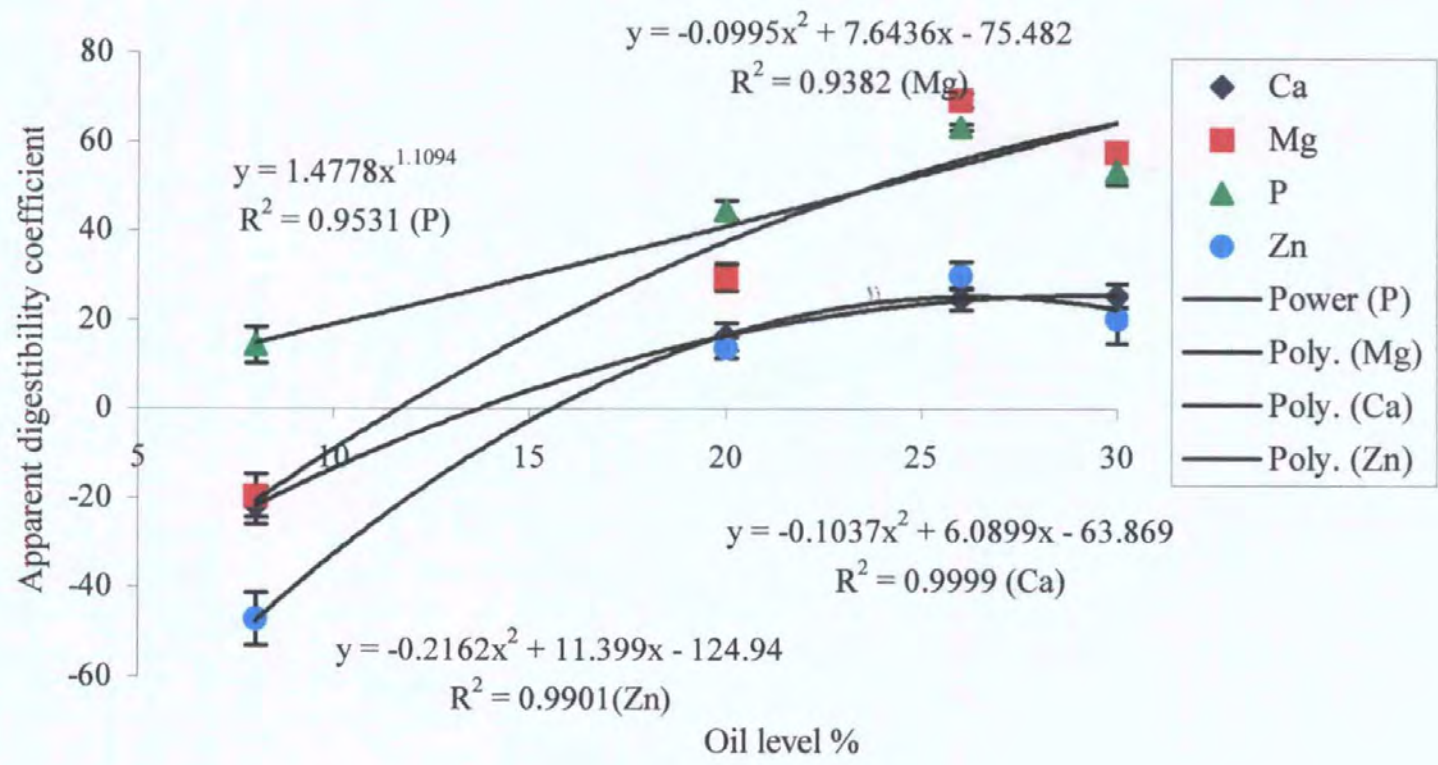


Figure 3.5 Apparent digestibility coefficients of minerals for 200g rainbow trout fed diets with varying oil level (n=9, error bars denote S.E).

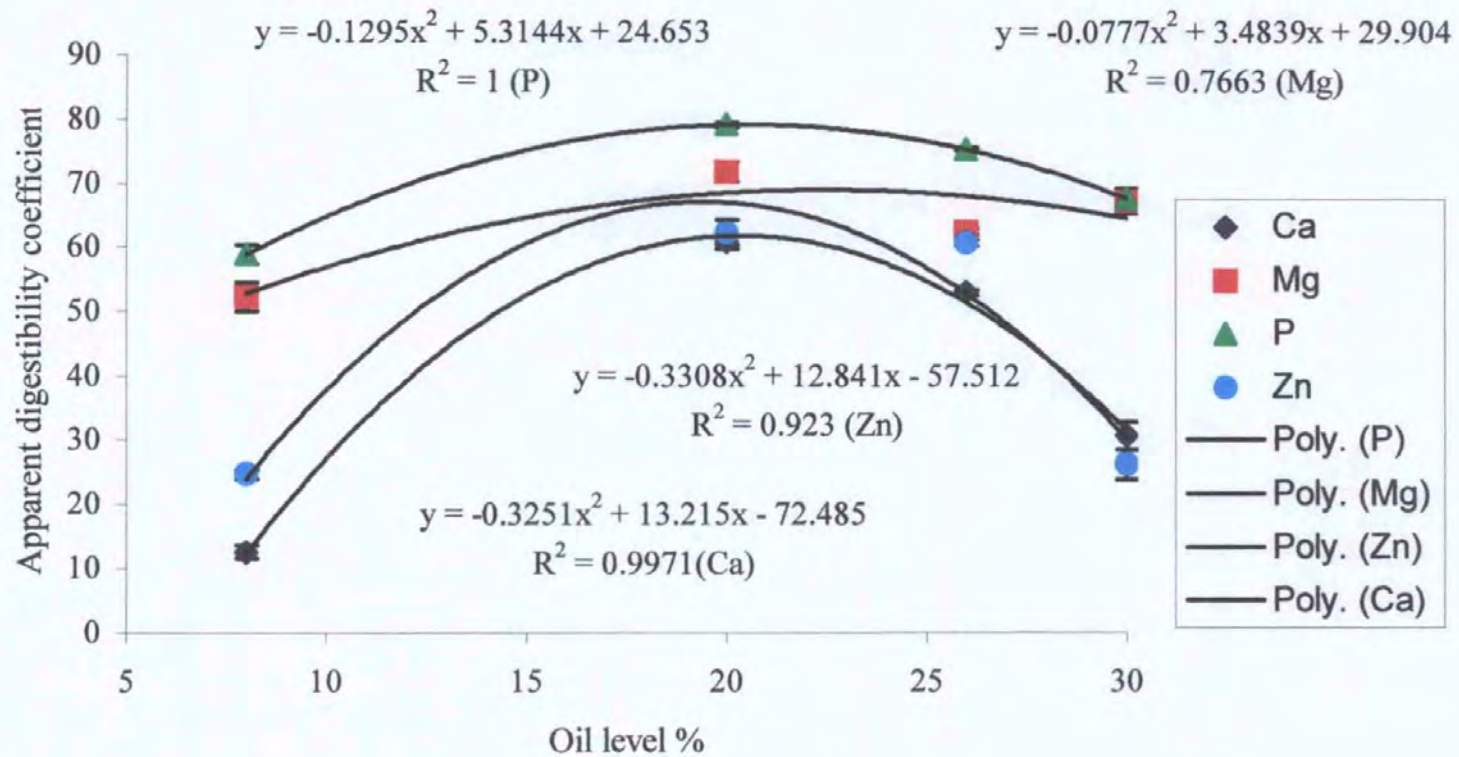


Figure 3.6 Apparent digestibility coefficients of minerals for 400g rainbow trout fed diets with varying oil level (n=3, error bars denote S.E).

The larger rainbow trout (200 and 400g, figures 3.5 and 3.6) utilised in this digestibility trial produced different digestibility profiles when compared to the smaller 50 and 100g fish. The general trend that was observed for the larger fish involved a significant increase in mineral digestibility for all minerals from the 8% to 20% oil diets. The 200g fish exhibited a slight increase in mineral digestibility from 20% to 26% dietary oil, conversely the 400g fish exhibited a slight decrease in mineral digestibility between these two respective dietary treatments. Both sets of trout then displayed a noticeable fall in apparent digestibility for all minerals on the 30% oil diet. With the exception of phosphorus digestibility, which follows a power curve for 200g fish, all other mineral digestibilities for 200 and 400g fish were fitted to polynomial parabolic curves. Compared with the smaller 50 and 100g fish, no clear distinction between P, Mg and then Ca and Zn digestibility values was observed. The only exception being that the 200g fish showed particularly poor digestibility resulting in negative figures. The results show that both dietary oil level and fish size have the ability to affect mineral digestibility, particularly in juvenile fish rainbow trout.

### **3.4 General Discussion**

#### ***3.4.1 The suitability of using fish meals from differing sources in diets for rainbow trout***

The primary aim of the first section of this experimental chapter was to assess the outcome of feeding juvenile rainbow trout defined 'off the shelf' commercially manufactured fish feeds. The diets varied in fishmeal source and phosphorus source, one being formulated with high quality brown fishmeal and supplemented with di-calcium phosphate (Royal Crystal), and the other with a mixture of brown fishmeal (22%) and an industrial white fishmeal (25%) with no mineral supplement (Hi-performance). The Hi-performance diet had significantly higher levels of ash, and all four minerals (P, Ca, Mg, Zn), whereas the lipid levels were lower when compared to the Royal Crystal diet. In general, the results from the first two experimental trials were very similar. Data obtained from the first 'in



house' trial was confirmed by the second trial conducted at the Sparsholt College fish trials unit. As these commercial diets do not produce any differences or impairment in growth and feed efficiency it can be suggested that both diets provide adequate levels of energy and nutrients to support growth and maintain health.

Whole body concentrations of P, Ca, Mg and Zn observed in the first two experimental trials, agree with those reported in various literature for rainbow trout (*Oncorhynchus mykiss*), (Shearer, 1984; Shearer, 1989; Shearer and Hardy, 1987; Skonberg *et al.*, 1997; Sugiura *et al.*, 2000b). Shearer *et al.*, (1994) investigated the body composition of Atlantic salmon, (*Salmo salar*) and reported similar mineral concentrations to those previously reported for rainbow trout, which also agree with those shown in this work. In general, the initial whole body concentrations were higher compared with the larger fish at the end of each trial. This pattern for phosphorus was observed by Ronsholdt, (1995) who stated that for rainbow trout, phosphorus content decreased with increasing K-factor which increased with weight, therefore phosphorus content indirectly decreases with increasing weight and volume. Various scientific investigations by Shearer in 1984 and later in 1994 also confirmed such findings for rainbow trout and Atlantic salmon (*S. salar*). It was observed that P and Ca concentrations in whole fish fed the Royal Crystal diet were higher than those in fish fed the Hi-performance formulation, and that fish fed the standard ash diet had significantly higher levels of all four minerals when compared with the high ash diet. These results suggest that even though these diets (Hi-performance and high ash) contained higher mineral levels, the retention of these minerals were at lower levels when compared with the 'standard' or control diets.

These results could be extrapolated further to suggest that it could be the ash level of the diet that influenced the mineral metabolism of these fish, which confirms the earlier findings of Shearer and Hardy, (1987) who found that high-ash diets could reduce whole

body concentrations of zinc. Sugiura *et al.*, (2000a) also reported that by using high-ash products such as white fishmeal (including skin and bone) in diets for rainbow trout the availabilities of phosphorus, calcium, magnesium, and zinc were appreciably depressed (11.8%, 4.9%, -8.1%, and -63.6% respectively), when compared with low-ash deboned white fishmeal (36%, 12.1%, 71.9%, and 11.9% respectively). A further study by Sugiura *et al.*, (2000a) demonstrated that when rainbow trout were fed diets containing incremental concentrations of fish bone at 0, 2, 5, and 10% inclusion levels. The apparent availabilities of phosphorus, calcium, magnesium and iron decreased (74%, 96%, 33% and 98% respectively) as fish bone content in the diet increased to 10%. Therefore, it could be concluded that as the bone fraction (ash level) of a diet increases, there is a relative decrease in mineral availability, which agrees with a similar statement from Rodehutschord *et al.*, (2000).

Research investigating the apparent availability of minerals in high-ash diets is limited, and the mechanisms responsible for reduced mineral availability cannot be accounted for, although the presence of high levels of Ca and P appear to be a primary contributing factor (Hardy *et al.*, 1984). In this series of studies availability of P, Ca, Mg and Zn was reduced when fish were fed the high-ash diet (11%). This is in agreement with previous reports by Satoh *et al.*, (1987a) and Shearer *et al.*, (1992) that high ash levels in the region of at least 12-18% of the diet result in a reduction in zinc availability.

One explanation for the reduced mineral levels in the presence of high ash, particularly the effects on zinc and magnesium, could be due to chelation with other compounds in the diet. Some molecules have the ability to form ionic complexes with certain minerals, and zinc is highly susceptible to this form of mineral bonding. For example, the phytate ion (which is present in all plant proteins) readily forms complexes with mono, di- and trivalent ions (including calcium, magnesium and zinc) (Reddy *et al.*, 1978; Roberts and

Yudkin, 1960). Phytate has a particular high affinity for zinc (Maddaiah *et al.*, 1964). Later research by Oberleas *et al.*, (1966) and Oberleas and Harland (1981) reported that phytate forms insoluble mineral complexes which are difficult for monogastric animals to absorb, which subsequently leads to decreased mineral absorption (Richardson *et al.*, 1985). Hydroxyapatite, which is formed mainly of tri-calcium phosphate and is the predominant component of the bone fraction in high ash diets, may have such mineral chelating capabilities, which would support the resulting low mineral availabilities associated with rainbow trout fed high ash diets.

It could be reasoned that as the primary source of phosphorus and calcium in high-ash diets is in the form of hydroxyapatite and tri-calcium phosphate, with the P and Ca components being bound together in a strong matrix. The digestive system of the fish may be unable to metabolise the individual minerals from this complex, leading to subsequent reduction in the digestibility of P and Ca. It has already been demonstrated that tri-, di- then monobasic phosphates are the sequence in which the phosphorus moiety is better utilised in fish (Lall, 1991; Ogino *et al.*, 1979; Rodehutscord *et al.*, 2000).

This theory may aid in explaining the differences in mineral availabilities between the first two experimental diets (Royal Crystal and Hi-performance), because percentage values for ash were fairly low (7.3 and 7.58%), and it was only P and Ca that were reduced.

Therefore, it would be difficult to attribute these reductions totally to mineral chelation, due to very high ash levels. However, the Royal Crystal diet was formulated to include an inorganic supplement of di-calcium phosphate, which was shown by Ogino *et al.*, (1979) to be more efficiently utilised by rainbow trout than tri-calcium phosphate. Therefore, the P and Ca in this diet were more easily utilised and hence digestibility figures were higher along with whole body mineral concentrations.

Rainbow trout fed both the Royal Crystal and standard ash diets exhibited significantly reduced values (approximately 20% lower) when compared with fish fed the Hi-performance or high-ash diets. This may indicate poorer availability of these minerals in the higher ash diets relative to the standard ash formulations. All digestibility/availability coefficients reported in these studies are apparent and therefore have not been corrected for endogenous losses. In addition to the digestibility data, faecal mineral concentrations also indicate that the absorption of minerals from both the Royal Crystal and standard ash diets is more efficient when compared to the Hi-performance and high ash diets as values for the latter diets were significantly higher. This also suggests that these minerals were less available when a high ash content is present in the diet.

In both experimental studies, mineral digestibility coefficients obtained were in general agreement with other published data with the exception of zinc, which had low ADC figures at 11.1%. For example, Sugiura *et al.*, (1998) observed that magnesium and phosphorus produced the highest digestibility coefficients (of 55.3 and 73.9% respectively) for salmonid fish with calcium and zinc producing lower values of 30.3 and 39.7%. These trends and relative values were also confirmed again by Sugiura *et al.*, in 2000 for rainbow trout (*O. mykiss*), and another recent study by Storebakken *et al.*, (2000) for Atlantic salmon (*S. salar*), although the apparent absorption from the control basal diet for magnesium was extremely negative at -500%.

Several of the ADC's presented in the results section of this study are negative values, particularly calcium and zinc, suggesting that larger proportions of these minerals were excreted than were initially presented to the fish via ingestion of the diets. The rationale for these negative apparent absorption estimates for Ca, Zn and occasionally Mg is that those minerals were taken up from the water primarily by drinking and/or uptake across the gill lamellae and are excreted via the intestine. Other examples of negative absorption

values for minerals have been reported for salmonids reared in freshwater by Lall, (1991), Nakamura and Yamada, (1980), Storebakken, (1985), Storebakken *et al.*, (1998) and Storebakken *et al.*, (2000) for water-borne calcium, as fish are particularly efficient at taking up this element. The uptake of zinc and magnesium from water has been also been demonstrated for salmonid fish by Shearer, (1995), Spry, (1998), Shearer and Asgard, (1992) and Sugiura *et al.*, (1998). Shearer, (1992) suggested that rainbow trout are able to meet their requirement for magnesium from either or both the diet or water, although the water-borne concentration of magnesium had to be 46 mg/l to be sufficient to meet the Mg requirement of the fish fed an Mg-free diet.

There is little recent evidence supporting the uptake of phosphorus from water, although Coffin *et al.*, (1949) reported that two different species of freshwater lake fish (*Fundulus* and *Notemigonus*) had taken up significant levels of water-borne radio labelled  $P^{32}$  after 50 hours of exposure. Coffin reported that these fish had either swallowed water or had received the phosphorus by eating plankton with the  $P^{32}$  incorporated into their tissues. Further 'dated' evidence of phosphorus uptake from water is for the goldfish (*Carrassius auratus*) by Asano and Ito, (1956) and Atlantic salmon (*S. salar*) from Srivastara, (1960). Solomalina and Arsan, (1980) established that in carp (*Cyprinus carpio*), the phosphorus uptake increases with a rise in water temperature and from a decrease the water calcium content.

It is evident that the level of free inorganic mineral ions in the water plays an important role in the final digestibility coefficients determined for diets and feedstuffs fed to fish. It appears that where a mineral is not present, is in an unavailable form or is perhaps energetically costly to digest from a diet, fish alternatively absorb that mineral where possible from the water. This gives rise to negative digestibility values, which can be highly variable. This variability may arise when comparing data with other published

work, which probably will have been conducted at a different research location (and probably country). Between locations the water will be from a different source i.e. mains water (as in an aquarium facility) vs. bore hole water (fish farm), or just in areas where water varies - such as 'hard' and 'soft' water. Such differences will lead to contrasting electrolyte levels. Therefore, it is important to consider the levels of water-borne minerals in the water source when explaining variance in mineral ADC's, as they may influence mineral digestibility in combination with the diet itself.

Net apparent mineral retention (NAMR) values were similar for rainbow trout in both the first and second trials. In each case the diet containing the standard level of ash (Royal Crystal and standard ash) had significantly higher NAMR values than the corresponding high ash diet (Hi-performance or high ash). Additionally, indicating an improved utilisation and retention of the minerals from diets containing standard ash levels. Similar values for phosphorus (35%) were reported by Shearer and Hardy, (1987) for trout fed diets containing de-boned fillet scrap. However, other published literature reports higher NAMR values of between 60-90% for phosphorus (Nordrum *et al.*, 1997) and 36-57% for magnesium (Shearer and Asgard, 1990), although mineral sources in these casein/gelatin diets for salmonids was purely from an inorganic source and hence incomparable with the data presented here.

Blood glucose levels obtained in this study ( $\approx 4$  mmol/l or 72 mg/dl) were in agreement with other salmonid data from Hille, (1982) and Warner *et al.*, (1978), but were near the low end of the range (72 – 218 mg/dl) reported by Miller *et al.*, (1983). This may be due to the fact that all fish were fasted for 24 hours prior to sampling therefore blood glucose would be reduced. Concentrations obtained for the blood plasma phosphorus were slightly higher at approximately 150  $\mu\text{g/ml}$  (5mMol/l) than those within the range 100 – 120  $\mu\text{g/ml}$  obtained by Sugiura *et al.*, (2000b), Vielma and Lall, (1998) and (Miller *et al.*, 1983).

Although values are within the range stated by Sakamoto and Yone, (1973) and Hille, (1982), discrepancies maybe due to variation in the homeostatic control of inorganic phosphorus during metabolism, a theory proposed by Gross, (1976).

Bile phosphorus levels were elevated in fish fed the Hi-performance diet. Bile fluid naturally contains inorganic salts, but there is little research showing variation in mineral levels between bile in fish, even though diet is known to be an important modulator in the process of bile formation and secretion (Tuchweber *et al.*, 1996). One of the natural functions of bile is to carry water insoluble waste that has been removed from the bloodstream by the liver. A suggestion could be made that perhaps the calcium phosphate salts (hydroxyapatite) present in the high ash diets have been absorbed into the bloodstream, but cannot be utilised by the fish as the molecule is too big. Hence these inorganic calcium phosphate salts are removed by the liver and then stored in the gallbladder with the bile to be released into the intestine and excreted. However, bile samples taken were not quantitative in relation to total volume of bile in the gallbladder, it has been noted that the production and flow rate of bile is increased when dietary oil levels are elevated (Tuchweber *et al.*, 1996; Szentmihalyi *et al.*, 2000). Since diet A (Royal Crystal) contained significantly higher lipid levels when compared with diet B (Hi-performance) a volume effect due to the variation in bile volumes collected may be attributable to the 'apparent' difference in the concentration of minerals determined between in these samples. For example, the bile from fish fed diet A maybe more dilute due to the larger volume produced, compared with the more concentrated but smaller volume of bile sampled from fish fed diet B. It should be noted that the total volume in the gallbladder was collected each time not a fixed aliquot. A further explanation of the relationship between minerals, bile and fat was reported by Xu *et al.*, (1998), when determining the fat digestibility in faeces of veal calves. High dietary calcium, reduced fat, Mg and P digestibility and led to increased bile excretion. It was proposed that the high Ca

increased the amount of mineral complexes in the intestine, because of the binding of bile acids, which would then exclude the bile acids from the process of fat digestion, and inhibit the reabsorption of bile acids.

Mineral levels in the different organs appear to reflect the requirement of that organ based on its function. As expected, the skeletal structures (the vertebrae) contained the highest levels of calcium and phosphorus as the basic physical component of bone is hydroxyapatite, (which is comprised of calcium and phosphorus) (Zerwekh, 1987). Lall, (1991) reported that 86-88% of body phosphorus in teleost fish is found in the skeleton. Additional sites of high calcium and phosphorus concentrations were the gills as samples included the gill arch in addition to the lamellae, and the skin complete with scale component. Vertebral and skin mineral concentrations for rainbow trout are within the range of those calculated by Skonberg *et al.*, (1997), although values reported by Vielma and Lall, (1998) are higher with respect to calcium, but not magnesium or phosphorus. The lower calcium values reported during this investigation may be due to instrumental drift at high concentrations, also due to the ICP-AES being highly sensitive and calcium a very abundant mineral. The results for this element were on occasion over the calibration range (up to 33%). These data were extrapolated assuming a linear calibration.

The calcium to phosphorus ratio for bone provides further evidence to indicate that the calcium concentrations reported during this trial may be underestimated. These ratios are reported to range from 1.5 to 2.1:1 for common carp (*C. carpio*), sea bream (*Sparus aurata*), rainbow trout and salmon (Lall and Bishop, 1977; Ogino and Takeda, 1978; Ogino *et al.*, 1979; Sakamoto and Yone, 1980; Watanabe, 1980a; Yone and Toshima, 1979). However, Ca:P for initial fish was 1.43:1 which although slightly low is acceptable but values for the 'final fish' (sampled at the end of trials) irrespective of diet source was approximately 1:1, suggesting that calcium values for the vertebrae of the fish sampled at



the completion of the trial are underestimates. In general, fish fed the Royal Crystal diet possessed higher mineral body distribution levels than those fed the Hi-performance diet with the exception of the skin. Indications imply that the retention of minerals into both the hard and soft organs was elevated in fish fed diets containing an inorganic mineral supplement thus enforcing the theory that this mineral source is more available to trout than a diet containing a high ash fishmeal instead of a mineral supplement. Conversely, the mineral concentrations for skin/scales were significantly increased in those fish fed the Hi-performance diet, a result which is difficult to explain.

It has been stated that skeletal structures represent a potential reservoir of calcium and phosphorus, with scale resorption being observed in response to starvation and sexual maturation (Carragher and Sumpter, 1991; Mugiya and Watabe, 1977; Persson *et al.*, 1999). Recently Skonberg *et al.*, (1997) demonstrated that the skin was the tissue most responsive to differences in dietary phosphorus level when compared to whole body, vertebrae, and blood plasma mineral and Alkaline phosphatase levels, suggesting that skin is a good indicator of the status of dietary phosphorus in rainbow trout. It was concluded that phosphorus, magnesium and calcium concentrations in the skin increased with increasing dietary phosphorus. Other authors such as Davis and Robinson, (1987) and Dougall *et al.*, (1996) have also found this response for the red drum (*Sciaenops ocellatus*) and striped bass (*Morone saxatilis*). The results discovered during the current trial also follow this trend in data, as fish fed the Hi-performance diet containing more phosphorus and possessed high mineral skin levels when compared with the Royal Crystal diet that contained lower mineral levels. However, this result does not confirm the theory that minerals in the Royal Crystal diet are generally in a more available form, as the higher skin mineral levels were noted in fish fed the Hi-performance diet where minerals were not supplemented to the diet in an inorganic 'available' form. Data relating changes in skeletal

structures to dietary mineral inclusion levels is limited and further research needs to be undertaken to elucidate these findings.

### *3.4.2 The effect of dietary oil level on mineral digestibility and fish size*

Research relating any influence of dietary oil level to mineral availability and digestion is limited therefore the majority of this discussion shall be speculative. Generally, increasing lipid levels in the diet resulted in higher mineral digestibility in juvenile fish (fish sizes 50 and 100g). The addition of oil to a diet is known to slow down the rate of food passage in the gut (increasing gut transit time) in mammals (Shafat and Rumsey, 1997) in addition to fish (Jobling, 1986), therefore the higher the dietary lipid level the longer the food bolus is retained in the digestive system. This may allow enhanced nutrient digestion, absorption and assimilation from the diet by increasing the length of exposure to digestive enzymes and duration for mineral uptake. Although it should be noted that absorption values for protein and calcium have been found to be only moderately increased when feeding high fat diets to avian species (Summers, 2000). These results may not be transferable to fish that have lower metabolic rates compared to terrestrial animals. A very recent study by Green *et al.*, (2002) found no significant effect of dietary lipid level on phosphorus retention when feeding rainbow trout diets formulated with varying levels of both phosphorus and lipid. However, this investigation appeared to focus primarily on the relationship between lipid protein (N) retention and excretion than links to mineral utilisation.

Higher dietary lipid will also provide the fish with increased energy levels (Phillips and Brockway, 1959), which in turn may supply more energy to the active transport systems involved in metabolism throughout the body of the fish. One such active system is associated with the absorption of minerals across the brush border membrane into cells from the lumen of the gastrointestinal tract. In higher vertebrates the absorption of

phosphorus across the intestine is dependent on a sodium gradient caused by the active co-transport of sodium, therefore the transport of phosphorus is secondary to that of sodium (Breves and Schroder, 1991; Murer and Hildmann, 1981; Peterlik *et al.*, 1981). A number of different sodium dependent phosphorus ion co-transport systems involved with phosphorus homeostasis have been identified in the past few years (Werner *et al.*, 1998), although the only evidence supporting this theory for fish was produced by Nakamura, (1985a), who reported a sodium-dependent absorption of inorganic phosphate in carp intestine. The intestinal absorption of calcium in mammals also requires energy via an ATP-ase pump to move Ca against an electro-chemical gradient, which is dependent on the luminal Ca concentration (Bronner, 1987b; Bronner *et al.*, 1981). Both magnesium and zinc ions also require energy to activate transport systems to assist in their transport across the gut mucosal interface (Bijvelds *et al.*, 1998; Glover and Hogstrand, 2002a; Glover and Hogstrand, 2002b). A high in the level of available energy may 'fuel' these active transport systems associated with mineral absorption across the gastrointestinal tract, accelerating the rate at which these minerals are taken up, resulting in increased apparent digestibility coefficients. This may also be linked to a greater physiological requirement for minerals due to the elevated plane of nutrition associated with faster growth on high fat diets.

During the digestive process the ingestion of fatty acids (lipids) both in quality and quantity stimulates the production and secretion of bile. Enhanced bile flow has been observed with high polyunsaturated fat intake and was attributed to both elevated bile acid dependent and bile acid independent flows (Tuchweber *et al.*, 1996). Tuchweber also established that diets enriched with fish oil as opposed to corn oil have been found to generate a greater flow of bile. Bile consists of water, cholesterol, lecithin, pigments, inorganic and bile salts and in the event of increased bile secretion the components of bile may be at higher levels. The sodium ion is known to be associated with the active

transport system of the gallbladder in rainbow trout (Grosell *et al.*, 2000), and is therefore present in the bile itself. Intestinal phosphate uptake is dependent on the electrolyte sodium, and an increase in bile sodium may result in an increase in the mineral transportation of phosphate ions across the gastrointestinal tract.

Further reasoning in explaining how increased oil levels can result in increased mineral uptake, could be related to the actual lipid itself. Many vitamins including A, D, E and K are fat soluble, having a lipophilic nature consequently increased lipid levels provides better absorption of these vitamins. Cholecalciferol is an active metabolite of vitamin D<sub>3</sub> and is known to stimulate intestinal calcium and phosphate absorption. This has been verified by DeLuca and Schones, (1976), Fernandez, (1995), Isselbacher, (1981), and Murer and Hildmann, (1981), therefore increased availability of cholecalciferol may result in a greater stimulation and influx of both calcium and phosphate ions.

Whereas increased level of dietary oil at all levels resulted in increased mineral digestibility for small sized fish (50 and 100g) the results for the 100 and 200g sized fish differed. Mineral digestibility increased for these larger fish only up to the 20% oil inclusion level in the diet, then digestibility generally plateaued (26% oil) and fell at 30% dietary oil. This superior mineral utilisation in smaller fish when compared to the bigger fish is consistent with the theory that smaller animals have a higher relative metabolic rate (Smith *et al.*, 1978). Diets containing higher levels of lipid provide the fish with increased dietary energy levels, as estimations of food energy for trout were determined by Phillips and Brockway, (1959) giving 3.9 kcal/g of protein, 1.6 kcal/g of carbohydrate and lipid the highest at 8.0 kcal/g. Consequently the juvenile fish are more metabolically active and demand more energy, as their requirements are greater than the larger fish in relative terms. The smaller fish would also be in their fastest growth period, needing a continual supply of minerals to support growth, metabolism and particularly bone mineralisation.

Alternatively the larger fish will be growing at a slower rate, with a lower requirement for mineral uptake and retention as stated by De Silva and Anderson, (1995).

In summary, there are a number of complex dietary components such as lipid level, ash content and nutritionally related factors that interact to modulate the absorption of trace elements in trout. Biotic factors such as fish size and stage of maturity, in addition to abiotic factors for example temperature, dissolved oxygen and concentration of water soluble minerals are also of considerable influence and should be considered in the interpretation of the results.

## **CHAPTER 4**

# **APPRAISAL OF FISHMEAL DIETS WITH AN INCREMENTAL INCLUSION OF AN INORGANIC PHOSPHOROUS SOURCE ON SELECTED MINERAL AVAILABILITY AND RETENTION FOR RAINBOW TROUT**

## CHAPTER 4

### APPRAISAL OF FISHMEAL DIETS WITH AN INCREMENTAL INCLUSION OF AN INORGANIC PHOSPHORUS SOURCE ON SELECTED MINERAL AVAILABILITY AND RETENTION FOR RAINBOW TROUT.

#### 4.1 Introduction

##### 4.1.1 Dietary minerals

Minerals such as phosphorus, calcium, magnesium and zinc are essential elements required by fish for many physiological and biochemical processes (Lall, 1989; Lall, 1991). Phosphorus and calcium are particularly important in the formation and maintenance of the skeletal system, as vertebrae maintain their rigidity from a solid phase of calcium phosphate. Additionally, phosphate is a vital component of ATP making it an essential factor in all energy-producing cellular reactions. Phosphorus deficiency signs in fish include impaired growth, poor feed efficiency and poor bone mineralisation, the latter being manifested in a variety of forms including lordosis (vertical curvature of the spine), scoliosis (lateral curvature of the spine) and enlarged vertebrae. Therefore, dietary minerals are required in quantities that can maintain and sustain optimum growth, tissue and formation, feed utilisation and various metabolic processes. This current chapter intends to focus primarily on the nutritional needs of rainbow trout for the mineral phosphorus whilst also observing the other macro-elements of interest, Ca, Mg and Zn.

Research by Ichii and Mugiya, (1983) and Templeton and Brown, (1963) using goldfish (*Carassius auratus*) and brown trout (*Salmo trutta*) respectively, have demonstrated that some minerals, such as calcium are readily absorbed from the surrounding water in addition to the diet. As fish are able to balance their requirements of this mineral from both sources, the dietary inclusion levels of calcium become less important. Some researchers have claimed

that a dietary calcium supplement may not be necessary for several fish species including carp (*Cyprinus carpio*) Ogino and Takeda, (1976), rainbow trout (*O. mykiss*) Ogino and Takeda, (1978), red sea bream (*Chrysophrys major*) Sakamoto and Yone, (1976), and guppy (*Poecilia reticulata*) Shim and Ho, (1989). However, this process highlights the need to ensure that dissolved minerals that are present only at negligible or low levels in the aquatic environment are also supplied in a suitable dietary form. One such mineral is phosphorus and coupled with the integral role that is played by this macro-element in the metabolism and physiology of vertebrates, it is essential that phosphorus is supplied at an appropriate level and in an available form in the diet (Hilton, 1989). Dietary phosphorus levels are often related to calcium due to the important and synergistic role these minerals play particularly in the process of bone mineralisation. It appears that the optimum dietary Ca:P ratio is important and should be considered when formulating diets, Sakamoto and Yone, (1973) established that a ratio of 1:2 was required by red sea bream (*Chrysophrys major*) and a 1:1 for eels (*Anguilla anguilla*), 1:3 for the American cichlid (*Cichlasoma urophthalmus*) by Chavez-sanchez *et al.*, (2000), and a ratio of 1:1 has also been suggested for brook trout (*Salvelinus fontinalis*) by Phillips *et al.*, (1958). However Nakamura, (1982) demonstrated that when calcium is in dietary excess over phosphorus an antagonistic effect has been seen in carp (*Cyprinus carpio*), which can lead to the inhibition of intestinal phosphorus absorption due to the formation of calcium phosphate in the digestive tract which is poorly digested (Andrews *et al.*, 1973). These findings suggest that the Ca:P ratio is important even though fish are able to absorb calcium readily from the surrounding water.

A dietary requirement for phosphorus has been recommended by NRC, (1993) for rainbow trout at an available level of 6mg P g<sup>-1</sup> diet when digestible energy (DE) content is 15 KJ g<sup>-1</sup>. Generally, salmonid fish have a typical dietary requirement ranging from 0.5 to 0.8% of the



diet (Ketola, 1975; Ketola and Richmond, 1994; Ogino and Takeda, 1978; Watanabe *et al.*, 1980). Other fish species comply with, or are near to, this range of phosphorus requirements include juvenile sunshine bass (*Morone chrysops* X *M. saxatilis*) (Brown *et al.*, 1993), common carp (*Cyprinus carpio*) (Kim *et al.*, 1998b; Ogino and Takeda, 1976), blue tilapia (*Oreochromis aureus*) (Robinson *et al.*, 1987), juvenile striped bass (*Morone saxatilis*) (Dougall *et al.*, 1996), channel catfish (*Ictalurus punctatus*) (Eya and Lovell, 1997; Lovell, 1978; Wilson *et al.*, 1982), American cichlid (*Cichlasoma urophthalmus*) (Chavez-sanchez *et al.*, 2000), eel (*Anguilla anguilla*) (Arai *et al.*, 1975a), gilthead sea bream (*Sparus aurata*) (Pimentel-Rodrigues and Loiva-Teles, 2001), milkfish (*Chanos chanos*) (Borlongan and Satoh, 2001) and the guppy (*Poecilia reticulata*) (Shim and Ho, 1989).

Calcium requirements have been estimated to be in a similar range to those for phosphorus from approximately 0.3 to 1.8% for several fish species (Andrews *et al.*, 1973; Chavez-sanchez *et al.*, 2000; Ogino and Takeda, 1976; Robinson *et al.*, 1987; Sakamoto and Yone, 1973). Although fish are able to utilise waterborne calcium it has been indicated that a dietary mineral supplement containing an easily digestible calcium source, (such as di-calcium phosphate) is still necessary in the feeds of some fish, even seawater species such as the Japanese flounder (*Paralichthys olivaceus*) (Hossain and Furuichi, 2000b), redlip mullet (*Liza haematocheila*) (Hossain and Furuichi, 2000a) and tiger puffer (*Takifugu rubripes*) (Hossain and Furuichi, 1998). Species that are reared in low calcium water may not be able to satisfy their metabolic calcium requirements and hence need a dietary mineral supplement, this has been demonstrated by Arai *et al.*, (1975a, 1975b) for the eel (*Anguilla anguilla*) and rainbow trout. Additionally Robinson *et al.*, (1984, 1986, 1987) published various literature relating to the necessity of dietary calcium for tilapia (*Tilapia aurea*), channel catfish (*Ictalurus punctatus*) and blue tilapia (*Oreochromis aureus*).

The optimum dietary requirement of fish for magnesium according to Shearer, (1989) using dose-response curves has been estimated to be 0.14% of the diet, which is higher than the values reported by Ogino *et al.*, (1978) and Knox *et al.*, (1981). These high values were possibly due to low bioavailability of the inorganic source or low levels of magnesium in the water (Shearer and Asgard, 1990), as fish are able to utilise water borne magnesium to a certain extent if dietary levels are insufficient.

The requirement for zinc by salmonid fish has been estimated to be within the range 15-57 mg kg<sup>-1</sup> (Maage and Julshamn, 1993; Ogino and Yang, 1978). However, the bioavailability of zinc can be reduced by dietary factors such as high ash and phytic acid content (Gatlin and Phillips, 1989; Shearer *et al.*, 1992), therefore practical zinc supplements are often three or four fold the actual required level, such as 150 mg kg<sup>-1</sup> for rainbow trout (Ketola, 1979) and 100 mg kg<sup>-1</sup> for Atlantic salmon (Maage *et al.*, 2001).

#### **4.1.2 Mineral supplements**

Even when mineral requirements have been accurately estimated and it is deemed necessary to include some sort of mineral supplement into the diet formulation, the most beneficial type of mineral supplement still has to be selected. The inorganic mineral supplement should provide the fish with the required minerals in the most biologically available form possible. Generally, the more soluble the supplement the more available the minerals are to the fish. Where phosphorus supplementation is concerned Ogino *et al.*, (1979) states that tri-, di- then monobasic phosphate salts are the sequence in which the phosphorus moiety is better utilised in fish with respective availabilities of 90%, 72% and 56% as quoted by Lall, (1991). Such findings were also confirmed by Rodehutsord *et al.*, (2000) in the rainbow trout, and Li *et al.*, (1996) and Robinson *et al.*, (1996) also established that dicalcium phosphate and diflourinated

phosphates are equally good at providing an adequate phosphorus source for channel catfish. Primary sodium phosphate has also been found to provide a highly available source of phosphorus for Atlantic salmon (Nordrum *et al.*, 1997). However, Satoh *et al.*, (1993) discovered that excessive dietary phosphorus induces poor growth in rainbow trout, and that the phosphorus portion of tricalcium phosphate may inhibit zinc availability.

#### **4.1.3 Dietary protein and minerals**

Currently the largest proportion of a modern aquafeed formulation utilises fishmeal as the main source of high grade protein. It also provides a substantial proportion of the macro-elements and some of the trace elements required by fish. However, fishmeal is one of the single most expensive aquafeed ingredients, and with fish stocks diminishing the world's fishmeal production is falling. Nutritionists are now searching for less expensive, more abundant, alternative protein sources. Research has so far considered soybean to be the most suitable substitute for fishmeal. However, research investigating the partial or total replacement of fishmeal with soybean is somewhat inconsistent. Some reported literature states that soybean products do not affect growth or feed utilisation in rainbow trout (Kaushik *et al.*, 1995). Alternatively, Kim *et al.*, (1998a) established a significant increase in growth for rainbow trout fed fishmeal compared to soy protein concentrate based diets and fish fed the latter diets exhibited greatly reduced phosphorus retention values. In previous research using carp (*Cyprinus carpio*) Kim *et al.*, (1995) concluded that more than a 25% substitution by full-fat soybean for fish meal could be negative on the growth and feed efficiency for these fish. However, at inclusion levels lower than 25% no significant differences were noted between the fish meal and soybean treatments. Generally, research indicates that partially or completely replacing fish meal in salmonid feeds with soybean protein is fairly unsuccessful at

high dietary inclusion levels (>40%) (Rumsey *et al.*, 1993), but can be relatively successful when included at levels lower than 40% of the feed.

Further reasoning for encouraging soybean to be utilised as a fishmeal substitute is its phosphorus level. When compared with fishmeal soybean contains lower levels of phosphorus which would aid in the reduction of this mineral being discharged in fish farm effluents. Fishmeal contains between 1.5-4.5% phosphorus. High quality herring fishmeal contains a fairly low level (1.8%) of phosphorus, whereas fishmeal comprised from filleted remains contains a higher 4.2% of phosphorus due to the large bone component of the meal. In fishmeals the majority of minerals are chiefly found in inorganic form that tends to be fairly available to fish from the gastro intestinal tract (Lall, 1991). Conversely, phosphorus in plant protein sources such as soybean are present in far lower concentrations ranging from 0.3-1.5%, however approximately two-thirds of this phosphorus is stored as phytate or phytic acid (Cromwell, 1992). Phytate is largely unavailable to fish and all monogastric animals as they do not possess the enzyme phytase responsible for hydrolysing this compound. It is evident that the bioavailability of phosphorus is highly variable between feed ingredients and inorganic supplements, therefore it is necessary when formulating diets containing high components of plant protein to assess the available mineral content, and accordingly supplement with an appropriate inorganic mineral source.

#### **4.1.4 Experimental aims**

Inorganic phosphorus sources are added in premixes for salmonids to compensate for the varying level and bioavailability in mixed ingredients. The aims of this study were to assess the apparent mineral digestibility and retention from feeding juvenile rainbow trout (*Onchorhynchus mykiss*) a specified reference diet, supplemented with an inorganic

phosphorus source. Phosphorus supplementation was in the form of monobasic calcium phosphate supplied at incremental levels, to provide dietary concentrations in excess of requirement. Various parameters including FCR, SGR, mineral digestibility and retention, elemental composition of blood plasma, bile, whole body and selected tissues were used to evaluate the dietary treatments.

## **4.2 Materials and Methods**

### **4.2.1 Experimental facilities**

Into each of twenty square 160L fibreglass tanks, 37 rainbow trout (*Onchorynchus mykiss*) with an average weight of approximately 25g were randomly stocked. Experimental facilities were as described in chapter 2 (page 49).

### **4.2.2 Diet formulation**

To assess the protein and mineral digestibility and absorption during this investigation a total of four commercially made test diets were produced using a pilot scale extruder at the Nutreco Aquaculture Research Centre (ARC), Stavanger, Norway with specification typical of those currently used in modern aquaculture practice i.e:- diets had defined a defined energy ratio, total protein and lipid levels and were presented as 3mm pellet size for trout used in this study. The fundamental aim of this study was to :-

1. Assess mineral availability and retention from diets based on a standard fishmeal protein source with either no or varying levels of phosphorus supplementation.

The four diets utilised were fed to fish in triplicate, various parameters including FCR, SGR and elemental composition of whole body and tissues were used to evaluate the dietary treatments.

Table 4.1  
Dietary formulation for test diets (No's 1-4) fed to rainbow trout.

Dietary Ingredient (%)	Diet 1 Fishmeal	Diet 2 Fishmeal + 0.27% P	Diet 3 Fishmeal + 0.6% P	Diet 4 Fishmeal + 0.9% P
Fish meal	40	40	40	40
Soya meal	24.41	24.41	24.41	24.41
Wheat	14.46	14.16	13.86	13.56
Fish oil	20.38	20.38	20.38	20.38
Mineral premix (P free) <sup>*1</sup>	0.25	0.25	0.25	0.25
Vitamin premix (P free) <sup>*1</sup>	0.25	0.25	0.25	0.25
DL-Methionine	2.0	2.0	2.0	2.0
Yttrium Oxide	0.05	0.05	0.05	0.05
'Aliphos'	0	0.90	2.64	3.96
Limestone flour	2.5	1.95	1.35	0.78

\* Vitamin/ mineral premixes supplied in excess of NRC (1993) salmonid requirements, P free.

<sup>1</sup> Specification according to TROUW Aquaculture UK Ltd

Aliphos supplement – Monobasic calcium phosphate

Table 4.2  
Proximate and mineral composition for all test diets (No's 1-4).

Component (%)	Diet 1	Diet 2	Diet 3	Diet 4
Protein	45.69	44.09	43.04	42.86
Lipid	19.63	21.26	20.52	21.23
Moisture	5.27	6.08	5.35	4.09
Ash	8.06	9.0	9.95	10.37
Ca	1.71	1.92	2.31	2.59
Mg	0.206	0.211	0.203	0.214
P	1.39	1.58	1.87	2.16
Zn	0.016	0.016	0.017	0.017
Ca:P	1:0.81	1:0.82	1:0.81	1:0.83
Yt O	0.054	0.058	0.057	0.06

The mineral supplement used was a granular mono calcium phosphate, which is commercially produced for animal feeds by the company 'Britphos' bearing the tradename of 'Aliphos'. In order to maintain a constant calcium to phosphorus ratio of approximately 1:1 for each dietary treatment, calcium carbonate (limestone flour) was added to each diet at appropriate levels. The diet formulations are presented in table 4.1 with the proximate composition and mineral composition profile being summarised in table 4.2.

#### ***4.2.3 Feeding, sampling and analysis***

The total tank biomass was calculated initially, with this value and the figures produced in the manufacturer's guidelines, it was estimated that fish should be fed initially at 3.0% for all six diets. Fish were fed twice daily at 9.30am and 4.00pm for a period of 12 weeks. At fortnightly intervals fish were re-weighed and feed rations adjusted to compensate for growth. Throughout the growth trial, weight gain, specific growth rate (SGR), feed intake, feed conversion ratio (FCR), any mortalities and water quality were monitored.

After fish had been allocated to tanks, surplus fish were used for initial analysis of whole body mineral composition. Methodologies for blood and various organ harvesting procedures are referred to in chapter 2. The rainbow trout were euthanised using 2-Phenoxy-ethanol (0.2ml/L), and culled in accordance with Schedule 1 of the Home Office Animals (Scientific Procedures) Act 1986. This procedure was repeated at the termination of the growth trial following a 24-hour starvation period trial with 6 fish per tank being utilised for organ harvesting. Bile from the gallbladder and blood samples via the caudal vein were also obtained from these fish and in addition to mineral analysis the blood samples were used to establish haematocrit, haemoglobin and glucose values. A further 6 fish per tank were removed for whole body mineral analysis. The remaining fish in each tank were stripped for



the collection of faecal matter these samples were subsequently oven dried before acid digestion and mineral analysis. Proximate composition and elemental analysis was conducted on all other appropriate samples following the procedures outlined previously in chapter 2.

### 4.3 Results

#### 4.3.1 Mortality, growth, and feed efficiency

Feed acceptance and general health was good for all tanks of fish, as a consequence the total mortality during the experiment was less than 1%. Mortalities were not treatment related and all fish appeared to be visually healthy at the end and throughout the experiment. In each case the weight of the dead fish was recorded and feed rations adjusted accordingly for the appropriate tank. The general growth and feed efficiency data including weight gain, FCR or SGR are summarised in table 4.3.

Table 4.3  
Weight gain, initial and final weight of experimental fish after 72 days of growth, (n=3 ± S.E).

Growth parameter	Diet 1	Diet 2	Diet 3	Diet 4
Initial weight, (g)	25.37 ± 0.1	24.67 ± 0.75	25.51 ± 0.24	25.31 ± 0.04
Final weight, (g)	147.69 ± 4.6 <sup>a</sup>	137.65 ± 2.5 <sup>a</sup>	142.82 ± 4.9 <sup>a</sup>	140.19 ± 7.5 <sup>a</sup>
Weight gain % (of initial weight)	482.14	457.97	459.86	453.89

Unlike superscripts denote a significant statistical difference,  $P < 0.05$  ANOVA.

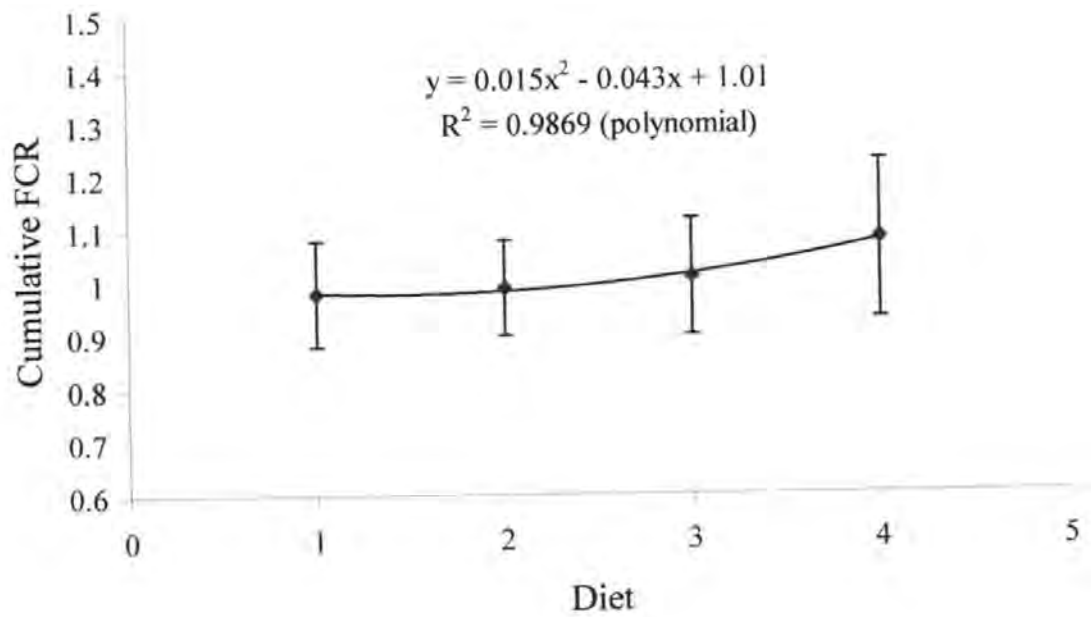


Figure 4.1  
Cumulative FCR of experimental fish after 72 days of growth, ( $n=3 \pm S.E.$ ).

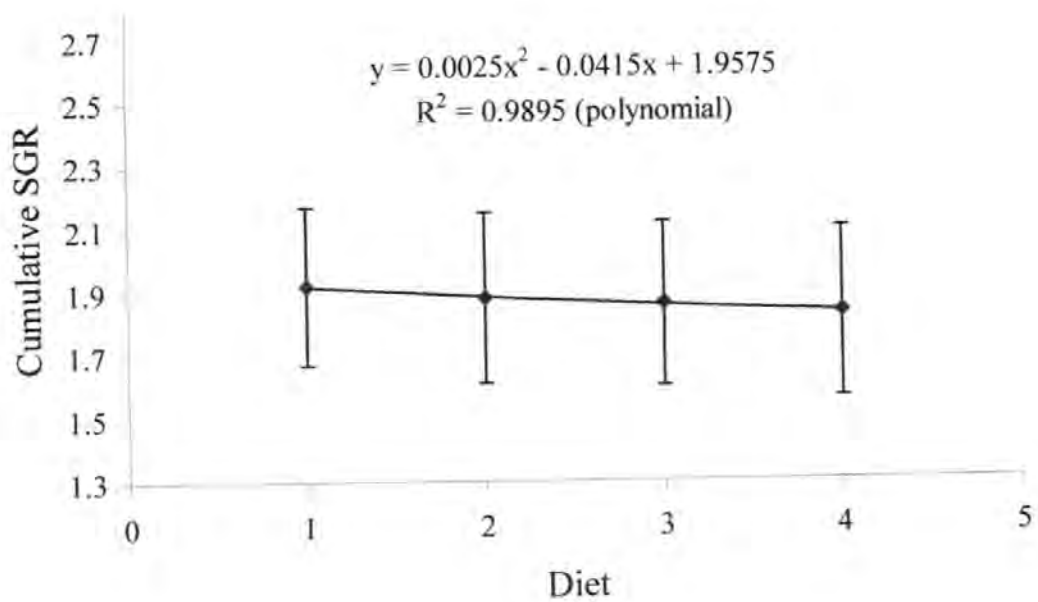


Figure 4.2  
Cumulative FCR of experimental fish after 72 days of growth, ( $n=3 \pm S.E.$ ).

Rainbow trout fed the fishmeal based diets (1-4) grew from 25 to approximately 140g during the course of the 3 month growth trial. No significant statistical differences were determined (possibly due to large standard error) between diets for the feed conversion ratio (FCR) or specific growth rate (SGR), which are displayed in figure 4.1 and 4.2. However, polynomial curves fitted to the data shows clear trend lines, suggesting a relationship between both FCR and SGR and dietary treatment. FCR appears to increase as dietary phosphorus increases, whereas SGR decreases with phosphorus increase.

#### **4.3.2 Mineral status – Whole body and tissue levels**

Dietary treatments had no significant effect on the phosphorus (P) concentrations of the average whole body mineral concentrations (Table 4.4), with all values ranging between 4111 – 4596 µg P/g wet weight for the initial fish and those fed the six experimental diets.

However, significant differences were noted for calcium which was lower in the body of fish fed diet 1 (3766µg Ca/g wet weight) in relation to all other fish sampled. The initial fish had significantly higher calcium levels than all other fish. Significant differences in body mineral distribution were also noted between fish fed the range of experimental diets for magnesium and zinc. For magnesium, significantly lower body levels (212.2µg Mg/g wet weight) were established in the initial fish, with higher levels of 337.7 µg Mg/g wet weight for fish fed diet 3. Again the initial fish exhibit a significant difference when compared to other fish, whole body zinc concentrations were higher (28.9µg Zn/g wet weight) than those for all other fish with the exception of fish fed diet 4.

Table 4.4

Mean whole body distribution of minerals ( $\mu\text{g/g-wet weight}$ ) in the rainbow trout fed four experimental diets, ( $n=11, \pm \text{S.E.}$ ).

Mineral ( $\mu\text{g/g-wet weight}$ )	Initial fish	Diet 1	Diet 2	Diet 3	Diet 4
Phosphorus	$4273 \pm 81^a$	$4596 \pm 226^a$	$4111 \pm 95^a$	$4340 \pm 153^a$	$4311 \pm 117^a$
Calcium	$5748 \pm 154^a$	$3766 \pm 261^b$	$5026 \pm 363^c$	$4678 \pm 164^c$	$4755 \pm 227^c$
Magnesium	$212.2 \pm 4.6^a$	$255.5 \pm 10.3^b$	$275.8 \pm 6.5^b$	$337.7 \pm 17.4^c$	$263.6 \pm 8.3^b$
Zinc	$28.9 \pm 1.1^a$	$24.0 \pm 2.1^b$	$23.2 \pm 1.0^b$	$26.1 \pm 1.0^{ab}$	$23.0 \pm 1.7^b$

Unlike superscripts denote a significant statistical difference  $P < 0.05$  ANOVA.

Table 4.5a.

Mineral concentrations (mg/g-dry weight) in the vertebrae of rainbow trout fed the four experimental diets, (n=12,  $\pm$  S.E).

Mineral (mg/g-dry weight)	Diet 1	Diet 2	Diet 3	Diet 4
Phosphorus	79.02 $\pm$ 0.47 <sup>a</sup>	76.09 $\pm$ 0.68 <sup>a</sup>	78.71 $\pm$ 0.72 <sup>a</sup>	82.61 $\pm$ 0.64 <sup>a</sup>
Calcium	131.25 $\pm$ 1.71 <sup>a</sup>	128.59 $\pm$ 1.45 <sup>a</sup>	122.23 $\pm$ 2.10 <sup>a</sup>	128.21 $\pm$ 1.97 <sup>a</sup>
Magnesium	2.38 $\pm$ 0.025 <sup>a</sup>	2.45 $\pm$ 0.017 <sup>a</sup>	2.41 $\pm$ 0.023 <sup>a</sup>	2.58 $\pm$ 0.031 <sup>a</sup>
Zinc	0.160 $\pm$ 0.004 <sup>a</sup>	0.158 $\pm$ 0.005 <sup>a</sup>	0.162 $\pm$ 0.002 <sup>a</sup>	0.155 $\pm$ 0.003 <sup>a</sup>

Unlike superscripts denote a significant statistical difference,  $P < 0.05$  ANOVA.

Tables 4.5a to 4.5c show the mineral concentrations from the vertebrae, spleen and kidney from the trial fish fed the four experimental diets. Results of the vertebral mineral concentrations are displayed in table 4.5a. As expected the concentrations for phosphorus and calcium are high ranging from 76.09 to 82.61mg/g-wet weight (phosphorus) and 122.23 to 131.25mg/g-wet weight (calcium), generally this mineral data gives a Ca:P ratio of 1.5:1. Overall the only significant differences in data were noted for the mineral phosphorus. No significant differences were noted between dietary treatments for calcium, zinc or magnesium in the vertebrae of these fish.

Phosphorus concentrations in the kidney of rainbow trout fed the 4 experimental diets (table 4.5b) ranged from 12.24 to 13.45mg/g-wet weight. Fish fed diets 1, and 2 had significantly lower phosphorus levels in their kidney when compared to diets 3, and 4. This trend was also seen for magnesium in the kidney of the trial fish. Where significant differences were noted between diets 3, and 4 which were higher in value (0.774, and 0.767mg/g-wet weight respectively), when compared with diets 1, and 2, the concentrations of which were 0.693 to 0.707mg/g-wet weight. Fish fed diet C had significantly higher levels of calcium in their kidney (0.678mg/g-wet weight), when compared with fish fed diets 1 to 3 (0.413-0.469mg/g-wet weight). No significant differences were established between the concentration of zinc in the kidneys of rainbow trout fed diets 1 to 4.

Table 4.5b.

Mineral concentrations (mg/g-dry weight) in the kidney of rainbow trout fed the four experimental diets, (n=12,  $\pm$  S.E).

Mineral (mg/g-dry weight)	Diet 1	Diet 2	Diet 3	Diet 4
Phosphorus	12.70 $\pm$ 0.35 <sup>a</sup>	12.24 $\pm$ 0.24 <sup>a</sup>	13.45 $\pm$ 0.22 <sup>b</sup>	13.08 $\pm$ 0.37 <sup>b</sup>
Calcium	0.413 $\pm$ 0.026 <sup>a</sup>	0.428 $\pm$ 0.026 <sup>a</sup>	0.469 $\pm$ 0.038 <sup>a</sup>	0.678 $\pm$ 0.078 <sup>b</sup>
Magnesium	0.707 $\pm$ 0.028 <sup>a</sup>	0.693 $\pm$ 0.018 <sup>a</sup>	0.774 $\pm$ 0.024 <sup>b</sup>	0.767 $\pm$ 0.015 <sup>b</sup>
Zinc	0.106 $\pm$ 0.006 <sup>a</sup>	0.096 $\pm$ 0.003 <sup>a</sup>	0.11 $\pm$ 0.005 <sup>a</sup>	0.114 $\pm$ 0.007 <sup>a</sup>

Unlike superscripts denote a significant statistical difference,  $P < 0.05$  ANOVA.

Table 4.5c.

Mineral concentrations (mg/g-dry weight) in the spleen of rainbow trout fed the four experimental diets, (n=12,  $\pm$  S.E).

Mineral (mg/g-dry weight)	Diet 1	Diet 2	Diet 3	Diet 4
Phosphorus	12.72 $\pm$ 0.21 <sup>ac</sup>	12.11 $\pm$ 0.42 <sup>bc</sup>	13.41 $\pm$ 0.23 <sup>a</sup>	13.10 $\pm$ 0.20 <sup>a</sup>
Calcium	0.185 $\pm$ 0.01 <sup>a</sup>	0.174 $\pm$ 0.017 <sup>a</sup>	0.191 $\pm$ 0.024 <sup>a</sup>	0.183 $\pm$ 0.31 <sup>a</sup>
Magnesium	0.714 $\pm$ 0.012 <sup>a</sup>	0.658 $\pm$ 0.02 <sup>bc</sup>	0.721 $\pm$ 0.014 <sup>a</sup>	0.686 $\pm$ 0.017 <sup>ac</sup>
Zinc	0.124 $\pm$ 0.013 <sup>a</sup>	0.099 $\pm$ 0.006 <sup>a</sup>	0.111 $\pm$ 0.01 <sup>a</sup>	0.127 $\pm$ 0.013 <sup>a</sup>

Unlike superscripts denote a significant statistical difference,  $P < 0.05$  ANOVA.



Phosphorus concentrations in the spleen of rainbow trout fed the 4 experimental diets (table 4.5c) were similar to those established for the kidney and ranged from 12.11 to 13.41 mg/g-wet weight. Significant differences in spleen phosphorus levels were noted between fish fed diet 2 when compared with diets 3 and 4, where values were 12.11 mg/g-wet weight vs. the higher 13.41 and 13.10 mg/g-wet weights respectively. A similar trend was seen for magnesium where significant differences were between the spleen of fish fed diet 2 (0.658 mg/g-wet weight) compared with the higher values of diets 1 and 3 (0.714 and 0.721 mg/g-wet weight). No significant differences were established between the concentration of zinc or calcium in the spleen of rainbow trout fed diets 1 to 4.

#### **4.3.3 Mineral status – Blood parameters, bile and HSI**

A range of biochemical assays to establish haemoglobin, haematocrit and glucose levels were performed on blood samples from fish fed the range of experimental diets. These results are shown in table 4.6, in addition to the hepato-somatic index (HSI).

Haematocrit percentages ranged between 33.43-35.77, haemoglobin values were 6.63-7.56 g/dL and glucose levels 4.29-4.62 mmol/L. No significant differences were noted between fish fed diets 1-4 for these three blood parameters. However, significant differences were noted for the HSI, where fish fed diet 1 possessed a significantly higher HSI than all other fish at 1.44.

Mineral concentrations in the blood plasma of fish fed the four experimental diets are displayed in table 4.6a. As the ICP-AES was used for the analysis of samples to ascertain mineral levels in the blood plasma it can be assumed that data shown is that of the total mineral concentrations, as opposed to just the inorganic portion.

Table 4.6

A range of blood plasma parameters and Hepato-somatic Index for rainbow trout fed four experimental diets, (n= 3, mean  $\pm$  S.E).

Parameter	Diet 1	Diet 2	Diet 3	Diet 4
Hepato-somatic Index	1.44 $\pm$ 0.04 <sup>a</sup>	1.21 $\pm$ 0.06 <sup>b</sup>	1.09 $\pm$ 0.03 <sup>b</sup>	1.14 $\pm$ 0.04 <sup>b</sup>
Haematocrit (%)	35.20 $\pm$ 0.56 <sup>a</sup>	35.17 $\pm$ 0.84 <sup>a</sup>	34.9 $\pm$ 0.49 <sup>a</sup>	35.77 $\pm$ 0.62 <sup>a</sup>
Haemoglobin (g/dL)	7.03 $\pm$ 0.23 <sup>a</sup>	7.36 $\pm$ 0.33 <sup>a</sup>	7.00 $\pm$ 0.10 <sup>a</sup>	7.56 $\pm$ 0.21 <sup>a</sup>
Glucose (mmol/L)	4.38 $\pm$ 0.14 <sup>a</sup>	4.29 $\pm$ 0.15 <sup>a</sup>	4.62 $\pm$ 0.14 <sup>a</sup>	4.47 $\pm$ 0.13 <sup>a</sup>
Calcium (mg/dL)	15.27 $\pm$ 0.93 <sup>a</sup>	15.81 $\pm$ 0.92 <sup>a</sup>	16.48 $\pm$ 1.06	14.13 $\pm$ 1.13 <sup>a</sup>
Magnesium (mg/dL)	3.09 $\pm$ 0.13 <sup>a</sup>	2.75 $\pm$ 0.06 <sup>a</sup>	2.56 $\pm$ 0.10 <sup>a</sup>	3.60 $\pm$ 0.32 <sup>b</sup>
Phosphorus (mg/dL)	55.56 $\pm$ 1.44 <sup>a</sup>	53.11 $\pm$ 1.14 <sup>a</sup>	53.25 $\pm$ 1.32 <sup>a</sup>	63.79 $\pm$ 0.96 <sup>b</sup>
Zinc (mg/dL)	1.41 $\pm$ 0.26 <sup>a</sup>	1.04 $\pm$ 0.04 <sup>a</sup>	0.93 $\pm$ 0.05 <sup>a</sup>	1.26 $\pm$ 0.09 <sup>a</sup>

Unlike superscripts denote a significant statistical difference,  $P < 0.05$  ANOVA.

For future reference it may be more accurate to utilise the whole blood for mineral analysis, because minerals may have been lost into the packed cell proportion of the sample during centrifugation to retain the plasma for analysis.

Phosphorus levels were by far the highest and ranged from 53.11 to 63.79mg/dL the only statistically different result was noted for fish fed the fishmeal diet with the highest level of mineral supplementation (diet 4). The same trend was noted for magnesium, where fish fed diet 4 had significantly higher plasma magnesium (3.60mg/dL) when compared with fish fed diets 1-3 (2.56-3.09mg/dL). No significant differences were seen between dietary treatments for fish fed diets 1 through 4 for plasma calcium (with values ranging from 14.13 to 16.48mg/dL) or zinc (0.93 to 1.41mg/dL).

Data referring to the bile mineral levels from the fish fed the 4 experimental diets is shown in table 4.7. Once again it should be noted that as analysis was carried using the ICP-AES therefore minerals levels displayed are total and not the inorganic fraction. Calcium was the most abundant mineral found in all bile samples with concentrations ranging from 72.93 to 87.30mg/dL. Bile magnesium and phosphorus concentrations were of similar magnitude ranging from 7.89 to 11.81mg/dL and 10.19 to 11.60mg/dL respectively. Significant differences were only noted between the bile from fish fed diet 1 for both calcium and magnesium, which were significantly lower when compared with fish fed diets 2, 3 and 4. Zinc was found to be present only at negligible levels in the bile of all fish sampled (0.11 to 0.14mg/dL).

Table 4.7

Mineral concentrations (mg/dL) in the bile of rainbow trout fed the four experimental diets, (n=3,  $\pm$  S.E).

Mineral (mg/g-dry weight)	Diet 1	Diet 2	Diet 3	Diet 4
Phosphorus	10.32 $\pm$ 1.27 <sup>a</sup>	10.19 $\pm$ 1.90 <sup>a</sup>	11.60 $\pm$ 1.03 <sup>a</sup>	11.36 $\pm$ 0.81 <sup>a</sup>
Calcium	72.93 $\pm$ 1.66 <sup>a</sup>	86.56 $\pm$ 3.60 <sup>b</sup>	87.30 $\pm$ 3.80 <sup>b</sup>	85.57 $\pm$ 3.60 <sup>b</sup>
Magnesium	7.89 $\pm$ 0.33 <sup>a</sup>	10.68 $\pm$ 0.70 <sup>b</sup>	11.08 $\pm$ 0.92 <sup>b</sup>	11.81 $\pm$ 1.18 <sup>b</sup>
Zinc	0.11 $\pm$ 0.04 <sup>a</sup>	0.12 $\pm$ 0.03 <sup>a</sup>	0.12 $\pm$ 0.06 <sup>a</sup>	0.14 $\pm$ 0.05 <sup>a</sup>

Unlike superscripts denote a significant statistical difference,  $P < 0.05$  ANOVA.

#### **4.3.4 Mineral Digestibility**

Faecal mineral concentrations from fish fed all dietary treatments are represented in figures 4.1, 4.2, 4.3 and 4.4 along with the relative dietary mineral level to allow for a direct visual comparison. Both phosphorus (P) and calcium (Ca) follow similar trends where there are clear significant differences between faecal samples of fish fed the set of four experimental diets. For calcium, excretory levels of this mineral from fish fed diet 3 and 4 (70.82 and 75.86mg/g-dry weight respectively) were significantly higher than diet 1 and 2 fed fish (58.40 and 60.07mg/g-dry weight respectively). Excretory levels of phosphorus from fish fed diets 3 and 4 (36.94 and 40.42mg/g-dry weight) were also significantly higher than those fed diets 1 and 2 (25.81 and 27.00mg/g-dry weight). These trends can clearly be seen in figures 4.1 and 4.2, with the mineral excretory levels imitating the differences in the dietary mineral levels between diets. Data presented for magnesium shows no significant differences between dietary treatments with values ranging from 4.01 to 4.23mg/g-dry weight (figure 4.3). The data obtained for zinc shows excretory levels in diet 1 of 0.54mg/g-dry weight was significantly higher than all other faecal samples from other dietary treatments. Rainbow trout fed diets 2, 3 and 4 all had similar excretory values for zinc ranging from 0.451 to 0.464mg/g-dry weight. These faecal concentrations are visually shown in conjunction with the relevant dietary zinc concentrations in figure 4.4.

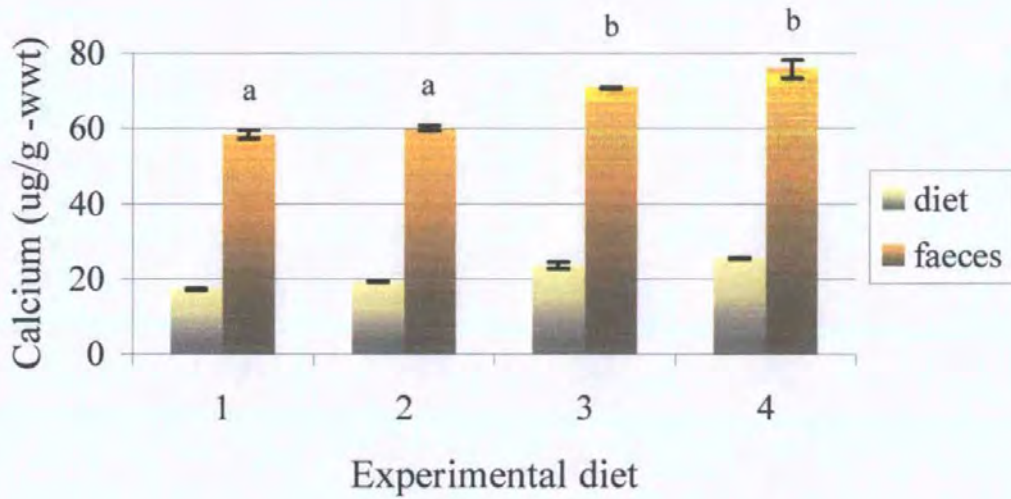


Figure 4.1

Calcium concentrations of the four experimental diets and the associated faecal material from rainbow trout fed those diets, (n=3,  $\pm$  S.E, unlike superscripts denote a significant statistical difference ANOVA  $P < 0.05$ ).

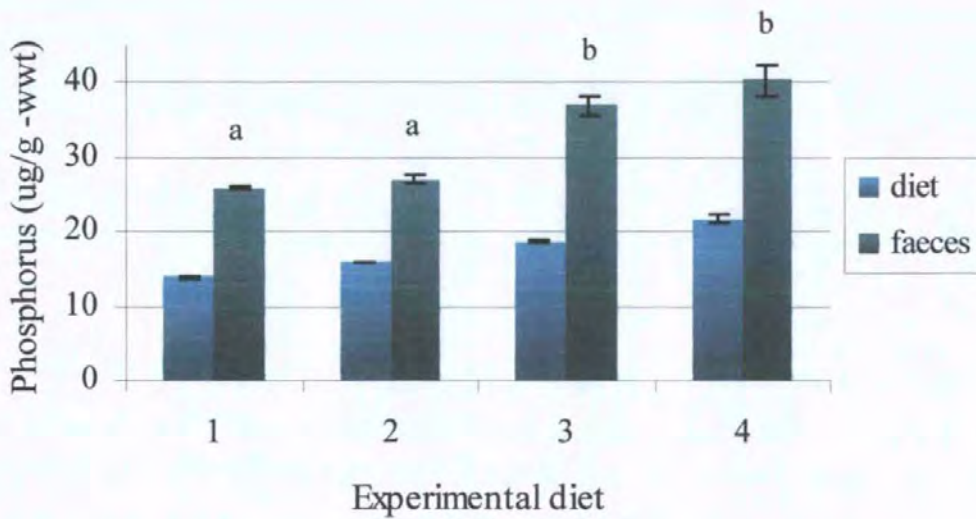


Figure 4.2

Phosphorus concentrations of the four experimental diets and the associated faecal material from rainbow trout fed those diets, (n=3,  $\pm$  S.E, unlike superscripts denote a significant statistical difference ANOVA  $P < 0.05$ ).

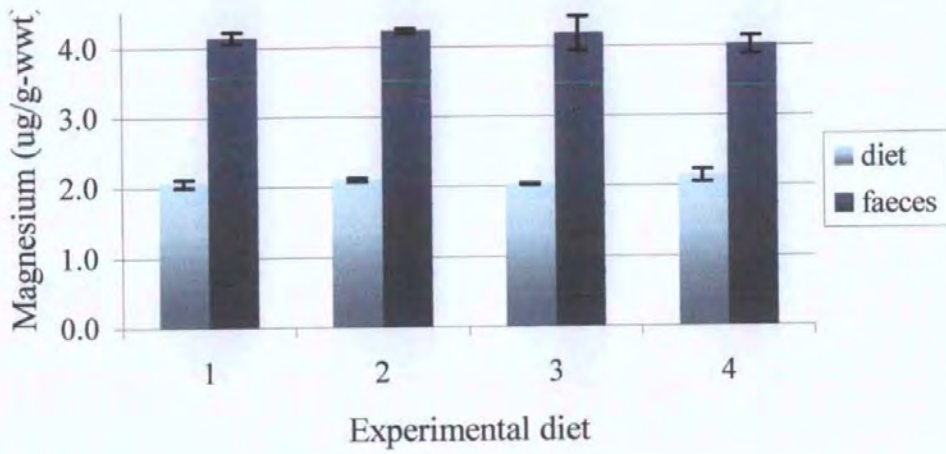


Figure 4.3  
Magnesium concentrations of the four experimental diets and the associated faecal material from rainbow trout fed those diets, (n=3,  $\pm$  S.E).

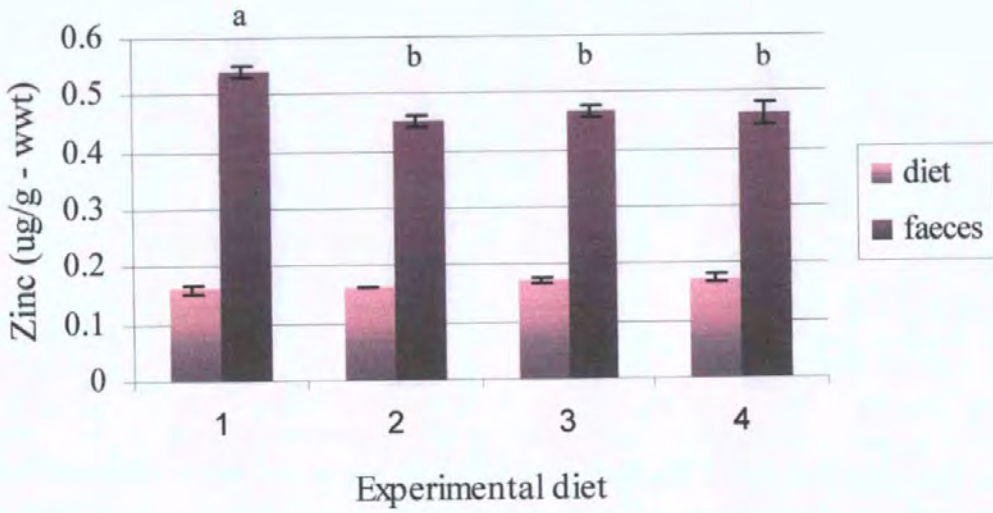


Figure 4.4  
Zinc concentrations of the four experimental diets and the associated faecal material from rainbow trout fed those diets, (n=3,  $\pm$  S.E, unlike superscripts denote a significant statistical difference ANOVA  $P < 0.05$ ).

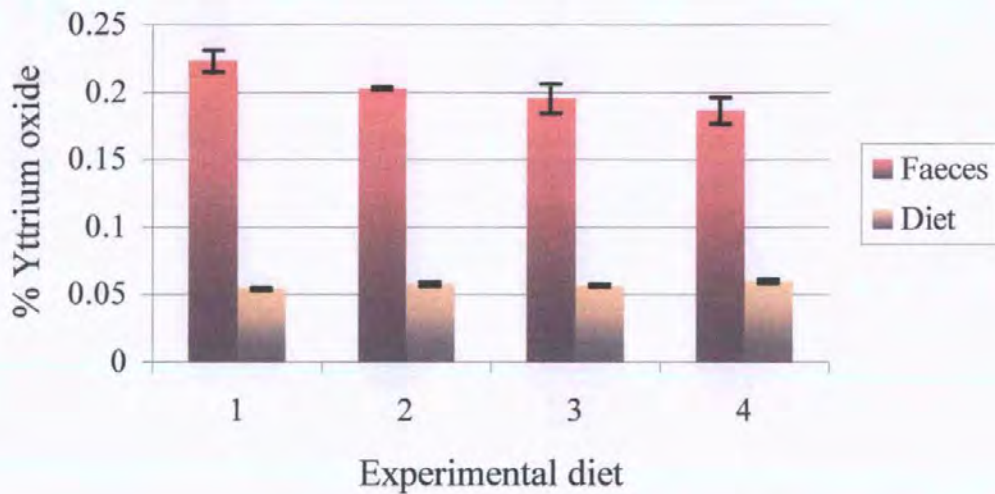


Figure 4.5

Yttrium oxide concentrations of the four experimental diets and the associated faecal material from rainbow trout fed those diets, (n=3,  $\pm$  S.E).

Figure 4.5 shows both dietary and faecal percentage concentrations of yttrium oxide in all four of the dietary treatments. Dietary levels were approximately the same at 0.05% for all four diets, whereas as the yttrium had been concentrated at least three to four times that of the dietary level when excreted by the fish. A trend can be seen between the four diets for excretory levels of yttrium, there is a general decrease from 0.22% to 0.18% in yttrium excretion as the mineral supplementation in the diet increases.



Table 4.8  
 Mineral apparent digestibility coefficients (%) for rainbow trout fed four experimental diets, (n=3,  $\pm$  S.E).

Mineral (%)	Diet 1	Diet 2	Diet 3	Diet 4
Phosphorus	54.68 $\pm$ 1.2 <sup>a</sup>	51.63 $\pm$ 0.8 <sup>a</sup>	47.64 $\pm$ 4.2 <sup>a</sup>	39.12 $\pm$ 3.7 <sup>b</sup>
Calcium	17.03 $\pm$ 1.2 <sup>a</sup>	11.05 $\pm$ 0.9 <sup>b</sup>	21.62 $\pm$ 2.0 <sup>a</sup>	3.52 $\pm$ 4.0
Magnesium	50.77 $\pm$ 2.2 <sup>a</sup>	42.90 $\pm$ 0.4 <sup>bc</sup>	46.70 $\pm$ 1.9 <sup>ac</sup>	39.20 $\pm$ 2.5 <sup>b</sup>
Zinc	18.44 $\pm$ 1.4 <sup>a</sup>	21.67 $\pm$ 0.7 <sup>a</sup>	28.90 $\pm$ 3.4 <sup>a</sup>	12.71 $\pm$ 4.1 <sup>b</sup>

Unlike superscripts denote a significant statistical difference ANOVA  $P < 0.05$ .

Table 4.8 shows the apparent mineral digestibility coefficients (ADC) from the four experimental diets fed to rainbow trout over the period of the growth trial. Phosphorus ADC's ranged from 39.12 to 54.68%, all diets were of similar percentages with the exception of fish fed diet 4 where the phosphorus digestibility from this feed fell to 39.2% and was significantly lower than all other diets. However, a clear decreasing trend can be noted for phosphorus digestibility when plotted against increasing dietary phosphorus, figure 4.6 shows this data fitted to a polynomial curve. Digestibility coefficients for calcium were the lowest ranging from 3.52 to 21.62% when compared with the ADC's for phosphorus, magnesium and zinc. As the data set of calcium apparent digestibility coefficients obtained for diet 4 comprised of several negative values giving a mean of 3.52%, a statistical analysis could not be applied to this data therefore, statistical analysis was only conducted on diets 1 to 3. Phosphorus digestibility from fish fed diet 2 was significantly lower (11.05%) than those fed diets 1 and 3 (17.03 and 21.62%).

Magnesium digestibility was highest from diet 1 at 50.77% and was significantly higher than diets 2 (42.9%), and 4 (39.20%). Additionally magnesium from diets 3 (46.70%) and 4 (39.20%) were significantly different from each other. Apparent digestibility coefficients calculated for zinc were similar for all dietary treatments with only one statistical difference being noted. Fish fed diet 4 had significantly reduced zinc digestibility (12.71%) when compared with the other three dietary treatments (18.44, 21.67 and 28.90% respectively).

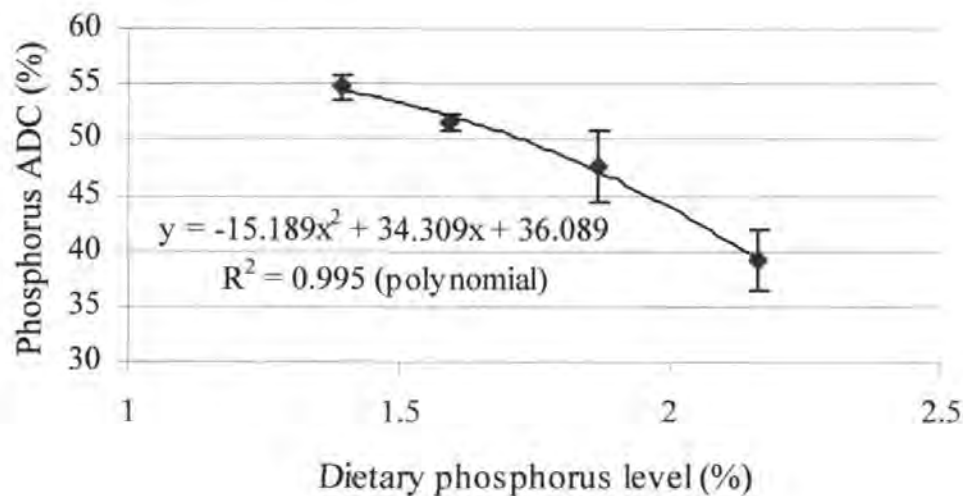


Figure 4.6  
Apparent digestibility coefficients of phosphorus against the dietary phosphorus levels included in experimental diets fed to rainbow trout (n=3,  $\pm$  S.E).

Percentage values established for the net apparent mineral retention are displayed in table 4.8. Apparent net phosphorus retention percentages range from 18.53 to 34.29% significant differences were established between nearly all diets, with the exception of diet 3 (23.39%) compared to diets 2 (26.4%) where no significant differences could be detected. Generally P retention decreased through diets 1 to 4. The data for diets 1 to 4 that were formulated with a fishmeal protein base and a range of mineral supplements, has been visually depicted in figure 4.7, from this graph the relationship between phosphorus dietary level and retention can easily be seen. An increase in dietary phosphorus relates to a reduction in the apparent net retention of this mineral.

Table 4.9

Net apparent mineral retention (%) for rainbow trout fed four experimental diets, (n=3,  $\pm$  S.E).

Mineral (%)	Diet 1	Diet 2	Diet 3	Diet 4
Phosphorus	34.29 $\pm$ 1.2 <sup>a</sup>	26.4 $\pm$ 1.62 <sup>b</sup>	23.39 $\pm$ 1.2 <sup>b</sup>	18.53 $\pm$ 0.48 <sup>c</sup>
Calcium	20.14 $\pm$ 2.07 <sup>a</sup>	24.65 $\pm$ 6.11 <sup>a</sup>	18.54 $\pm$ 1.07 <sup>a</sup>	16.10 $\pm$ 2.22 <sup>a</sup>
Magnesium	13.06 $\pm$ 0.35 <sup>a</sup>	13.66 $\pm$ 0.44 <sup>a</sup>	17.38 $\pm$ 2.33 <sup>b</sup>	11.91 $\pm$ 0.44 <sup>a</sup>
Zinc	14.75 $\pm$ 0.15 <sup>a</sup>	13.53 $\pm$ 0.66 <sup>a</sup>	14.51 $\pm$ 0.08 <sup>a</sup>	11.69 $\pm$ 0.17 <sup>a</sup>

Unlike superscripts denote a significant statistical difference ANOVA  $P < 0.05$ .

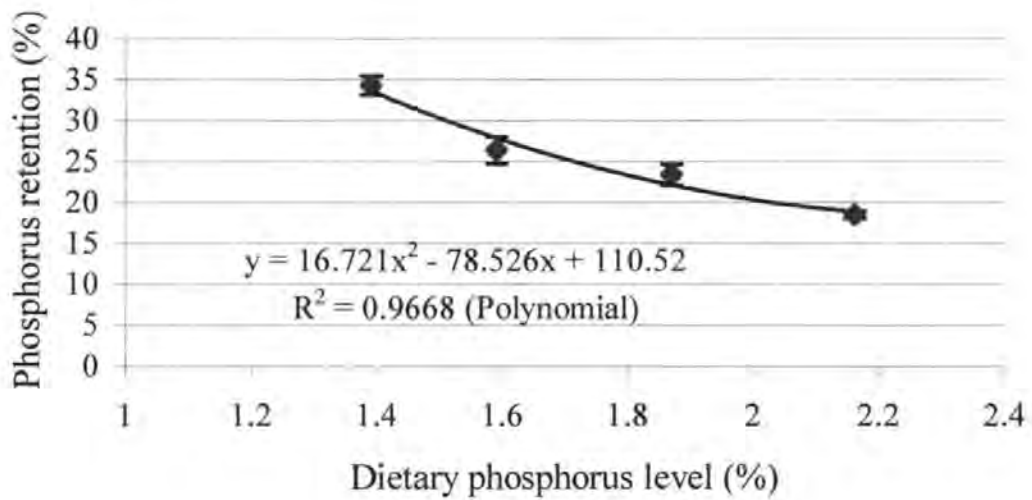


Figure 4.7  
Phosphorus retention against the dietary phosphorus levels included in experimental diets fed to rainbow trout, (n=3, ± S.E).

In general there were no significant differences between mineral retention data from fish fed the four experimental diets varying in mineral supplementation level. Apparent net calcium retention percentages range from 16.1 to 24.65% for the experimental diets 1 to 4, with no significant differences noted between treatments. Data ranged from 11.91% to 17.38% for the apparent net magnesium retention from experimental diets 1 to 4 fed to rainbow trout, with fish fed diet 3 having a significantly higher magnesium retention of 17.38% when compared with fish fed diets 1, 2 and 4. The apparent net zinc retention percentages resembled those of magnesium ranging from 11.69 to 14.75%, with no significant differences between dietary treatments.

## 4.4 Discussion

### 4.4.1 Growth and body mineral status

Experimental diets were formulated to reflect those of commercially based feeds with a predominant fishmeal component, in addition to a mineral supplementation of mono-basic calcium phosphate. It should be realised that mineral levels of phosphorus and calcium were in excess of dietary requirement. This fact should be kept in mind when interpreting results from this investigation. However these diets clearly demonstrate the ease with which diets with high mineral contents can be produced. This is particularly so as the base diet received no mineral supplementation yet contained a P level of 1.39%, thus indicating that fishmeal quality is essential when considering dietary mineral levels.

Whole body concentrations of the minerals observed in this investigation generally agreed with those reported in various published literature for salmonid fish. Phosphorus concentrations were between 4111 and 4596 $\mu\text{g/g}$  and Ca ranged from 3766 to 6645  $\mu\text{g/g}$  agreeing with other data for salmonid fish from Asgard and Shearer, (1997), Baeverfjord *et al.*, (1998), Nordrum *et al.*, (1997) Shearer and Asgard, (1990) and Skonberg *et al.*, (1997) and Ogino and Takeda, (1976) for carp (*Cyprinus carpio*). Higher body levels of P and Ca minerals have been listed for the rainbow trout (*O. mykiss*) by Hardy *et al.*, (1991), for the sunshine bass (*Morone chrysops* X *M. saxatilis*) Brown *et al.*, (1993) and tilapia (*Oreochromis aureus*) Robinson *et al.*, (1987). Magnesium (212.2 to 384 $\mu\text{g/g}$ ) were similar to those found for rainbow trout by Baeverfjord *et al.*, (1998), Shearer, (1989), Shearer and Asgard, (1990) Shearer and Asgard, (1992) and Skonberg *et al.*, (1997), although slightly lower than values published by Robinson *et al.*, (1987) for the tilapia and Atlantic salmon (*Salmo salar*) Nordrum *et al.*, (1997). Finally zinc (Zn) concentrations were between 21.5 to 28.9 $\mu\text{g/g}$ ,

similar to those established by Hardy and Shearer, (1985), Shearer and Asgard, (1990) and Maage *et al.*, (2001) for rainbow trout and Atlantic salmon.

The initial whole body concentrations for Ca and Zn were higher compared to the larger fish at the end of each trial, which has also been observed by Shearer (1984) and Shearer *et al.*, (1994). However no differences were noted between initial and 'final' fish for whole body P as observed by Ronsholdt, (1995). This is possibly due to the fact that P content indirectly decreases with increasing weight and volume and the 'final' fish during this trial only averaged an mean weight of 130g, in production terms is still a fairly moderate size for rainbow trout that can be 300-400g at market. Dietary mineral levels, particularly P, appeared to have no obvious effect on whole body P levels which contradicts findings by Skonberg *et al.*, (1997) who ascertained that whole body P concentrations were responsive to dietary P intake. Additionally whole body P as well as Ca and magnesium (Mg) have been used as an indicators of dietary mineral status by other researchers Hardy *et al.*, (1991); Ogino and Takeda, (1978), although Robinson et al (1987) found no relation between dietary Ca and whole body concentrations of Ca in tilapia (*Oreochromis aureus*). Shearer and Asgard (1990) showed that a secondary indicator of magnesium mineral status such as whole body concentration is useful in determining the relative availability of that mineral to the fish from various dietary sources. However in many other types of research study the mineral levels assessed usually encompass the whole estimated range for that particular mineral, for example between 0.25 to 1.15% for P in salmonid fish. These mineral ranges are commonly formulated into semi-purified diets using casein/gelatin as the protein source, therefore the inorganic supplement is fundamentally the only available mineral source and hence results can be directly related to the dietary mineral level. Using an inorganic mineral supplement formulated into diets using fishmeal as the primary protein ingredient was held to be important

in this study, because the essence of the programme was to evaluate mineral interactions in feeds similar to practical commercial feeds. This could lead to a discussion involving the origin of mineral utilised by the fish – the fishmeal or the supplement and were there any interactions. However due to the unexpectedly high P levels in the diets this was not possible, as results obtained for fish fed diet 1 demonstrate the high level of minerals from the fishmeal alone was sufficient for optimal growth and health of the fish. This reasoning may aid in explaining why body mineral concentrations did not reflect the dietary mineral levels.

Mineral vertebral concentrations did not reflect dietary mineral levels. Vertebral mineral concentrations were similar to other published data (Dougall *et al.*, 1996; Ogino and Takeda, 1976; Ogino *et al.*, 1979; Porn-Ngam *et al.*, 1993; Robinson *et al.*, 1987; Sakamoto and Yone, 1978; Vielma, 1998; Vielma and Lall, 1998a; Maage *et al.*, 2001). Consequently bone minerals levels did not reflect dietary levels unlike alternative data that reports such associations. Bone phosphorus and calcium have been found to be reflective of dietary level of that mineral for various fish species (Robinson *et al.*, 1987; Sakamoto and Yone, 1978; Vielma and Lall, 1998a), in addition to zinc (Maage and Julshamn, 1993; Maage *et al.*, 2001). As differences during this investigation were only noted for P it is possible that other minerals such as Ca and Mg could be absorbed from the water if dietary levels were insufficient. Both magnesium and zinc levels during this study were constant and consequently no differences were noted between zinc concentrations in the whole body or vertebrae. Vertebral levels of P and Ca may not have reflected the dietary P and Ca levels for diets 1 to 4 due to the high minerals content of these diets. Such levels were probably adequate at supporting bone mineralisation even in the basal diet (number 1).



Brown et al (1993) conducted a trial to assess the dietary P requirement of juvenile sunshine bass (*Morone chrysops* X *M. saxatilis*), by feeding them a basal diet formulated with red drum muscle as opposed to a casein diet. Bone composition was found to be influenced by dietary P, although there was an inability to induce severe dietary P deficiencies due to the relatively high level of P present in the basal diet. This latter point highlights the difficulty in formulating a 'realistic' feed using an animal based protein source to produce diets with a low P level without relying on a casein based diet. However in order to formulate diets that have a high protein content (45%) and a low phosphorus content (less than approximately 0.8%) a casein or egg albumin protein source must be used due to the naturally low levels of phosphorus in this protein source.

Vielma and Lall (1998) stated that bone ash content is a sensitive criterion for evaluating P utilisation in addition to many other researchers Asgard and Shearer, (1997), Ketola, (1975), Ketola and Richmond, (1994) and Watanabe *et al.*, (1980). Results from the current study support this theory. Fish fed diets 1 to 4 had higher overall growth and elevated levels of P in their vertebrae. Studies indicating that excessive dietary levels of P, Ca and Mg can reduce zinc availability, growth and vertebral Zn content have been reported by Andrews *et al.*, (1973) for catfish (*I. punctatus*), and Porn-Ngam *et al.*, (1993), and Spinelli *et al.*, (1983) for rainbow trout (*O. mykiss*). Although a relationship akin to this was not established during this investigation, this was possibly because the mineral supplement added had a high bioavailability and when in excess passed through the digestive tract without interacting with the zinc portion of the diet.

Mineral composition of the kidney indicated increased levels of P, Ca and Mg in the diets containing the highest mineral levels (namely 3, and 4). As the kidney is the primary route for

urinary excretion it is possible that minerals from these diets are in excess and/or are unavailable and are therefore being excreted. This suggestion could be clarified if urine samples had been collected from the fish and analysed for mineral content. Data for spleen mineral levels appears somewhat variable with no obvious trends.

#### **4.4.2 Blood and bile mineral status**

Both blood glucose and haematocrit levels obtained in this study were within the normal range for haematological and blood chemistry characteristics for salmonids (Hille, 1982; Houston and Smeda, 1979; Miller *et al.*, 1983; Robinson *et al.*, 1996; Warner *et al.*, 1978). Dietary treatments appeared to have no effect on these parameters, which was expected as the fish employed throughout this growth trial maintained in good health, with these blood parameters reflecting this observation. Whereas a reduction in haematocrit level, has been stated by Andrews *et al.*, (1973) as a clinical sign of P deficiency in channel catfish, also variations in blood glucose often reflect stress. Again haemoglobin concentrations are comparable with those published by Miller *et al.* (1983) and Houston and Smeda (1979) and are within the normal range published for rainbow trout. Sakamoto and Yone, (1977) established that an elevation in the hepatosomatic index related to a decrease of dietary P level, results from this investigation partially agree with this theory. As fish fed diet 1 had a significantly higher HSI when compared to all other results, diet 2 also appears to follow this trend.

The range of mineral concentrations for calcium, magnesium and zinc obtained for the blood plasma from fish fed the 6 experimental diets were in accordance with those obtained by Brown *et al.*, (1993), Miller *et al.*, (1983), Spry *et al.*, (1988), Warner *et al.*, (1978), Yone and Toshima, (1979), Dougall *et al.*, (1996), Maage *et al.*, (2001), Sugiura *et al.*, (2000c) and Vielma and Lall, (1998b). However the plasma concentrations ascertained for phosphorus

were extremely elevated (approximately 55mg/dL, or 18mmol/L) when compared to other published data. However it should be noted that analysis of these samples produced total mineral levels not just the inorganic portion, additionally the blood plasma as opposed to the serum or whole blood was utilised. As P has such a high metabolic demand it is possible that the organic proteinaceous fractions incorporating bound P were present in the sample. Published data for blood plasma is generally for inorganic P and ranges between 4 - 25 mg/dL (Brown *et al.*, 1993; Dougall *et al.*, 1996; Miller *et al.*, 1983; Sugiura *et al.*, 2000c; Vielma and Lall, 1998a; Vielma and Lall, 1998b; Warner *et al.*, 1978; Yone and Toshima, 1979). It would be reasonable to expect total P levels to be higher than those of just inorganic P, which may explain to the high range found in this study.

During a growth trial with rainbow trout, Rodehutscord, (1995) established that inorganic phosphate levels related to dietary concentration at low levels, and as the dietary level increases the equivalent plasma concentration rises, reaching a plateau. This plateau was found to be at dietary level of approximately 6g/kg of P in the dry feed and resulted in a 5mmol/L inorganic phosphate level in the blood plasma. This 'plateau' effect was also noted by Brown *et al* (1993) and Vielma (1998), who suggested it as evidence of homeostatic control, or it could be saturation of the physiological transport mechanisms with inorganic phosphorus. It seems that rainbow trout are able to maintain high plasma P as long as dietary P is adequate, which may explain the high P plasma levels for this investigation due to the high dietary mineral levels of P and Ca. Variable plasma phosphate concentrations can also be attributed to the time lapsed since last feeding (Rodehutscord, 1996). Serum phosphorus has been shown to increase linearly with phosphorus supplementation for channel catfish Eya and Lovell, (1997) and for rainbow trout by Sugiura *et al.*, (2000b). A linear increase was not observed during this trial, although plasma P levels for fish fed diet 4 that received the highest

level of inorganic supplement were significantly higher when compared to all other treatments. Both Mg and Zn in plasma, have been found to be negatively influenced by increased levels of dietary P and Ca (Porn-Ngam *et al.*, 1993; Vielma and Lall, 1998b), and results from this trial indicate that fish fed the basal diet that received no mineral supplementation had the highest levels of plasma zinc. Overall no obvious trends can be identified throughout the mineral blood plasma data, which could possibly be a result of variation in the samples. Or more likely that mineral levels were so high in the diets (for P and Ca) fish were able to sustain constant high plasma levels through homeostatic control exhibiting a 'plateau' effect, or the saturation of basic physiological processes with these elements.

Levels of mineral supplementation appeared to have no effect bile phosphorus and zinc concentrations. However, calcium and magnesium levels were significantly reduced in fish fed diet 1, the diet with no additional mineral supplementation. Bile fluid naturally contains inorganic salts, but there is little research showing variation in mineral levels between bile in fish, even though diet is known to be an important modulator in the process of bile formation and secretion (Tuchweber *et al.*, 1996). One of the natural functions of bile is to carry waste that has been removed from the bloodstream by the liver into the gallbladder. If levels of calcium and magnesium were in excess, it is feasible that they are being removed from the blood to be excreted via the bile, which may explain elevated mineral levels in the bile of fish fed the diets containing the additional mineral supplementation. It should additionally be highlighted that the bile samples taken were not quantitative in relation to total volume of bile in the gallbladder, as it has been noted that the production and flow rate of bile varies when dietary components change (Tuchweber *et al.*, 1996).

#### 4.4.3 *Apparent mineral digestibility and retention*

As to be expected faecal concentrations reflected dietary levels of P, Ca and Mg, the exception to this theory was the mineral zinc. Both P and Ca followed a similar excretory trend with the fish fed the highest mineral supplemented fishmeal diets excreting the highest levels of minerals. The variability in the levels of mineral excretion can be explained by a few fundamental physiological theories. The excreted minerals are either biologically unavailable to the fish, in excess of their dietary requirements, or the fish are unable to utilise them in such high levels i.e the membrane co-transporters in the gut maybe flooded or the rate of passive diffusion is at its peak capacity. As fish were provided with excessive levels of P and Ca it is probably valid to state that optimal growth and bone mineralisation was reached for fish fed diets 1 to 4 therefore mineral levels excreted were excessive combined with any unavailable minerals and natural endogenous losses. Dietary zinc levels were constant however faecal zinc concentrations were not reflective of this, as excretory patterns appeared similar to that of Ca and P. This may indicate that there could be possible physiological interactions between Ca, P and Zn. Similar findings on this possible relationship have been published by Andrews *et al.*, (1973), Porn-Ngam *et al.*, (1993), Richardson *et al.*, (1985), and Spinelli *et al.*, (1983). The latter author established that dietary P in excess of the required level led to a reduction in Zn absorption, whereas excessive Ca did not. However excessive P together with Ca, again led to a reduction in Zn absorption, growth and Zn content. As a component of this work this researcher also assessed the effect of Ca to P ratios, and concluded that a ratio of 1:1 was optimal for lessening deleterious affects on Zn fed to rainbow trout. In this current study the Ca:P ratio was maintained at 1 throughout the range of dietary treatments, and yet differences in Zn excretory rates were encountered between diets, perhaps indicating that during this trial it was the total dietary Ca and P levels playing of a role in influencing Zn rather than the Ca:P ratio. Further interactions between the dietary minerals can be noted when reviewing both the

apparent digestibility and retention data. Zinc absorption and retention appears to increase with dietary mineral supplementation level indicating a possible agonistic effect which is reversed to an antagonistic effect when mineral supplementation is at its highest, where the Zn ADC and retention values are reduced. This same trend was also noted for magnesium, which also suggests the vulnerability of this mineral to interact with high dietary levels of Ca and P. Mineral interactions may be particularly apparent if the Ca and P in question is from a hard tissue origin such as hydroxyapatite, tricalcium phosphate or excessive levels of an inorganic source (Sato *et al.*, 1993), as these mineral salts have the ability to chelate with other free mineral ions. An interesting study was conducted by Paripatananont and Lovell, (1995) who established that using 'pre-chelated' mineral sources in diets for channel catfish (*I. punctatus*) resulted in higher mineral digestibility coefficients, when compared to use of standard inorganic mineral supplements, for example zinc proteinate vs. zinc sulfate. These results show the potential use of chelated mineral sources as opposed to inorganic mineral sources in dietary premixes.

Apparent mineral digestibilities for magnesium and phosphorus were the highest approximately ranging between 40 and 55%, calcium and zinc had similar lower digestibility coefficients ranging between 3 and 28%. These results obtained during this growth trial were in general agreement with other published mineral digestibility data (Kim and Ahn, 1993; Sugiura *et al.*, 1998; Bai *et al.*, 2001; Storebakken *et al.*, 2000; Sugiura *et al.*, 2000a). Most dietary related trials that are involved with the estimation of mineral requirements are usually formulated using a casein/gelatin protein base. In this type of diet the only source of P is from an inorganic supplement result in far higher mineral digestibility coefficients such as 93% (Baeverfjord *et al.*, 1998). High ADC's are the result of the inorganic mineral source being highly available to the fish, there are also no other mineral components of the diet for

supplemented minerals to interact with. In these diets the primary protein source is casein which is practically 'mineral free' and therefore provides limited available minerals in the way fishmeal would. Additionally researchers often quote digestibility coefficients for individual dietary components such as the mineral supplement which are considerably higher than those obtained for the diet as a whole. These are the primary reasons help in explaining why ADC's obtained from commercial or commercial like aquafeeds often produce much lower mineral digestibility coefficients.

Overall, the fish fed diet 4 had reduced digestibility coefficients for all minerals, suggesting a possible 'mineral overload', due to the over-supplementation of minerals to these fish.

Various mineral interactions and chelation effects are probably the cause for this, with all minerals bound to such a degree that the digestibility suffered and as a result their bioavailability became limited. A further explanation that may play a role in influencing mineral digestibility from the experimental feeds is the dietary protein source. Although the experimental diets primarily utilised fishmeal as a protein base, they all contained 24% soybean meal, in which anti-nutritional factors such as phytate occur. It is known that the phytate component of plant proteins has a high affinity for zinc and to a certain extent magnesium, and can form a stable complexes (Maddaiah *et al.*, 1964; O'Dell and Savage, 1960; Reddy *et al.*, 1978; Reinhold *et al.*, 1973). Research by Oberleas *et al.*, (1966) and Oberleas and Harland, (1981) has illustrated that these mineral complexes are insoluble and difficult to absorb across the gastrointestinal tract of vertebrates, and consequently leads to a decrease in the absorption of this mineral. A decrease in both the magnesium and zinc digestibility lead to reduced retention of these minerals due to the presence of phytate in the diet is clearly shown in the results from this study, and has been noted in other literature for chinook salmon (*Oncorhynchus tshawytscha*) by Richardson *et al.*, (1985).

As expected apparent net mineral retention values were lower than that of the corresponding digestibility coefficients but are in agreement with those of Hardy and Shearer, (1985) for rainbow trout, Kim *et al.*, (1998a), Kim *et al.*, (1998b), Kim *et al.*, (1995) for carp, and Lovell, (1978) for work with channel catfish. However, retention data presented for fish fed casein/gelatin diets with varying mineral levels is higher and ranges between 46 to 91% for salmonids (Asgard and Shearer, 1997; Hardy *et al.*, 1991; Nordrum *et al.*, 1997).

Phosphorus retention exhibited an obvious trend that showed an increase in dietary P resulting in a reduced retention percentage for the four trial diets. However it should be remembered that since the range of experimental diets contained high levels of dietary P and even the base diet was adequate in providing enough minerals for optimal growth, this would suggest that in general dietary minerals levels were in excess. Therefore the minimum levels for P and to a certain extent Ca were above the dietary requirement and hence retention cannot be used to accurately measure mineral availability as the excess is excreted and relative retention decreases as the dietary concentration is increased. This was also found by Nordrum *et al* (1997). Alternatively, Hardy *et al* (1991) established a dose-dependent relationship between apparent phosphorus retention and dietary phosphorus level, which is probably true at levels close to that of requirement, but this relationship cannot be directly compared with this work due to the excessive levels under question. Further work would be necessary to ascertain at what dietary inclusion level of P this relationship is expressed, and at what P levels the relationship ceases.

To conclude, dietary mineral levels added to the diets fed to rainbow trout in this study were adequate to meet optimal growth and bone mineralisation. Net apparent mineral retention is not a good indicator of mineral bioavailability when dietary mineral levels are in excess, under



the same dietary conditions both whole body and blood plasma levels were poor at reflecting dietary mineral levels, but vertebrae appeared to be a little more sensitive. Careful consideration when formulating aquafeeds is essential when trying to optimise both the protein and mineral status of the fish.

For these reasons, the following chapter details investigations to ascertain the more detailed role of the gastrointestinal tract of rainbow trout with respect to differential absorption of dietary protein and minerals. Digestibility characteristics of key feed ingredients are of fundamental importance and the focus of the work was directed towards establishing availability of the major elements of interest from a selection of feed ingredients and mineral sources.

## **CHAPTER 5**

**PART A STUDIES TO DETERMINE THE DIFFERENTIAL  
ABSORPTION OF DIETARY PROTEIN AND MINERALS IN  
THE GASTROINTESTINAL TRACT OF  
RAINBOW TROUT**

**PART B PROTEIN AND MINERAL DIGESTIBILITY OF  
SUITABLE ALTERNATIVE FEEDSTUFFS AND VARYING  
DIETARY MINERAL SOURCES AND ADDITIVES FOR  
RAINBOW TROUT**

## CHAPTER 5

### PART A. STUDIES TO DETERMINE THE DIFFERENTIAL ABSORPTION OF DIETARY PROTEIN AND MINERALS IN THE GASTROINTESTINAL TRACT OF RAINBOW TROUT

#### 5.1 Introduction

##### 5.1.1 Structure of the digestive tract

The digestive systems of fish are subject to large inter-species variations, which are usually dependent on the mode of nutrition and composition of diet. The major divisions of the teleost alimentary canal are mouth, buccal cavity, pharynx, esophagus, stomach, intestine, rectum and related organs (Fänge and Grove, 1979). Most teleost fish including salmonids possess a digestive canal that is structurally divided into functionally different parts; i.e:- the esophagus, stomach and intestine, with the liver and gallbladder as associated organs. The pharynx passes into a short but muscular esophagus of which the mucosa is dominated by goblet cells, which are capable of producing large quantities of mucous acting as a lubricant to achieve liquifaction and to facilitate food transit. A typical salmonid stomach is 'J' shaped and has a lining of columnar epithelial cells, throughout which are scattered goblet cells. Tubular glands along with neck cells are also evident and primarily found in the cardiac and fundic regions of the stomach. The tubular glands are mainly comprised of gastric gland cells, which are similar to chief cells in other animals, and in 1955, Weinreb and Bilstad established that they are active in both acid production and the synthesis of pepsinogen. Generally, the stomach is the site of mixing and primary digestion of food (Horn, 1998), and terminates in a pyloric sphincter, which consists of a thick circular smooth muscle layer, into the pyloric region of the intestine.

Among vertebrates, teleost fish are unique in being the only species to possess appendages called caeca at the gastrointestinal junction (Kent, 1983). Kapoor *et al.*, (1975) stated that

these blind ended appendages are present in over 60% of all known fish species but their exact function is still not known. Pyloric caeca can be highly variable in nature, particularly in teleost fish where reported numbers are between none to numerous (>1000). The number and size varies considerably between fish group, species and even within species. The intestinal caeca consist of well developed musculature consisting mainly of circular muscle fibres, the inner epithelium contains goblet cells but studies have indicated that they lack cells that have the ability to secrete digestive enzymes. Although it has been indicated that caeca supplement the digestive function of the stomach or intestine by increasing the surface area for digestion and absorption (Buddington and Diamond, 1987; Thorarensen *et al.*, 1991), these speculative views have yet to be confirmed experimentally. In teleosts the intestine varies greatly and can be short and straight or comprised of various loops and folds, and is usually related to the species dietary preferences (Fange and Grove, 1979). The intestinal mucosa is lined by simple columnar epithelium with a scattering of goblet cells, the former of which possess a brush border of microvilli typical of absorptive tissues. The submucosa is thin and contains collagen and elastic fibres, blood vessels and nerves. The musculature consists of inner circular and outer longitudinal smooth muscles. The terminal part of the hind gut leading to the rectum is demarcated from the mid intestine by banding of striated smooth muscles, and is typically enriched with goblet cells, this rectum region widens as it leads to the anus from where solid waste is excreted.

### **5.1.2 Protein Digestion**

Digestive proteolytic enzymes that act on the peptide links of proteins are secreted into the lumen of the alimentary canal from various origins but predominantly the gastric glands. Gastric fluid has been found to be highly acidic in many teleost fish between pH 2 and 4 (Kapoor *et al.*, 1975). However, the majority of intestinal enzyme activity occurs in optimal conditions between pH 6 and 8. This is due to the release of bicarbonate ( $\text{HCO}_3^-$ )

and alkaline bile salts which neutralise the hydrochloric acid (HCl) in preparation for alkaline digestion, (De Silva and Anderson, 1995; Fange and Grove, 1979). The secretion of HCl as a proportion of the gastric fluid into the stomach occurs usually in response to the presence of food. Another important component of gastric fluid is a zymogen called pepsinogen, which is inactive. However, pepsinogen is converted to the active pepsin by acid hydrolysis, and is undoubtedly the primary protease enzyme encountered.

Consequently, most of the initial digestion of protein occurs in the stomach as a result of the action of pepsin. The endopeptidase activity of the gastric fluid renders proteins soluble and more readily digested by subsequent pancreatic and intestinal proteases.

Further protein degradation occurs in the anterior intestine where trypsin and chymotrypsin play an important role in breaking down soluble proteins into polypeptides. These polypeptides are hydrolysed further by pancreatic carboxypeptidases and intestinal peptidases secreted from the exocrine pancreas and intestinal fluids respectively.

Consequently, the digesta mixture present in the intestinal lumen comprises mainly low molecular weight peptides and amino acids as well as undigested protein.

A comprehensive review of carbohydrate and protein digestibility and absorption was written by Alpers in 1994, where it is stated that protein absorption mechanisms are diverse and complicated. This is mainly because fish are able to absorb proteinaceous components either as amino acids, peptides or whole proteins (Smith, 1989). Free amino acids are absorbed actively into enterocytes by sodium linked transporters and carrier molecules. Such systems were also found to exist for small un-hydrolysed peptides (Ash, 1985). Large peptides and proteins can enter directly into the blood stream via a paracellular route, or by pinocytosis into enterocytes where deamination or transamination usually occurs (Jobling, 1995).

### 5.1.3 Mineral Absorption

The dietary requirement for various minerals particularly phosphorus has been studied for many species of fish including Atlantic salmon (*Salmo salar*) (Asgard and Shearer, 1997; Ketola, 1975), striped bass (*Morone saxatilis*) (Dougall *et al.*, 1996), sunshine bass (*Morone chrysops* X *M. saxatilis*) (Brown *et al.*, 1993), American cichlid (*Cichlasoma urophthalmus*) (Chavez-sanchez *et al.*, 2000), red drum (*Sciaenops ocellatus*) (Davis and Robinson, 1987), channel catfish (*Ictalurus punctatus*) (Lovell, 1978; Wilson *et al.*, 1982), and rainbow trout (Ketola and Richmond, 1994; Rodehutsord and Pfeffer, 1995b). However, little research has focused on the intestinal absorption of inorganic phosphate ( $P_i$ ) in fish, whereas the mechanisms of intestinal  $P_i$  transport has been established for other vertebrates as a result of research conducted by Borowitz and Ghishan, (1989), Breves and Schroder, (1991), Schroder *et al.*, (1990), and Sirazi-Beechey *et al.*, (1996). Recently, a paper by Avila *et al.*, (2000) described the mechanism of intestinal  $P_i$  transport in the rainbow trout by manipulating dietary levels of phosphorus. It was established that in the small intestine of trout inorganic phosphate uptake was strongly inhibited by phosphonoformic acid, a competitive inhibitor of mammalian inorganic phosphate transport, as well as the absence of  $Na^+$ . This may indicate the presence of a Na-dependent inorganic phosphate carrier transporting phosphorus across the intestine, which is regulated by dietary phosphorus levels. This is a physiological process similar to that found in birds and fish in general.

In mammals and birds, intestinal  $P_i$  absorption has been found to occur via two physiological mechanisms: 1) a passive non-saturable transport and 2) a secondary active sodium coupled transporter (Breves and Schroder, 1991). This latter mechanism has been shown specifically to decrease with increasing dietary phosphorus or nutritional status in vertebrates by Lee, (1986), and Murer *et al.*, (1994). This process has been demonstrated for other minerals such as iron, zinc, calcium and copper (Ferraris, 1994), where

transporters of essential minerals are down-regulated with dietary concentration (Karasov and Diamond, 1987). When dietary mineral concentrations are high, physiological requirements can be satisfied absorbed primarily by passive absorptive processes or few active transporters, where as during low dietary levels more transporters are needed to achieve this uptake minerals against a concentration gradient.

Phosphorus is thought to be regulated by several mechanisms throughout the vertebrate body that are homeostatically controlled, an integral part of such regulation appears to be the role of vitamin D and its active metabolites. Vitamin D is known to regulate plasma  $P_i$  concentration by regulating intestinal and renal handling of  $P_i$  in mammals. For example, if phosphorus plasma decreases the production of an active metabolite of vitamin D called calcitriol (1,25-dihydroxycholecalciferol) is stimulated and released into the circulatory system. Calcitriol stimulates the active sodium-coupled  $P_i$  transport system located in the upper small intestines, which in turn increases the  $P_i$  absorption from the gastro-intestinal tract. This process assists in maintaining plasma phosphorus at normal physiological levels (Schroder *et al.*, 1996). However, when levels of dietary phosphorus are sufficient, vitamin  $D_3$  and its metabolites do not appear to stimulate  $P_i$  absorption by the intestine in the rainbow trout (Avila *et al.*, 1999).

The homeostatic control of calcium in vertebrates is also mediated in a similar way to phosphorus by calcitriol (Avila *et al.*, 1999; Schroder *et al.*, 1996), and is often simultaneous in effect (Schroder *et al.*, 1996). Calcitriol stimulates the production of a calcium binding protein that has been proposed to facilitate the movement of calcium ions across intestinal biomembranes into enterocytes. It also appears that, regardless of dietary mineral level, intraperitoneal injections of cholecalciferol or calcitriol increase the intestinal  $Ca^{2+}$  uptake in goldfish (Fenwick, 1984).

It appears that there are many factors influencing the absorption of minerals across the gut, many of which are complicated and inter-dependent. This qualitative study aims to investigate the relative levels of mineral and protein absorption throughout the gastrointestinal tract of the rainbow trout by paying particular attention to four different regions of the gut deemed to be of major importance in the digestion sequence of rainbow trout.

## **5.2 Materials and Methods**

### **5.2.1 Experimental facilities**

As this fundamental study required a small number of fish to be sacrificed, and be of a short time period; 'stock' fish that were already held in the aquarium facility at the university were utilised. This investigation involved the determination of absorption profile, and as a consequence large fish were utilised, to ensure that a sufficient faecal sample size was obtained and to minimise handling stress during faecal collection. In five square 160L fibreglass tanks, 15 rainbow trout (*Oncorhynchus mykiss*) with an average weight of 350g were being held, a random selection of these fish were used for this study.

The fibreglass tanks were suspended over three linked tanks containing mechanical filtration, biological filtration and cooling compartment. Together they comprised a closed re-circulation system with additional mechanical filtration being provided by a Hi-Rate sand filter. Freshwater flowed into each tank at a rate of 13L/minute, and water temperature was maintained at  $15\pm 1^{\circ}\text{C}$  by the use of a submerged coolant system. Various water parameters were monitored including phosphorus, nitrite, nitrate and ammonia/ammonium and these were all held within ranges tolerated by rainbow trout. The pH levels were maintained within the range of 6.5-7.5 by the use of calcium carbonate and 'magnospheres' to aid in the buffering capacity of the water. Photoperiod was maintained at a 12hr light: 12hr dark regime, by timed lighting.



### 5.2.2 Diet formulation and sample collection

Table 5.1  
Dietary formulation for standard expanded test diet, fed to rainbow trout.

Dietary component	(%)
Peruvian fishmeal	17.36
Soya meal (full fat)	21.40
Maize gluten meal	20.00
Whole wheat	30.61
Vitamin/mineral premix	0.62
Remix *	10.00

\*Remix:- This is the added fraction obtained from industrial sweepings of previous diet production in the feed mill.

Table 5.2  
Proximate and mineral composition for the 'standard expanded' test diet.

Dietary Component	(%)
Protein	46.52
Lipid	7.82
Moisture	6.41
Ash	10.00
Calcium	0.782
Magnesium	0.175
Phosphorus	0.740
Zinc	0.022

To assess protein and mineral uptake in the digestive system of the rainbow trout, a commercially available floating pellet diet (4mm) was utilised. The feed was a 'standard expanded' variety and is frequently used for stock fish as a maintenance diet in farms operating under extensive conditions. The test diet formulation is presented in table 5.1. with the protein and mineral composition profile being summarised in table 5.2.

### ***5.2.3 Feeding, sampling and analysis***

Fish in the five experimental tanks were fed twice a day to satiation at 10.00am and 4.00pm, for a period of 4 days. On the 5th day the fish were fed in the morning and three hours after the feeding of the last meal two fish from each of the five tanks were randomly chosen for sacrifice. The rainbow trout were over-anaesthetised using 2-Phenoxy-ethanol (0.2ml/L), and culled in accordance with Schedule 1 of the Home Office Animals (Scientific Procedures) Act 1986. Dissection was conducted by an initial dorso-ventral incision from the anus to vent, thus opening the body cavity. Once exposed, fat deposits were gently excised from the abdomen. When all the internal organs and structures could be clearly seen, metal hair pins were used to segregate the digestive system into four sections; the stomach, pyloric region (including caeca), the mid gut and hind gut and anus (refer to Figure 5.1). The whole digestive tract was then carefully surgically excised from the carcass, by cutting through the oesophagus above the stomach and dissecting downwards ceasing at the anus. Dissection was close to the last meal to ensure that the whole gut would be full of digesta. The whole gastrointestinal tract including the attached stomach was laid flat in a plastic tray, bagged and labelled then immediately frozen at -17°C.



Figure 5.1  
Gastrointestinal tract of rainbow trout showing four segmented sections. (Photographs courtesy of Mr Paul Russel)

To obtain the digesta, samples were removed from the freezer and cut into the four sections segregated by the dividing hair-pins. The individual sections were then allowed to partially defrost and the contents removed by laterally cutting through the gut wall, digesta was then freeze dried after which samples were acid digested by microwave and the mineral content determined by ICP-AES. Additionally, the protein analysis was carried out by the kjeldahl method (refer to chapter 2 for methods).

Statistical analysis was employed to test for any significant differences between all gut regions with respect to mineral and protein concentrations in digesta. Differences among the digesta from the four areas of the gut were tested by single factor ANOVA followed by a Student-Newman-Keuls (SNK) multiple stage test. Differences were considered significant at  $P < 0.05$ .

### **5.3 Results**

Concentrations of the mineral and protein faecal fractions taken from the digesta throughout the gastro-intestinal tract of the fish are shown in Figures 5.2a to e. Mineral concentrations were calculated as absolute taking into account the varying amounts of digesta in each of the gut sections.

The four bar-graphs illustrating the mineral concentrations throughout the gastro-intestinal tract clearly all follow the same trend. Mineral levels are the highest in the stomach decreasing considerably to approximately a tenth of the concentration in the pyloric gut region. These levels then increase slightly between the pyloric and mid gut regions and again between the mid and hind gut, final concentrations are approximately a third of the values obtained for the stomach region. The exception being for zinc which was approximately half of the initial diet concentration, thus illustrating a lower digestibility for zinc.

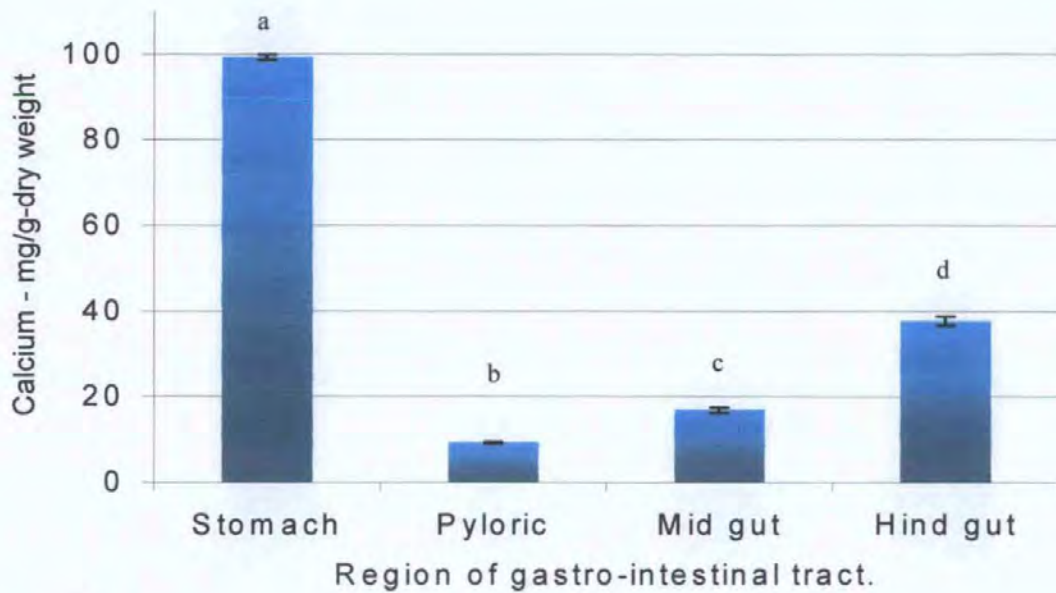


Figure 5.2.a.  
 Concentrations of calcium in the digesta distributed throughout the four regions of the gastro-intestinal tract of rainbow trout fed to satiation with a 'standard expanded' diet ( $n=5 \pm S.E$ ). Unlike superscripts denote a significant statistical difference (ANOVA, SNK,  $P < 0.05$ ).

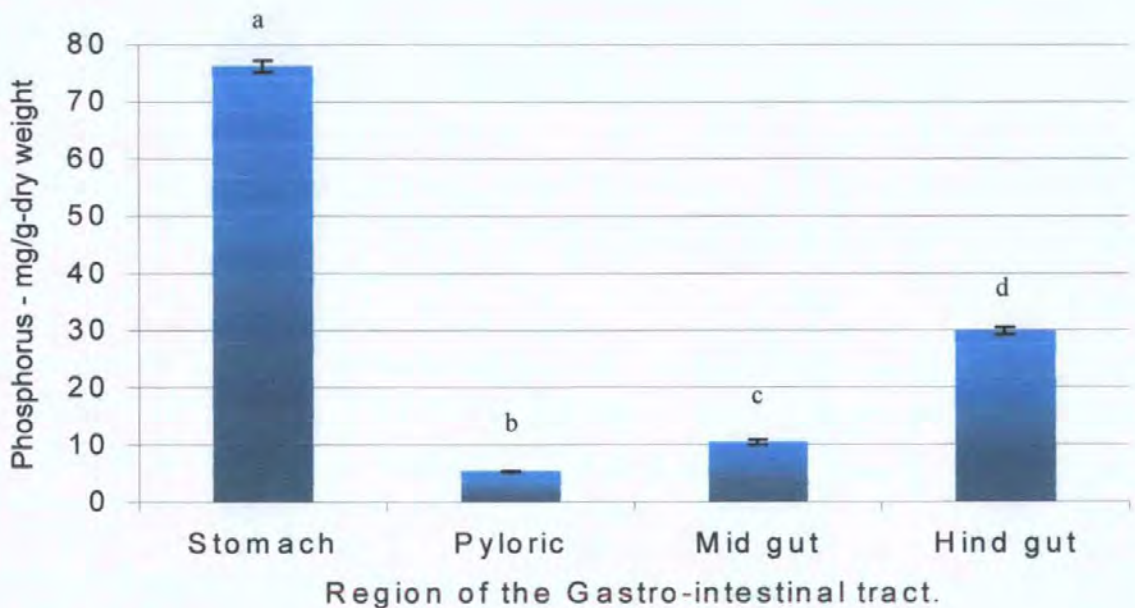


Figure 5.2.b.  
 Concentrations of phosphorus in the digesta distributed throughout of the four regions of the gastro-intestinal tract of rainbow trout fed to satiation with a 'standard expanded' diet ( $n=5 \pm S.E$ ). Unlike superscripts denote a significant statistical difference (ANOVA, SNK,  $P < 0.05$ ).

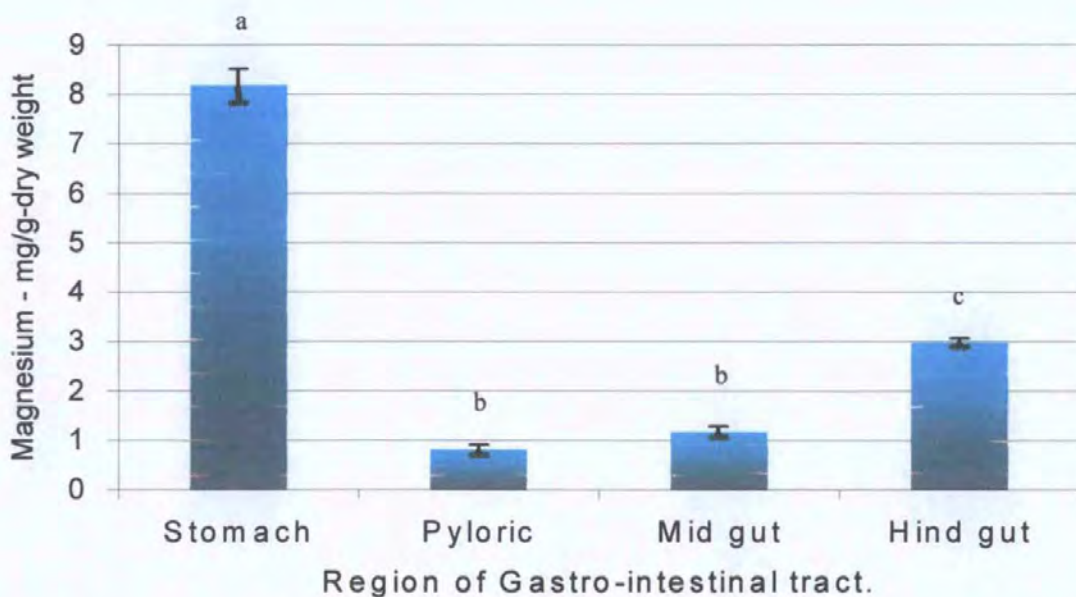


Figure 5.2.c.

Concentrations of magnesium in the digesta distributed throughout the four regions of the gastro-intestinal tract of rainbow trout fed to satiation with a 'standard expanded' diet ( $n=5 \pm SE$ ). Unlike superscripts denote a significant statistical difference (ANOVA, SNK,  $P < 0.05$ ).

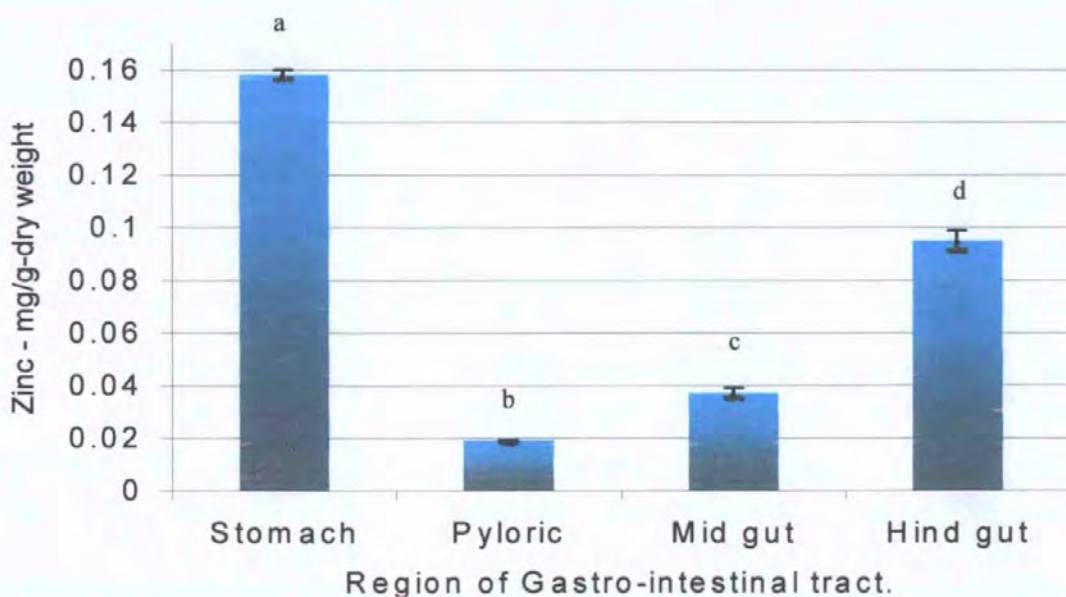


Figure 5.2.d.

Concentrations of zinc in the digesta distributed throughout the four regions of the gastro-intestinal tract of rainbow trout fed to satiation with a 'standard expanded' diet ( $n=5 \pm SE$ ). Unlike superscripts denote a significant statistical difference (ANOVA, SNK,  $P < 0.05$ ).

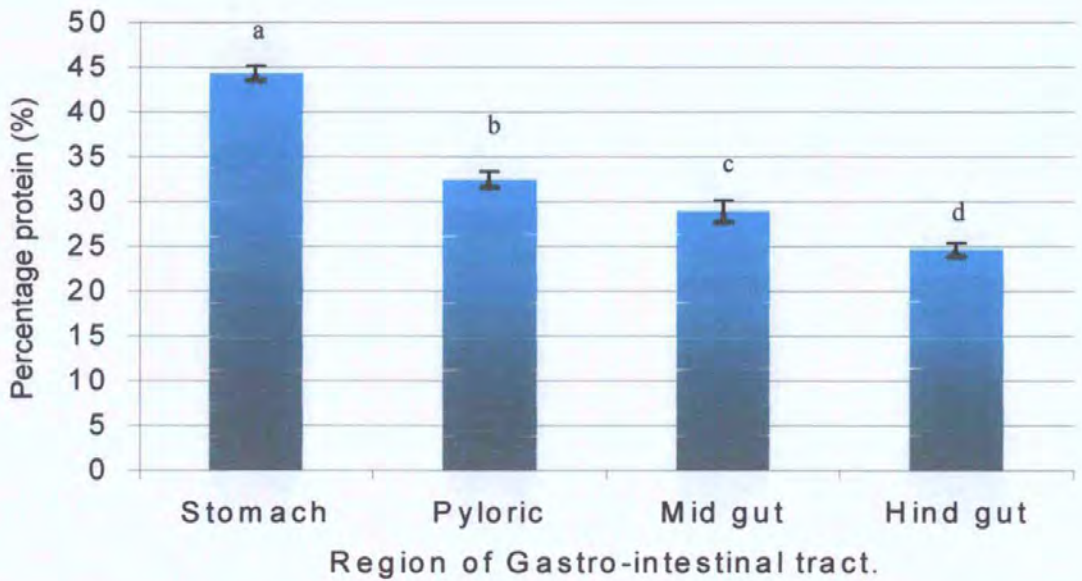


Figure 5.2.e.

Percentage values of protein in the digesta distributed throughout the four regions of the gastro-intestinal tract of rainbow trout fed to satiation with a 'standard expanded' diet ( $n=5 \pm SE$ ). Unlike superscripts denote a significant statistical difference (ANOVA, SNK,  $P < 0.05$ ).

Comparatively, percentage protein values do not generally follow the same pattern as the mineral data. However, the largest decline in protein concentration is located between the stomach and pyloric region which is similar to results produced for the minerals, subsequently values steadily decline to a value approximately half that of the initial percentage.

Mineral and protein concentrations in the digesta are significantly different between each of the four regions of the gastro-intestinal tract (ANOVA, SNK,  $P < 0.05$ ). The only exception, being the concentration of magnesium between the pyloric and mid regions of the gastrointestinal tract.

## 5.4 Discussion

The investigation outlines a preliminary approach to obtain semi quantitative data with respect to the differential absorption profiles of dietary protein and selected minerals relevant to the general programme of research. It should be noted that a dietary marker was not included in the experimental design due to the commercial nature of the test diet. Incorporation of an inert marker such as chromic oxide ( $\text{Cr}_2\text{O}_3$ ), yttrium or ytterbium oxide would have allowed detailed digestibility coefficient assessment of digesta for each segment of the gastrointestinal tract. The relative marker/nutrient (i.e: protein and mineral) ratio would have thus provided the exact degree of absorption (or apparent digestibility coefficients) for the nutrients assessed. However, the use of markers is controversial and there is doubt in the scientific literature whether any specific marker stated provides accurate quantitative data (Bowen, 1978; Tacon and Rodrigues, 1984). In the present experiment, it would have been possible to add a marker to the feed either by external application or by pre- grinding the diet and reconstituting the diet with a specified marker at a concentration similar to those described previously. However, the diet used was a commercial source and typical of those presently available for trout. It should also be noted that the feeding regime employed maintained a continuous feed intake consistent with the optimum daily ration appropriate to rainbow trout in the early phases of production. This is somewhat different from the discrete single meal intakes often reported in experiments with fish to provide information on gastric evacuation rate, digestion and nutrient absorption. Continuous feeding is more relevant to typical production (on farm) conditions; also this type of feeding may be reflective of the natural feeding habits of rainbow trout in the wild. Hence, the more rapid transit of digesta associated with frequent, daily meal intakes may provide a more realistic basis for the comparison of mineral composition in different regions of the gastrointestinal tract. This is especially true of the digesta associated with the large intestine segment relative to the faecal composition prior to elimination from the body.



Although semi quantitative, the relative mineral composition indicates interesting sequential events occurring during the course of the translocation of the digesta. There was a consistent marked reduction in the absolute macro element levels for calcium, phosphorus, magnesium and zinc from the stomach compartment to the hind gut with a particular reduction evident for the pyloric 'section' where maximum absorption of each mineral seemed to occur. A consistent trend for each case was the tendency for a rise in concentration for the mid gut and a larger increase in the hind gut. This effect was probably a combination of some re-absorption of minerals due to the flux of water and electrolytes across the gut lumen in association with the vascularisation of the distal region of the gut and also further nutrient absorption of protein, lipid and carbohydrate components of the feed. This may have resulted in a "concentration effect." The true digestibility or absorption of each element would have been masked by the continuous movement of digesta and the interpretation of this data is made difficult because it is a qualitative assessment of a 'snapshot' event rather than based on the final composition of faeces resulting from the meal. It can be argued that more direct measurements could have been obtained from either total collection of faeces by the use of specialised metabolism chambers or removal from the fish by other means such as the stripping of faeces as used in previous experiments. However, the study has clearly demonstrated the internal complexities associated with mineral absorption and the considerable differences between the regions of the gut.

Presently there is a limited amount of published data relating to mineral absorption across the different sections of the gastrointestinal tract in fish. However, whilst assessing the digestion and digestibility in gilthead sea bream (*Sparus aurata*), Fernandez *et al.*, (1998) calculated the ADC values for various components including phosphorus from several regions of the intestine using chromic oxide as the marker. It was established that although significant differences were noted between the stomach and faecal samples, generally no

significant increase in values was noted along the intestine. Fernandez *et al.*, (1998) concluded that the majority of phosphorus absorption takes place in the stomach or anterior region (which was described as being the first 3cm after the stomach and pyloric caeca), even though ADC values were not calculated during this study these results are directly comparable. Further results by Fernandez (1998) found decreases in the phosphorus absorption in the medial and posterior region of the intestine of gilthead sea bream for most of the experimental diets, this finding also agrees with those found in the current study. In summary it was suggested that as the majority of phosphorus absorption took place in the stomach and pyloric regions of the intestine as opposed to the medial and posterior regions. It is feasible that the pH conditions in the stomach and pyloric caeca maybe more suitable for the solubility and assimilation of phosphates than other regions of the gut.

Lied *et al.*, (1992) investigated protein digestibility in Atlantic cod (*Gadus morhua*) using the 'external' dietary markers chromium (III) oxide and titanium (IV) oxide, in addition to 'internal' mineral markers that were already integral components of the diets. Preliminary analyses of gastrointestinal contents from cod showed that minerals such as calcium, iron and zinc were concentrated along the alimentary canal, suggesting that these elements are ingested much in excess of what can be absorbed, similar findings to those from the present study. As a consequence of these findings, Lied (1992) evaluated these mineral macro-elements from the feed as potential internal markers. Identical estimates of protein digestibility were obtained when calcium and zinc were used as 'internal' markers, when compared to results produced for use of titanium (IV) oxide as an inert 'external' marker to measure digestibility.

The stomach profile is obviously closer to that of the feed employed for the investigation and the profile of the digesta content of the hind gut would indicate that an appreciable

amount of calcium and phosphorus as well as magnesium and zinc remains unabsorbed. This has obvious relevance to the question of environmental impact of phosphorus losses to the surrounding water body and intensive rearing of salmonids at high density on feeds containing phosphorus from different sources, and suggests that minerals are ingested in excess of what can be absorbed. The implications for other trace elements such as zinc are also important. Zinc in addition to the other minerals in this study is an essential element for fish and factors that may alter the bioavailability include, meal size, feeding frequency, fish size, temperature, and diet composition (Lall, 1989). It is known that several minerals may compete for absorption and various antagonistic effects can occur. However when the uptake pathways of mineral active transport systems become saturated at high luminal concentrations, many minerals are able to diffuse passively across the intestinal basolateral membrane. Therefore two components are usually involved in the uptake of minerals across the intestine, and this has been demonstrated for zinc in the winter flounder (*Pseudopleuronectes americanus*) by Shears and Fletcher, (1983) and the rainbow trout Glover and Hogstrand, (2002). Zinc in particular is known to have significant absorptive interactions with other dietary cations forming tightly bound chelates, often resulting in a lower digestibility (Hardy and Shearer, 1985; Knox *et al.*, 1984; Wekell *et al.*, 1986). The type of mineral chelate produced is particularly dependent on the form in which the zinc is fed, being either from an organic or inorganic source.

Similarly, there was an obvious absorption of protein across the regions of the intestine, but this experiment was not able to provide absolute digestibility coefficients for the protein in the feed due to the absence of a suitable marker. However, the concentration of protein in digesta sampled in the hind gut was half that of the stomach. This would indicate an appreciable level in the resulting faeces. Interestingly there was no evidence of any increase at any location subsequent to the stomach. It can be inferred that protein digestion and subsequent absorption is a uniform process, post stomach to pyloric region

where the largest decrease in protein content was noted. This confirms results found by Lied *et al.*, (1992) for Atlantic cod who stated that protein digestion and absorption took place mainly in the pyloric caeca and first 3cm of the intestine, with smaller increases being observed in the posterior and rectal regions of the gut. This pattern of a progressive increase in protein digestibility for samples taken simultaneously along the gut was also found by Fernandez *et al.*, (1998) for gilthead seabream (*S. aurata*) and rainbow trout by Austreng, (1978). It should be noted that the digesta of the hind gut is likely to contain endogenous protein losses such as the sloughing of epithelial cells, enzyme and mucosal secretions, blood components, and possibly milt depending on the age of the fish. Hence, the protein digestibility of the diet may be higher than estimated. A more accurate estimation of undigested protein could therefore be established by utilising a diet comprised of a pure protein source such as casein or albumin, providing a specified protein content of uniform composition at a low protein intake.

Clearly the digestion and absorption mechanism in fish is a complex process that is affected by a number of factors. The differentiation of the gastrointestinal tract is an important feature as well as the composition of the diet. Comparative studies should attempt to compare the assimilation of discrete meal intakes with that of a continuous feeding regime and complemented with standard digestibility trials. More detailed studies would also be able to discriminate between the endogenous losses of macro and trace elements within the gastrointestinal tract and allow for a more accurate measurement of the true availability of the nutritionally important elements from the diet. In this respect the following section aims to address this subject by assessing the protein and mineral digestibilities of various alternative feed ingredients in addition to several dietary mineral sources in feeds for the rainbow trout.

## CHAPTER 5

### **PART B. PROTEIN AND MINERAL DIGESTIBILITY OF SUITABLE ALTERNATIVE FEEDSTUFFS AND VARYING DIETARY MINERAL SOURCES AND ADDITIVES FOR RAINBOW TROUT**

#### **5.5 Introduction**

Feed formulation necessitates the development of balanced diets that meet the full nutritional requirements of fish. This must be achieved with a complement of selected feed ingredients, supplements and additives at an economic cost.

Feed ingredients are chosen on the basis of their digestible energy and protein content and quality of essential amino acids. Although these ingredients contribute an appreciable level of macro and trace elements to the diet, the feed is always supplemented with an appropriate trace element mix to provide the requirements met by the NRC (1993) recommendation for fish, including salmonids such as trout and salmon.

Therefore, it is imperative that the digestibility of nitrogen and the major elements in various feeds are determined as well as the availability of these minerals in organic supplements. This sub-chapter addresses experimental studies to ascertain the digestibility and mineral availability of important feed ingredient sources destined for rainbow trout diets.

##### ***5.5.1 The use of alternative protein sources from plants***

Fish nutritionists are increasingly turning to alternative protein sources that are more cost effective and sustainable in terms of supply to meet the needs of various aquafeed formulations (Rumsey, 1993; Hardy and Kissil, 1997). In this respect, considerable interest has been placed upon the use of plant proteins as an alternative to fishmeal with predominant attention being on soybean products. Soybean protein sources are available

in several forms mainly full fat and dehulled, solvent extracted and also as protein concentrates or isolates. Soy protein concentrate (SPC) has been used successfully as a partial replacement for fishmeal in rainbow trout (*Oncorhynchus mykiss*) by Kaushik *et al.*, (1995), Medale *et al.*, (1998), and Stickney *et al.*, (1996), juvenile red drum (*Sciaenops ocellatus*), Davis *et al.*, (1995) and gilthead seabream (*Sparus aurata*), Kissil *et al.*, (2000). On the other hand a full replacement of fish meal by SPC has produced contradictory results with Medale *et al.*, (1998) finding reduced growth, but Kaushik *et al.*, (1995) reported no negative effects when fed to rainbow trout. Feeds containing high levels of SPC often have reduced palatability which would contribute to a low feed intake leading to reduced growth according to Stickney *et al.*, (1996). Hajen *et al.*, (1993b) demonstrated that accurate digestibility figures for commercial sources of soybean meal and soybean protein isolate could not be calculated due to the poor appetite of chinook salmon (*Oncorhynchus tshawytscha*) fed diets containing these protein sources at a 30% inclusion level. Although apparent digestibility coefficients of 77.0% and 86.3% respectively for soybean and soybean protein isolates were quoted for chinook salmon (*O. tshawytscha*).

In comparison, rainbow trout (*Oncorhynchus mykiss*) appear to adapt to diets containing high levels of soybean and there have been many reports on the successful use of soybean in diets for rainbow trout by Dabrowski *et al.*, (1989), Smith *et al.*, (1988) and Rumsey *et al.*, (1993). Although in most of these investigations soybean was included at levels lower than 40%, in addition to other protein sources in the feed. When 40% or more of the dietary protein component is replaced with non-solvent extracted soybean meal, a suppression of growth has been observed in rainbow trout by Pongmaneerat and Watanabe, (1992) and Olli and Krogdahl, (1994). The resulting reduced growth is related to the presence of anti nutritional factors (ANF's) in the soy meal. In addition, the production method of soybean meals may also play an important role in their acceptance to fish, since soybean meals that are not extracted by alcohol often exhibit a bitter flavour (Rackis *et al.*,

1970). Therefore low palatability due to a bitter or lack of flavour may explain reduced growth due to feed intake restriction. Refstie *et al.*, (1997), demonstrated that rainbow trout were able to adapt to diets containing up to 60% soybean meal after a period of 'acclimation' time. Fish then continued to grow equally as well as trout fed a fish meal control diet, indicating that this species of fish is highly adaptable to changes in dietary composition.

Although various soybean products have been the primary alternative plant protein, many other products have been investigated including rapeseed meal, cottonseed meal (Tacon, 1994), linseed and sesame seed meal (Hossain and Jauncey, 1989a). Burel *et al.*, (2000) conducted an investigation feeding extruded peas and lupin to both rainbow trout and turbot (*Psetta maxima*) with lupin being the more promising fishmeal substitute producing protein ADC's of 96% and 98% with respect to each fish species evaluated. It appears that plant sources can be utilised as an alternative protein source to fishmeal, and support adequate growth and feed utilisation, providing that inclusion levels are limited to below 40-50% of the diet. This figure depends greatly on fish species, type and quality of alternative protein source, protein processing technique and other dietary components.

As the majority of phosphorus in plant feedstuffs is in the form of phytic acid, and is unavailable to fish (Riche and Brown, 1996), it is imperative to try and ensure that the availability of phosphorus is kept constant across sources and concentrations when formulated into various test diets. Otherwise the dietary phosphorus sources may shift from available to unavailable forms within the matrix of the diet.

### **5.5.2 The use of alternative protein sources from animal origin**

The use of animal by-product meals as a replacement for fishmeal in aquaculture diets has been studied in recent years and in general these investigations have proved to be positive

with respect to fishmeal substitution. Typically, feather meal and meat and bone meal have been the most commonly used types of rendered animal protein feedstuffs used in fish nutrition. Recently, the use of these ingredients has been limited or avoided for many reasons, usually linked to poor product quality and low digestibility. Additionally, in recent years European legislation has banned the use of meat meals and by products for the purpose of animal feed, due to inter species disease risks.

However the increased use of these products in other continents is becoming a reality despite the problems encountered in Europe. As a result of improved manufacturing techniques feather meal and meat and bone meals are highly digestible source of protein for fish (Bureau *et al.*, 1999). The latter author determined the ADC of protein for 20 rendered animal protein ingredients for salmonids, feather meals varied between 81–87%, poultry by-product meals were between 87-91%, meat and bone meals varied between 83-89%, and the ADC for the blood meals varied between 82-99%. These results indicated a significant improvement in the digestibility of animal protein ingredients, probably as a result of improved manufacturing practices and processing conditions (Bureau *et al.*, 1999). A further trial by Bureau *et al.*, (2000), demonstrated that up to 15% feather meal and up to 24% meat and bone meal can be incorporated into diets fed to rainbow trout without having any significant affect on growth, and with only a slight reduction in feed efficiency.

Further evidence to demonstrate the potential of meat products in aquafeeds was provided by Stone *et al.*, (2000), who fed silver perch (*Bidyanus bidyanus*) diets containing up to 30% meat meal which proved to be digested well by this fish species. However compared with fishmeal, the meat products contained lower levels of many amino acids, particularly lysine (Stone *et al.*, 2000), and the excess levels of bone (high ash content) in the meal could have acted as an anti-nutritional factor resulting in poor digestibility. A particularly



comprehensive research study on the digestibility of 29 alternative ingredients for Australian perch (*Bidyanus bidyanus*) was conducted by Allan *et al.*, (2000). The results showed that although fishmeal produced very high ADC's for both protein and energy, other ingredients including some animal meals were very similar to the performance of fishmeal. Some of the ingredients with high protein content i.e. blood meal and feather meal produced higher protein ADC's, but the disadvantage with these highly digestible ingredients is their inferior amino acid profile in comparison to fishmeal. Alternatively, meat and bone meal produced a relatively low protein digestibility of 71.5%, probably due to the high ash fraction of this diet.

### **5.5.3 Mineral supplementation**

Ingredients with low phosphorus availability are avoided in aquatic feeds and diets are often supplemented by chemical, physical or enzymatic treatments in order to increase phosphorus availability (Sugiura *et al.*, 1998a).

Improved availability and reduction of dietary phosphorus is probably the most economical approach to lower P pollution, although care must be taken to provide adequate P to the fish to support growth and maintain physiological processes (Asgard and Shearer, 1997). Phosphorus requirements for rainbow trout range from 2.4 to 5.9g/kg (Rodehutscord, 1996). Fishmeal is the main source of minerals in aquafeeds, but fishmeals also vary greatly in source and quality, and as a consequence digestibility coefficients for phosphorus differ considerably between raw materials. Additionally, apparent mineral availabilities obtained as a result of feeding fish meals are not particularly high, for example Nordrum *et al.*, (1997) estimated a 51% availability for phosphorus from fish bone meal fed to Atlantic salmon, (*S. salar*), and Lovell, (1978) estimated a 40% phosphorus availability when channel catfish (*I. punctatus*) were fed fish meal diets. The levels of phosphorus contained in plants being considered as alternative protein sources,

are notably lower than many fish meals and hence would make them ideal to incorporate into aquafeeds, however plant products particularly soybean vary greatly in their anti-nutritional factors (ANF's). Probably the most important ANF relating to phosphorus availability in soybean, is phytate; a cyclic inositol compound bound with six phosphate groups. Phytate is unavailable to fish as they do not possess the enzyme phytase needed to cleave the compound, therefore diets containing high levels of soy bean may have insufficient phosphorus availability to satisfy the phosphorus requirements of the fish. Therefore it is necessary to supplement diets with inorganic salts, especially as the phosphorus availability from these inorganic salts is much higher than from fishmeal and plant protein sources alone (Lall, 1991).

Ogino *et al.*, (1979) investigated the availability of several inorganic phosphates to common carp and trout, and established that primary calcium phosphate, primary sodium and potassium phosphate was utilised effectively with apparent digestibilities of 94, 98 and 98% respectively. Secondary and tertiary calcium phosphates were found to be low in availability with digestibility coefficients of 71 and 64% respectively, these values confirmed earlier estimates by Lovell, (1978) for channel catfish. Although this result was not confirmed by Nordrum *et al.*, (1997) who found that retention of P sources varied from 86% for primary calcium phosphate, to 91% for secondary calcium phosphate and 131% for primary sodium phosphate (this high value being attributed to improved availability of P in the basal diet). Further trials involving channel catfish have also been conducted to assess the efficacy of inorganic salts. It was concluded that dicalcium phosphate and de-fluorinated phosphates were equally efficacious for use as phosphorus sources for channel catfish by Li *et al.*, (1996). The method for establishing mineral bioavailability from these inorganic sources was determined by an analytical process, the solubility of these inorganic salts was measured in neutral ammonium citrate, DCP had a NAC solubility of 90.7% and DFP 85.4%. Robinson *et al.*, (1996) has suggested that de-fluorinated phosphate maybe

more desirable than dicalcium phosphate due to its lower solubility in water (Robinson *et al.*, 1996).

The particular variety of inorganic phosphorus salt is also thought to affect the availability of other minerals, specifically zinc. Satoh *et al.*, (1993) fed rainbow trout diets containing various types of phosphate for 27 weeks and reported that growth was found to be reduced by various kinds of phosphates and bone meal, with the exception of  $\text{CaHPO}_4$  (monocalcium phosphate). The supplementation of diets with  $\text{Ca}(\text{H}_2\text{PO}_4)$  (dicalcium phosphate) induced short body dwarfism, whilst  $\text{Ca}_3(\text{PO}_4)_2$  (tricalcium phosphate) and bone meal caused eye lens cataracts, and lowered vertebrae zinc content. The di- and tri-basic forms of calcium phosphate were also the supplements fed to fish that exhibited the lowest Zn absorption, indicating that tricalcium phosphate may inhibit Zn availability (Satoh *et al.*, 1993). Therefore it seems necessary to assess inorganic supplements when formulating fish feeds, to optimise feed utilisation and minimise levels of mineral excretion.

#### **5.5.4 Supplementation with non-mineral feed additives**

Dietary supplements can be used to increase the availability of phosphorus in feed ingredients, with the overall aim of reducing the phosphorus output in discharge waters. In recent years phytase has been investigated as a potential additive to feeds, particularly those containing plant proteins. Phytase is the enzyme responsible for hydrolysing phytate into a phosphorus form that is available to fish (Cain and Garling, 1995; Jackson *et al.*, 1996; Rodehutsord and Pfeffer, 1995a; Schafer *et al.*, 1995; Vielma *et al.*, 2000). There is limited research conducted directly utilising fish species with studies that include the supplementation of the exogenous enzyme phytase to the diet. The majority of primary data concerning the use of phytase in animal feeds has resulted from the use of poultry (Mohammed *et al.*, 1991; Qian *et al.*, 1997).

Presently, fishmeal is the primary ingredient in salmonid feeds consequently phosphorus from this source contributes the major proportion of phosphorus in the diet. However, phosphorus in fishmeal is not always efficiently utilised in fish. Sugiura *et al.*, (1998a) stated that during digestibility trials the apparent availability of phosphorus from fishmeal ranged from 11.8-50.2% (unpublished data). Therefore, it would seem important to improve the phosphorus availability from fishmeal in order to aid in reducing the phosphorus levels in fish farm discharges.

Sugiura *et al.*, (1998a) conducted an investigation to determine if a range of feed supplements with the potential to improve dietary mineral availability from fishmeal, had any measurable effect. Of the 11 supplements tested, citric acid yielded the only significant result by increasing mineral availability, a further study established that diets supplemented with 5% citric acid greatly influenced the availability of calcium and phosphorus. More recently Vielma *et al.*, (1999) supplemented diets fed to rainbow trout with citric acid and found that the utilisation of phosphorus was improved, although the increase in body P content was not significant.

A further feed additive that has been found to affect mineral metabolism is vitamin D<sub>3</sub> or cholecalciferol. Research into the effect of this vitamin is limited in fish nutrition, although it has been suggested that vitamin D<sub>3</sub> may have a similar role in fish as in warm blooded animals. In poultry, vitamin D<sub>3</sub> was found by Wasserman and Taylor, (1973) to positively affect the transport of phosphorus across the intestine, and in general the role of vitamin D<sub>3</sub> is to elevate the plasma calcium and phosphorus to a level that will support normal bone mineralisation (Deluca, 1979).

The amino acid phenylalanine may be linked to the transport of phosphorus uptake in the gastro-intestinal tract, by its effects on the enzyme alkaline phosphatase. Taylor, (1974), carried out physiological trials involving the everted gut sacs from the ileum of rachitic chicks, to monitor phosphate transport. It was initially established that vitamin D had a positive effect on phosphate transport in the intestine, which was independent of calcium, and the addition of L-phenylalanine to the incubation medium increased mucosal uptake and storage of phosphate by an unknown mechanism, and may act a calcium chelator. Although these effects were noted in poultry, comparative trials using fish have not been conducted.

#### ***5.5.5 Methods of digestibility determination***

The determination of digestibility coefficients can be calculated as a result of various methods for the whole diet or one particular component of the diet. Over recent years, technological advances especially in industrial processing have produced feedstuffs of increased quality, and although a range of methods have been developed for the deduction of digestibility figures, these fundamental procedures of making an accurate measurement for fish diets can prove to be very difficult. The principal problem occurs as a result of the aquatic environment in which the fish are contained hence, the measurement of feed intake and output of faeces is technically difficult. The main reason being due to the fact that nutrients especially minerals, can leach from the diets and voided faeces once in contact with the water. Such leaching of nutrients can lead to a 'false' increase in the ADC obtained as stated by Austreng, (1978), and De la Noue and Choubert, (1986).

In general, digestibility measurements can be made via a direct method involving the quantitative collection of faeces that correspond to a number of meals (Ogino *et al.*, 1973; Post *et al.*, 1965). Alternatively an indirect method has been developed to obviate the problems occurred during quantitative collection, by introducing an inert marker into the

feed which is used as an internal tracer through the fish. The marker is then analysed in both the feed and the faeces and then digestibility for a given nutrient can then be calculated. Both methods are commonly used, but both encounter the same problem in obtaining representative fish faeces (De la Noue and Choubert, 1986).

Many techniques have been employed in research to collect faeces. These have included:- stripping by applying abdominal pressure (Hajen *et al.*, 1993a; Nose, 1967; Windell *et al.*, 1978), anal suction (Windell *et al.*, 1978), intestinal dissection (Austreng, 1978) (Hajen *et al.*, 1993a; Windell *et al.*, 1978), metabolic chambers (Post *et al.*, 1965), faecal settling (Cho and Slinger, 1979), filtration of tank water (Choubert *et al.*, 1979). One of the most commonly utilised methods for direct faecal collection from rainbow trout is by stripping, this is when abdominal pressure is applied to the hind gut region of the fish, forcing the expulsion of faeces and was established by Austreng, in 1978. Not unlike many other techniques and procedures the stripping method for faecal collection has various advantages and disadvantages. An advantage to this method is that the same fish can be used repeatedly and due to the direct collection of the faeces no contact is made with the water, hence nutrient leaching cannot occur. However, handling stress and sudden defecation during anaesthetisation can lead to underestimation of ADC values as suggested by Hajen *et al.*, (1993a), and Spyridakis *et al.*, (1989). Additionally it is difficult to separate faecal and urinary waste hence contamination of the sample can occur. The principal objection to any method of direct intestinal faeces collection is that faecal matter maybe removed prior to the natural course of digestion and hence reducing the retention time, absorption and simulating a nutrient with poor digestibility (Vens-Cappell, 1985).

The Guelph system is a mechanical method that fundamentally collects faeces naturally excreted from the fish. Typically, fish are contained in tanks with sloping bottoms which lead to a central drainage channel, effluent water is then directed through a column where

faecal matter settles and the excess water becomes waste (Cho and Slinger, 1979). A comparative study involving the Guelph system, stripping and intestinal dissection was conducted by (Hajen *et al.*, 1993a). Indications showed that stripping and dissection yielded lower ADC's than faeces collected by the Guelph system, but nutrient leaching from faeces in the Guelph system resulted in ADC values which were erroneously high, since nutrient losses are treated as if absorbed by the fish. As a consequence of these problematic methods it was decided to collect faecal matter by the stripping method during this series of investigations. This was due to the reasoning that the primary concern of this research is in mineral nutrition and most inaccuracies involving minerals are in connection with techniques which allow faecal/water contact.

The indirect method of digestibility measurements involves the incorporation of an inert marker into the fish feed to follow the progress of digestion, (Cho *et al.*, 1982).

Digestibility coefficients can then be calculated by assessing the relative change in marker concentration between the faeces and the diet, in comparison to the concentration changes between diet and faeces for the nutrient being monitored (Maynard and Loosli, 1969). At present, chromic oxide ( $\text{Cr}_2\text{O}_3$ ) is the preferred external dietary marker for estimating digestibility in both terrestrial animals and fish (Austreng, 1978; McDonald *et al.*, 1977). However research by Bowen, (1978) has indicated that chromic oxide passes along the gastro-intestinal tract at a different rate compared to the digesta. This usually results in a poor quantitative recovery of the marker relative to the digesta or faeces. Additionally chromic oxide is believed to possess carcinogenic properties, these problems have caused questions to arise as to the suitability of this marker for fish trials. In recent years the use of yttrium oxide as an inert marker to measure digestibility has been introduced and it is this marker that will be used during this series of experiments to estimate digestibility. Other 'non' chemical markers have been used to assess digestibility, including acid washed

sand and polyethylene, although results were found to be erratic and un-reproducible (Tacon and Rodrigues, 1984).

In certain practical situations it is not always possible to add 'external' markers into the feeds, so interest turned to natural or 'internal' markers. For example, acid insoluble ash (De Silva and Perera, 1983), cellulose (Buddington, 1979) and crude fibre (De Silva and Perera, 1983; Tacon *et al.*, 1983b), although these methods are generally unaccepted.

It was felt that after a review of the relevant literature, several of the key themes could be developed to determine the digestibility profile of phosphorus and nitrogen in selected feed ingredients and additives used in practical fish feeds. The aims of this study were to:-

- 1 Determine the apparent digestibility coefficients (ADC's) of protein and minerals, for a range of commercially available alternative protein sources.
- 2 To calculate the mineral digestibility for standard basal diets that had been formulated to contain a range of mineral supplements.
- 3 Thirdly a range of additives, specifically vitamin D, citric acid and phenylalanine were incorporated into the basal diet aimed at increasing mineral availability by influencing gastrointestinal absorption.

All faeces were collected by the stripping method, using yttrium oxide as the inert marker.

## **5.6 Methods**

### **5.6.1 Experimental facilities**

During this digestibility trial larger fish were utilised, to ensure a sufficient faecal sample size was obtained and to minimise handling stress during faecal collection. Therefore, 15 rainbow trout (*Oncorhynchus mykiss*) with an average weight of 246g were stocked into each of eighteen square 160L fibreglass tanks. As these fish were only being used for



faecal collection and being fed variations on standard salmonid feeds, the same fish were utilised for all five sections of this trial. Consequently, the initial weight of the fish increased during each section of the trial with average fish weights being 250g, 268g, 287g and 305g respectively for the following four dietary sets being evaluated.

The fibreglass tanks were suspended over three linked tanks containing mechanical filtration, biological filtration and cooling compartment. Together they comprised a closed re-circulation system with additional mechanical filtration being provided by a Hi-Rate sand filter. Prior to grading, during an acclimation period fish were treated with an anti-parasite agent. Freshwater flowed into each tank at a rate of 13L/minute, and water temperature was maintained at  $15\pm 1^{\circ}\text{C}$  by the use of a submerged coolant system. Various water parameters were monitored including phosphorus, nitrite, nitrate and ammonia/ammonium and these were all held within ranges tolerated by this fish species. The pH levels were maintained within the range of 6.5-7.5 by the use of calcium carbonate and 'magnospheres' to aid in the buffering capacity of the water. Photoperiod was maintained at a 12hr light: 12hr dark regime, by timed lighting.

### ***5.6.2 Diet formulation and preparation***

The various alternative protein sources were to be assessed by the substitution method, which involves making a standard reference diet and then substituting a proportion of it with the test ingredient, in this case the ratio was 57% reference diet and 38% test ingredient. All experimental diets used for this series of investigations were made 'in-house.' All dry ingredients were weighed and mixed together in the bowl of a Hobart food processor, when the mixture was uniformly blended the oil was added. De-ionised water was then slowly added during mixing until the mash was moist enough for extrusion. The diet mixture was then extruded to a size of 2-3mm, the moist strands were spread over foil

trays and dried in a fan assisted drying cabinet. Once dry the strands were broken into uniform size pellets and stored in airtight containers.

Table 5.3.a

Dietary formulations for reference and test diets, fed to rainbow trout.

Dietary component	Reference diet (%)	Test Diet (%)
Test ingredient*	-	38
Fishmeal LT94 (Icelandic)	60	36
Cod liver oil	10	6
Cornstarch/Dextrin (2:1)	25	15
Vitamin/mineral premix**	4	4
$\alpha$ - cellulose	0.75	0.75
Yttrium oxide marker	0.25	0.25

\* Alternative protein sources utilised as the test ingredient are as follows;

Feather meal - Prosper de Mülder Ltd, UK

Poultry by product meal - Prosper de Mülder Ltd, UK

Blood meal – American Protein Corporation, Des Moines, Iowa, USA

Maize gluten meal – TROUW Aquaculture Ltd, UK

Norsea fishmeal, white fish offal meal – TROUW Aquaculture Ltd, UK

Special 'G' fishmeal, low ash fish hydrolysate – Sopropêche, Boulogne-sur-mer, France

Sunflower meal – TROUW Aquaculture Ltd, UK

Icelandic fishmeal (LT94), low temperature fishmeal – TROUW Aquaculture Ltd, UK

Krill meal – TROUW Aquaculture Ltd, UK

Soya protein concentrate – Central Soya, DK, European Protein

Hi pro soyabean meal (solvent extracted) – TROUW Aquaculture Ltd, UK

Full fat soyabean meal – TROUW Aquaculture Ltd, UK

\*\* Vitamin/ mineral premixes supplied in accordance with NRC salmonid requirements

The test diet formulations are presented in tables 5.3 (a, b, c and d) with the protein and mineral composition profile being summarised in tables 5.4 (a, b and c). Icelandic fishmeal was the primary protein source in the reference diet, this diet was prepared fresh each time a new set of experimental diets was made, hence there were several reference diets (each one was made at a different time period).

Table 5.4.a  
Protein and mineral composition for the individual alternative protein sources.

Feed Ingredient	Ca (%)	Mg (%)	P (%)	Zn (%)	Protein (%)	Ash (%)
Icelandic fishmeal	4.32	0.24	2.39	0.069	73.38	12
Feather meal	0.95	0.04	0.30	0.089	83.42	1.6
Poultry by product meal	5.29	0.13	2.42	0.083	67.45	12.1
Blood meal	0	0.03	0.36	0.004	95.99	0.8
Maize gluten meal	3.63	0.07	0.56	0.006	62.50	5
Norsea fishmeal	6.52	0.22	3.21	0.068	76.26	18
Special 'G' fishmeal	0.45	0.06	0.71	0.011	74.20	5
Sunflower meal	0.69	0.71	1.29	0.107	38.30	5
Krill meal	3.85	0.46	1.55	0.037	61.08	13
Soya protein concentrate	0.43	0.34	0.69	0.070	66.20	6.5
Hi-pro soyabean meal	0.41	0.31	0.68	0.044	50.61	7
Full fat soyabean meal	0.36	0.23	0.69	0.039	37.49	6.5

Table 5.4.b.  
Protein and mineral composition of experimental diets containing a range of alternative protein sources.

Diet containing feed ingredient	Calcium (%)	Magnesium (%)	Phosphorus (%)	Zinc (%)	Yttrium oxide (%)	Protein (%)
Reference diet	1.954	0.139	1.243	0.104	0.658	46.70
Feather meal	1.423	0.092	0.760	0.098	0.620	60.70
Poultry meal	3.187	0.145	1.738	0.098	0.609	52.66
Blood meal	1.356	0.092	0.847	0.095	0.616	57.11
Poultry + Blood meal	2.343	0.122	1.334	0.094	0.635	66.30
Feather + Blood meal	1.537	0.099	0.853	0.099	0.675	59.41
Reference diet	2.124	0.138	1.453	0.094	0.112	49.55
Maize gluten	1.286	0.093	0.900	0.093	0.133	46.74
Norsea fish meal	3.051	0.144	1.739	0.091	0.112	46.88
Special G fishmeal	1.452	0.310	1.210	0.094	0.110	49.64
Sunflower fishmeal	1.416	0.085	0.979	0.097	0.109	38.34
Reference diet	2.695	0.131	1.416	0.115	0.098	49.50
Krill meal	2.506	0.252	1.199	0.084	0.104	45.75
Soya protein concentrate	2.097	0.182	0.967	0.115	0.112	47.22
Hi pro soya	2.077	0.180	1.008	0.101	0.115	41.89
Full fat soya	1.923	0.148	0.959	0.090	0.119	37.91

Diets formulated to contain mineral supplements and feed additives utilised maize gluten as a base protein as presented in table 5.3.b. Throughout the range of mineral supplements used, the highest Ca to P ratio was 1.3 : 1, this ratio was then held throughout all diets by supplementing with calcium chloride (table 5.3.c). Alpha-cellulose was used as a filler, which is known not to affect digestibility in such low inclusion levels (Davies, *pers comm*). All diets were made 'in-house' to methods outlined in chapter 2.

Table 5.3.b  
Basal dietary formulation for experimental diets fed to rainbow trout containing various mineral supplements.

Dietary component	Diet composition (%)
Maize gluten	53
Gelatinised wheat starch	9.6
Fish oil	10
Corn oil	5
Vitamin premix <sup>1</sup>	2
Mineral premix <sup>2</sup>	2.1
Amino acid premix <sup>3</sup>	8.5
Alpha cellulose / Mineral supplement <sup>4</sup>	9.9
Yttrium oxide marker	0.1

1 Vitamin premix as supplied by TROUW Aquaculture in accordance with NRC guidelines.

2 Mineral premix – KCl (1.5%), MgO (0.3%), NaCl (0.3%), MnSO<sub>4</sub>, ZnSO<sub>4</sub>, FeSO<sub>4</sub> (trace).

3 Amino acid premix – L-lysine (3%), Methionine (0.6%), Threonine (0.8%), Arginine (1%), Histidine (0.4%), Phenylalanine (1.5%), Leucine (1%).

4 Alpha cellulose to supplement ratio – see table below.

Table 5.3.c

Formulation of the mineral supplement component added to experimental diets, fed to rainbow trout containing each source.

Mineral Supplement	Supplement %	CaCl %	$\alpha$ - cellulose %
MCP	3.509	1.715	4.59
MDCP	3.653	1.215	5.046
DCP	3.96	0.0281	5.926
DCPDH	4.396	-	5.518
MP	5.926	3.988	-

MCP	Mono-calcium phosphate	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$
MDCP	Mono-dicalcium phosphate	$\text{CaHPO}_4$
MDCP	Mono di-calcium phosphate	$\text{CaHPO}_4 \cdot \text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$
DCPDH	Di-calcium phosphate dihydrate	$\text{CaHPO}_4 \cdot 2 \cdot \text{H}_2\text{O}$
MP	Magnesium phosphate	$\text{Mg}_3(\text{PO}_4)_2$

Table 5.3.d

Basal dietary formulation for experimental diets fed to rainbow trout containing various feed additives.

Dietary component	Diet composition (%)
Fishmeal (LT94)	40
Hi-pro soya bean	25
Fish oil	10
Cornstarch	10
Dextrin	5
Vitamin/Mineral premix	4
Alpha cellulose	5.75
Yttrium oxide marker	0.25

Table 5.5c

Protein and mineral composition of experimental diets containing various mineral supplements and additives.

Basal diet + supplement	Ca (%)	Mg (%)	P (%)	Zn (%)	YtO (%)	Protein (%)
Reference diet	2.245	0.187	1.255	0.096	0.112	37.88
MCP	1.816	0.225	1.057	0.041	0.111	37.42
DCP	1.816	0.229	1.045	0.042	0.114	36.92
MDCP	2.053	0.237	1.220	0.042	0.120	38.44
DCPDH	2.074	0.227	1.244	0.047	0.117	36.21
MP	1.897	1.892	1.105	0.044	0.111	36.75
Reference diet	2.518	0.214	0.895	0.087	0.095	37.39
Phytase	2.326	0.201	0.869	0.086	0.094	37.88
Phenylalanine	2.443	0.212	0.893	0.090	0.097	37.17
Phen' + Vit D <sub>3</sub>	2.409	0.219	0.913	0.085	0.096	38.07
Vit D <sub>3</sub>	2.379	0.219	0.885	0.088	0.099	38.42
Citric acid	2.314	0.209	0.912	0.087	0.094	39.60

Feed additives were substituted in the place of a proportion of the alpha-cellulose as follows to produce 4 experimental diets:- citric acid at 5%, vitamin D<sub>3</sub> at 0.05% (400,000 U/G), L-Phenylalanine at 1.5%, vitamin D<sub>3</sub> and L-Phenylalanine combination at 1.55% (0.05 + 1.5%). The diet containing phytase was produced from the reference diet, after drying this diet was coated with a gelatin mixture containing an active 50,000 IU of phytase (trade name of Nautophos supplied by BSAF, Germany).

### **5.6.3 Feeding, sampling and analysis**

The total animal biomass was calculated for each of the tanks, with this value and the figures produced in the manufacturer's guidelines, it was estimated that fish should be fed 1.3% of their body weight per day, this rate subsequently fell to 1.0% as the fish grew. Fish were fed once a day at 4.00pm, for a period of 10 days, on the 11th day 17 hours after the feeding of the last meal fish were manually stripped, all fish were stripped in this time frame in order to maintain continuity. Faeces were then immediately frozen at -17°C prior to the freeze drying process, after which samples were acid digested by microwave and the yttrium and mineral content determined by ICP-AES. Additionally, the protein analysis was carried out by the kjeldahl method (refer to chapter 2 for methods).

## **5.7 Results**

Fish readily accepted all experimental diets with the general feeding reaction being good. No mortality was encountered during the period of the digestibility trial. For statistical purposes data was analysed in 'blocks' representing each set of diets (comprising of 5 diets for the protein data and 6 diets for the supplement data, each block is differentiated by a horizontal line in the table) for one time period. Therefore significant differences can only be stated for data within blocks, although general trends and predictions are made between block data. Statistical analysis was carried out using the 'GMAV 5' statistical software package to conduct 'nested' two-way ANOVA's on the data in question, followed by SNK



multiple range testing. A probability level of  $P < 0.05$  was considered statistically significant.

Apparent digestibility coefficients of protein for diets containing all of the alternative protein sources ranged between 77.74 to 94.47% and are reported in table 5.5. The diet containing the blood meal protein produced the highest ADC of 92.48% for the first set tested, this was the only coefficient higher than the reference diet (90.43%). The apparent digestibility coefficients for each particular feed ingredient in this first set of diets also confirmed blood meal to be the most highly digestive protein source with a value of 100%. This ADC was significantly higher than all other ingredients in this set. Feather meal, poultry meal and the feather/blood meal combination of feed ingredients all resulted in similar ADC's ranging between 73.78-76.35%, in comparison the reference diet had a significantly lower ADC value of 66.07%.

Apparent digestibility coefficients calculated for the second block of diets, indicated no significant difference within the set the data ranging between 89.16-90.38%. Although the Norse fishmeal had a significantly lower ADC of 63.27% when presented as an individual feed ingredient and compared within its set. Significant differences in ADC values were noted in the third block of diets with two of the soya bean protein sources 87.00% (Hi pro) and 88.32% (full fat) being significantly lower when compared to the reference diet of 91.23%. Conversely soya protein concentrate (SPC) was significantly higher than the reference at 94.47%. These results were mimicked with the ADC figures for the individual feed ingredients.

Table 5.5

Apparent digestibility coefficients of protein for each experimental diet, and individual alternative protein source, (n=3,  $\pm$  S.E).

Diet/Protein source	Apparent protein digestibility of diet (%)	Apparent protein digestibility of feed ingredient (%)
Reference diet	90.43 $\pm$ 0.16 <sup>c</sup>	66.07 $\pm$ 0.28 <sup>b</sup>
Feather meal	77.74 $\pm$ 1.34 <sup>b</sup>	73.78 $\pm$ 2.56 <sup>a</sup>
Poultry meal	82.28 $\pm$ 0.91 <sup>a</sup>	76.18 $\pm$ 1.88 <sup>a</sup>
Blood meal	92.48 $\pm$ 0.34 <sup>d</sup>	100.00 $\pm$ 0.62 <sup>c</sup>
Feather + Blood meal	82.67 $\pm$ 0.79 <sup>a</sup>	76.35 $\pm$ 1.23 <sup>a</sup>
Reference diet	89.32 $\pm$ 0.09 <sup>a</sup>	74.14 $\pm$ 0.16 <sup>a</sup>
Maize gluten	89.16 $\pm$ 0.50 <sup>a</sup>	76.42 $\pm$ 1.0 <sup>a</sup>
Norsea fish meal	89.41 $\pm$ 1.04 <sup>a</sup>	63.27 $\pm$ 1.68 <sup>b</sup>
Special G fishmeal	90.25 $\pm$ 1.06 <sup>a</sup>	75.25 $\pm$ 1.86 <sup>a</sup>
Sunflower meal	90.38 $\pm$ 0.25 <sup>a</sup>	76.02 $\pm$ 0.66 <sup>a</sup>
Reference diet	91.23 $\pm$ 0.26 <sup>a</sup>	69.91 $\pm$ 0.46 <sup>a</sup>
Krill meal	92.68 $\pm$ 0.49 <sup>a</sup>	72.09 $\pm$ 0.96 <sup>ac</sup>
Soya protein concentrate	94.47 $\pm$ 0.29 <sup>b</sup>	75.33 $\pm$ 0.54 <sup>c</sup>
Hi pro soya	87.00 $\pm$ 1.05 <sup>c</sup>	56.03 $\pm$ 2.29 <sup>b</sup>
Full fat soya	88.32 $\pm$ 0.86 <sup>c</sup>	54.89 $\pm$ 2.29 <sup>b</sup>

Unlike superscripts denote a significant statistical difference,  $P < 0.05$  ANOVA, each block of data is divided by a horizontal line.

Table 5.7

Apparent digestibility coefficients for the mineral component of each experimental diet, containing an alternative protein source, (n=3,  $\pm$  S.E).

Diet/Protein source	Calcium (%)	Magnesium (%)	Phosphorus (%)	Zinc (%)
Reference diet	16.00 $\pm$ 4.74 <sup>a</sup>	47.09 $\pm$ 3.83 <sup>a</sup>	47.10 $\pm$ 2.68 <sup>a</sup>	7.31 $\pm$ 1.99 <sup>a</sup>
Feather meal	12.80 $\pm$ 2.68 <sup>a</sup>	55.67 $\pm$ 6.84 <sup>ab</sup>	48.06 $\pm$ 1.32 <sup>a</sup>	20.29 $\pm$ 5.77 <sup>a</sup>
Poultry meal	29.49 $\pm$ 2.49 <sup>b</sup>	46.99 $\pm$ 1.52 <sup>a</sup>	45.49 $\pm$ 1.73 <sup>a</sup>	8.31 $\pm$ 2.79 <sup>a</sup>
Blood meal	26.64 $\pm$ 2.43 <sup>b</sup>	63.35 $\pm$ 2.08 <sup>b</sup>	59.95 $\pm$ 1.76 <sup>b</sup>	41.59 $\pm$ 1.81 <sup>b</sup>
Feather + Blood meal	-8.14 $\pm$ 6.73	49.71 $\pm$ 1.83 <sup>a</sup>	18.43 $\pm$ 2.22 <sup>c</sup>	19.38 $\pm$ 3.31 <sup>a</sup>
Reference diet	16.51 $\pm$ 1.56 <sup>a</sup>	34.54 $\pm$ 1.79 <sup>b</sup>	44.93 $\pm$ 1.49 <sup>a</sup>	9.72 $\pm$ 2.24 <sup>a</sup>
Maize gluten	10.98 $\pm$ 3.11 <sup>a</sup>	59.83 $\pm$ 2.68 <sup>a</sup>	47.33 $\pm$ 1.67 <sup>a</sup>	8.38 $\pm$ 0.50 <sup>a</sup>
Norsea fish meal	11.41 $\pm$ 1.03 <sup>a</sup>	41.86 $\pm$ 1.36 <sup>c</sup>	28.40 $\pm$ 1.18 <sup>b</sup>	2.17 $\pm$ 1.04 <sup>b</sup>
Special G fishmeal	2.81 $\pm$ 1.20 <sup>b</sup>	88.03 $\pm$ 0.98 <sup>d</sup>	59.99 $\pm$ 0.88 <sup>c</sup>	6.67 $\pm$ 1.34 <sup>a</sup>
Sunflower fishmeal	-3.76 $\pm$ 1.98	-214.73 $\pm$ 10.94	24.05 $\pm$ 1.88 <sup>d</sup>	-7.06 $\pm$ 1.43
Reference diet	22.87 $\pm$ 2.24 <sup>b</sup>	31.19 $\pm$ 1.69 <sup>a</sup>	37.93 $\pm$ 2.33 <sup>b</sup>	3.53 $\pm$ 2.15 <sup>b</sup>
Krill meal	40.05 $\pm$ 1.28 <sup>a</sup>	32.86 $\pm$ 2.46 <sup>a</sup>	51.82 $\pm$ 1.29 <sup>a</sup>	12.26 $\pm$ 2.51 <sup>a</sup>
Soya protein concentrate	22.04 $\pm$ 1.74 <sup>b</sup>	29.30 $\pm$ 1.78 <sup>a</sup>	36.49 $\pm$ 2.22 <sup>b</sup>	3.78 $\pm$ 2.24 <sup>b</sup>
Hi pro soya	22.36 $\pm$ 1.26 <sup>b</sup>	29.18 $\pm$ 4.79 <sup>a</sup>	42.70 $\pm$ 2.26 <sup>c</sup>	16.84 $\pm$ 1.34 <sup>c</sup>
Full fat soya	12.03 $\pm$ 1.57 <sup>c</sup>	13.63 $\pm$ 4.20 <sup>b</sup>	37.50 $\pm$ 1.62 <sup>bc</sup>	-6.25 $\pm$ 1.79

Unlike superscripts denote a significant statistical difference,  $P < 0.05$  ANOVA, data with negative values were excluded from statistical analysis.

Apparent digestibility coefficients for the minerals, calcium, magnesium, phosphorus and zinc from the individual feed ingredients are reported in table 5.6. Most statistical software packages are unable to analyse negative data therefore the few negative data values in table 5.6 were excluded from the analysis as a whole, hence they have no allocated lettering in the table above. Once again the data was analysed in 3 blocks, therefore direct comparisons can only be significant within these blocks, although general trends can be noted between the blocked data. In the first block both calcium and zinc produced fairly low ADC values between -8.14 and 29.49% with the exception of a zinc value of 41.59% calculated for the blood meal diet. Whereas the minerals magnesium and phosphorus gave higher ADC results ranging between 45.49 to 63.35% with the exception of a phosphorus value of 18.43% for the combined feather and blood meal diet, in fact all mineral data for this diet was low in comparison with the other 4 diets. In general, few significant differences were noted for this data and it could be suggested that the primary result established was that all mineral digestibility was significantly higher for the blood meal diet, with all other diets being similar to the reference diet.

The second block of data produced more varied results. The diet containing sunflower meal produced the lowest ADC values indicating the poorest mineral digestibility for this protein source. Digestibility figures for calcium were similar 10.98%, 11.41% and 16.51% for maize gluten, Norsesea fish meal and the reference diet respectively, the exception being 2.81% for the Special 'G' fishmeal diet which was significantly lower than the other diets. Significant differences between digestibility values were noted between all diets for magnesium the lowest being 34.54% for the reference diet, then 41.86% for Norsesea fish meal, 59.83% for maize gluten and 88.03% for Special 'G' fish meal. Results for phosphorus showed no significant difference between maize gluten and the reference diet, Norsesea fish meal was significantly lower at 28.40% and Special 'G' significantly higher at

59.99%. The only significant difference noted for the zinc digestibility data was a low value of 2.17% for Norsesea fish meal when compared to the other three diets.

Results in the third block of data show that the diets containing soya protein concentrate and Hi Pro soya along with the reference diet had no significant differences between them for calcium digestibility. The full fat soya diet was found to be significantly lower at 12.03% and the krill meal diet significantly higher at 40.05%. This pattern was similar for the mineral magnesium with the full fat soya diet being significantly lower than the other diets, which were not significantly different from each other. Digestibility trends were similar for the minerals phosphorus and zinc, the soya protein concentrate, full fat soya and reference diets bared no significant difference, with the Hi pro soya and krill meal diets possessed significantly higher digestibility coefficients for these two minerals.

Table 5.7 reports the apparent digestibility data for the mineral component of each individual alternative protein source. Due to the fact that results displayed several negative ADC values throughout the table a statistical analysis was unable to be performed on the data set as a whole (Sokal and Rohlf, 1995). Generally data obtained for the mineral digestibility from individual protein sources is very varied including several negative coefficients and with a few values being extreme. Negative digestibility figures for calcium were calculated for the combined feather and blood meal diet, and diets containing maize gluten, Special 'G' fish meal, sunflower meal and full fat soya. The highest calcium digestibilities were for the soya protein concentrate and Hi pro soya protein sources at 67.16% and 72.08% respectively, followed by blood meal 52.67%, krill meal 44.64% and poultry meal at 37.90% all other values ranged between 5.76% and 16.15% the latter of which being the reference diet. Only two negative magnesium digestibility coefficients were reported, and once again they were for sunflower meal and the full fat soya protein sources.

Table 5.7

Apparent digestibility coefficients for the mineral component of each individual alternative protein source.

Diet containing feed ingredient	Calcium (%)	Magnesium (%)	Phosphorus (%)	Zinc (%)
Reference diet	8.20 ± 5.65	30.76 ± 5.82	27.74 ± 3.67	12.48 ± 7.89
Feather meal	8.05 ± 3.33	121.72 ± 28.0	27.86 ± 8.79	45.83 ± 16.68
Poultry meal	37.90 ± 3.95	60.92 ± 4.34	49.74 ± 3.27	12.17 ± 8.71
Blood meal	52.67 ± 5.78	210.1 ± 19.27	127.44 ± 10.9	2314.1 ± 113
Feather + Blood meal	-82.72 ± 28.22	103.21 ± 15.6	-140.6 ± 15.09	83.67 ± 18.47
Reference diet	9.46 ± 2.08	24.53 ± 2.96	32.49 ± 2.50	18.33 ± 5.34
Maize gluten	-39.85 ± 15.23	96.30 ± 9.26	16.43 ± 7.03	111.63 ± 18.3
Norsea fish meal	5.76 ± 1.26	36.47 ± 2.32	8.47 ± 1.68	-12.54 ± 3.65
Special G fishmeal	-96.53 ± 10.21	1109.2 ± 13.9	124.85 ± 3.95	45.41 ± 9.72
Sunflower fishmeal	-97.70 ± 10.62	-78.33 ± 3.43	-31.68 ± 3.76	-29.67 ± 3.42
Reference diet	16.15 ± 3.68	19.19 ± 2.41	25.45 ± 3.64	9.54 ± 7.80
Krill meal	44.64 ± 2.19	33.90 ± 3.53	53.47 ± 2.62	50.78 ± 7.28
Soya protein concentrate	67.16 ± 22.11	23.31 ± 2.51	17.92 ± 8.23	12.75 ± 4.28
Hi pro soya	72.08 ± 16.69	24.87 ± 7.32	48.26 ± 8.87	87.86 ± 8.08
Full fat soya	-88.51 ± 22.28	-3.41 ± 7.02	20.47 ± 5.95	-53.38 ± 10.78

Magnesium digestibility values varied considerably with the highest figures being reported for Special 'G' fish meal (1109.2%), blood meal (210.1%), feather meal (121.72%), feather and blood meal mix (103.21%), maize gluten (96.3%) and poultry meal (60.92%). All other ADC values were fairly similar ranging from 19.19% for the reference diet to 36.47% for the Norsesea fish meal diet. Digestibility coefficients for phosphorus ranged between -140.6% to 127.44% although generally the bulk of results fell between 8.47% and 53.47% with the higher values representing the blood meal, Special 'G' fishmeal, krill meal, poultry meal and Hi-pro soya diets. The lower results were the sunflower meal, Norsesea fishmeal and full fat soya meal diets. Zinc followed a similar trend with values ranging between -53.38% for full fat soya to an extreme high of 2314.1% for blood meal, and once again the higher values obtained were for the blood meal, maize gluten, Hi-pro soya and Special 'G' fish meal diets. The lowest coefficients were for the diets utilising full fat soya, sunflower meal and Norsesea fishmeal as the alternative protein source. To summarise the diets that produced the highest digestibility of minerals when presented to rainbow trout were ones comprised of the following alternative protein sources; blood meal, Special 'G' fishmeal, Hi-pro soya, krill meal, maize gluten, feather meal, poultry meal and soya protein concentrate. With the lowest mineral digestibility coefficients being observed for the diets containing sunflower meal, Norsesea fishmeal, full fat soya and Icelandic (LT 94) fishmeal (this was the primary component of the reference diet).

Table 5.8 shows the apparent digestibility coefficients for the mineral and protein components of the experimental diets containing mineral supplements and dietary additives. Protein digestibility coefficients for diets containing the mineral supplements ranged between 81.32% to 93.33%, diets with additional mono-calcium phosphate (MCP), mono di-calcium phosphate (MDCP) and magnesium phosphate were not significantly different from the reference diet of 92.17%. Although the diet with calcium phosphate dihydrogen (CPDH) was significantly lower than the reference at 87.99%, and the di-

calcium phosphate (DCP) lower still at 81.32%. Clear statistical differences between protein digestibility coefficients for diets containing dietary additives, could only be distinguished between the following diets;- phenylalanine and vitamin D, phytase and phenylalanine and phenylalanine/vitamin D with vitamin D (with the former of the two being statistically higher than the latter). Generally these results indicate that diets supplemented with phenylalanine had slightly lower protein digestibility.

Apparent mineral digestibility coefficients for diets containing mineral supplements were fairly similar for the four elements analysed. In each case the reference diet (containing no additional minerals) produced significantly lower digestibility values, for calcium MP (59.00%), MCP (54.63%) and MDCP (54.12%) were significantly the highest, with CPDH significantly lower at 44.05%, DCP (31.23%) and reference diet (26.35%) lower still. Magnesium digestibility values were significantly higher (71.33% - 58.48%) for all supplemented diets when compared to the reference diet but not different from each other. This pattern was repeated for the phosphorus digestibility coefficients with all diets being significantly higher than the reference diet (39.56%), although the DCP diet (50.32%) was higher than the reference diet but significantly lower than the others (61.97% - 62.18%). Zinc digestibilities for MP (41.20%), MDCP (42.20%) and CPDH (50.24%) were significantly higher than all other diets, with MCP (29.01%), DCP (23.18%) and the reference diet (13.73%) being significantly poorer in descending order. It appears that minerals were most available and hence most digestible from diets containing supplements of MDCP, MP, than MCP and CPDH, with mineral availabilities being considerably lower in diets containing DCP or no mineral supplement (reference diet).



Table 5.8

Apparent digestibility coefficients for the mineral and protein components of experimental diets, containing supplements (n=3 ± S.E).

Basal diet + supplement	Ca (%)	Mg (%)	P (%)	Zn (%)	Protein (%)
Reference	26.35 ± 5.4 <sup>b</sup>	44.30 ± 2.1 <sup>b</sup>	39.56 ± 4.1 <sup>b</sup>	13.73 ± 0.6 <sup>b</sup>	92.17 ± 0.6 <sup>a</sup>
MCP	54.63 ± 3.5 <sup>a</sup>	60.02 ± 6.3 <sup>a</sup>	62.18 ± 4.7 <sup>a</sup>	29.01 ± 6.4 <sup>a</sup>	92.97 ± 0.5 <sup>a</sup>
DCP	31.23 ± 3.9 <sup>b</sup>	58.48 ± 8.4 <sup>a</sup>	50.32 ± 4.5 <sup>c</sup>	23.18 ± 0.8 <sup>c</sup>	81.32 ± 1.5 <sup>b</sup>
MDCP	54.12 ± 4.2 <sup>a</sup>	69.07 ± 3.3 <sup>a</sup>	62.02 ± 4.1 <sup>a</sup>	42.20 ± 6.0 <sup>d</sup>	91.07 ± 0.3 <sup>a</sup>
CPDH	44.05 ± 3.1 <sup>c</sup>	71.33 ± 1.9 <sup>a</sup>	61.97 ± 2.8 <sup>a</sup>	50.24 ± 2.9 <sup>d</sup>	87.99 ± 1.3 <sup>c</sup>
MP	59.00 ± 0.4 <sup>a</sup>	60.68 ± 0.2 <sup>a</sup>	59.63 ± 1.0 <sup>a</sup>	41.20 ± 3.8 <sup>d</sup>	93.33 ± 0.3 <sup>a</sup>
Basal diet + additive					
Reference	11.39 ± 1.1 <sup>a</sup>	31.21 ± 3.3 <sup>a</sup>	39.72 ± 0.7 <sup>a</sup>	14.25 ± 1.9 <sup>a</sup>	90.12 ± 0.60 <sup>ac</sup>
Phytase	13.46 ± 3.2 <sup>a</sup>	35.16 ± 2.6 <sup>a</sup>	39.15 ± 2.8 <sup>a</sup>	15.46 ± 2.7 <sup>a</sup>	91.81 ± 0.89 <sup>a</sup>
Phen	17.76 ± 5.8 <sup>a</sup>	34.41 ± 3.0 <sup>a</sup>	36.54 ± 4.4 <sup>a</sup>	17.17 ± 4.1 <sup>a</sup>	87.97 ± 0.89 <sup>bc</sup>
Ph + Vit D <sub>3</sub>	16.11 ± 4.6 <sup>a</sup>	40.49 ± 3.3 <sup>a</sup>	43.71 ± 3.1 <sup>a</sup>	25.9 ± 2.0 <sup>ab</sup>	89.13 ± 0.29 <sup>bed</sup>
Vit D <sub>3</sub>	17.71 ± 1.8 <sup>a</sup>	38.34 ± 0.8 <sup>a</sup>	44.21 ± 2.4 <sup>a</sup>	28.01 ± 3.4 <sup>c</sup>	91.88 ± 0.08 <sup>a</sup>
Citric acid	26.31 ± 2.2 <sup>b</sup>	47.24 ± 5.3 <sup>b</sup>	59.07 ± 2.2 <sup>b</sup>	17.30 ± 2.8 <sup>b</sup>	90.86 ± 0.64 <sup>ad</sup>

Unlike superscripts denote a significant statistical difference,  $P < 0.05$ , ANOVA.

MCP -	Mono-calcium phosphate
DCP -	Di-calcium phosphate
MDCP -	Mono di-calcium phosphate
DCPDH -	Di-calcium phosphate dihydrate
MP -	Magnesium phosphate
Phen -	Phenylalanine
Vit D -	Vitamin D <sub>3</sub> (cholecalciferol)

Mineral digestibilities for diets containing the dietary additives showed few statistical differences, with the diet supplemented with citric acid displaying the only significant difference giving higher coefficients than all other diets for calcium, magnesium and phosphorus. Analysis produced for zinc digestibilities indicates that diets containing vitamin D or the vitamin D and phenylalanine combination were significantly higher than all other diets. It should be noted that whilst not significantly different diets containing vitamin D did produce elevated mineral digestibility coefficients for both magnesium and phosphorus.

## **5.8 Discussion**

### **5.8.1 Protein digestibility from diets containing plant proteins**

In this study three different soya bean protein sources were utilised and the determined apparent protein digestibility coefficients for the diets as a whole ranged from 87-89.16%. Though digestibility estimates for the individual ingredients were variable soya protein concentrate was high at 75.33%, but significantly lower were 56.03% and 54.89% for Hi-pro soya (solvent extracted) and full fat soya bean meal respectively.

The majority of published material relating to the digestibility of these protein sources refers to the digestibility of the whole diet as opposed to the digestibility of the feed ingredient alone. Accounting for this type of data presentation, results from this study are in agreement with those for salmonids (Aksnes and Opstvedt, 1998; Gomes *et al.*, 1995; Medale *et al.*, 1998; Morales *et al.*, 1999; Oliva Teles *et al.*, 1994; Pongmaneerat and Watanabe, 1992; Refstie *et al.*, 1997; Sanz *et al.*, 1994; Storebakken *et al.*, 1998a). In addition to other fish species such as tilapia (*Oreochromis aureus x O. niloticus*) (Degani *et al.*, 1999), greenback flounder (*Rhombosolea tapirina*) (Bransden and Carter, 1999), Murray cod (*Maccullochella peelii peelii*) (Abery *et al.*, 2002), Australian silver perch

(*Bidyanus bidyanus*) (Allan *et al.*, 2000), Asian seabass (*Lates calcarifer*) (Boonyaratpalin *et al.*, 1998), red drum (*Sciaenops ocellatus*) (Gaylord and Gatlin, 1996), common carp (*Cyprinus carpio*) (Kim *et al.*, 1998a) which have all been found to have digestibility coefficients close to those presented in this investigation. For all examples cited digestibility coefficients for soya bean diets ranged between 84-96% indicating that these protein sources are general highly digestible relative to the standard fishmeal protein source. Sullivan and Reigh, (1995) established a digestibility coefficient of 79.95% purely for the soybean meal as an ingredient when fed to hybrid striped bass (*Morone saxatilis* X *Morone chrysops*) at a 30% inclusion level. Gomes da Silva and Oliva-Teles, (1998) also established an ADC of 88.6% for solvent extracted soybean as an individual feedstuff fed to seabass (*Dicentrarchus labrax*), both of these findings are substantially higher when compared to those for the current study.

Although the digestibility level of the soybean diet as a whole regularly has a direct relationship with the level of soybean in the test diet, it has been demonstrated that inclusion of soybean protein sources at relatively high levels may exert negative effects on feed utilisation and growth. This was illustrated by Kim *et al.*, (1995) when feeding carp, (*Cyprinus carpio*) more than 25% full fat soybean. Soybean is far less palatable when compared to fishmeal often exhibiting a bitter taste, which can result in a poor feed intake in some fish species. It is often necessary to incorporate feed attractants into diets formulated with high levels of soybean, although some fish are able to adapt to the feed. In particular rainbow trout have been found to rapidly adapt to changes in dietary soybean meal illustrating that this species is flexible to changes in feed formulations (Refstie *et al.*, 1997).

It appears that channel catfish, (*Clarius gariepinus*) have the best ability to utilise soybean meal without any apparent adverse effects, with suggestions that this protein source could

be used to completely replace fish meal in catfish feeds according to Robinson and Li, (1994). Generally to predict the level at which soybean products can replace fishmeal in aquafeeds appears to be dependant on fish species with warm water species being more capable of dealing with soybean as a primary protein source than cold water species.

The type of processing technique also appears to have a bearing on the outcome of the protein digestibility figure, many different forms of soybean are available in the feed industry for example solvent-extracted, extruded, steamed, soya protein concentrate and soaked raw full-fat. In general the majority of soybean products formulated into aquafeeds have been found to be highly digestible, however the raw soybean has been shown to be poorly utilised resulting in a protein ADC of 73.7% when fed to Asian seabass (Boonyaratpalin *et al.*, 1998). Whereas soy protein concentrates are produced to deactivate anti-nutritional factors (ANF's) and excessive fibre, and have been shown to give better growth and digestibility coefficients for rainbow trout than other soy bean products (Kaushik *et al.*, 1995; Murai *et al.*, 1989; Olli and Krogdahl, 1994). This was also observed during this investigation, as the soy protein concentrate (SPC) diet yielded the highest ADC than any other diet (including the fishmeals). The SPC was also highly digestible when assessed on an individual ingredient level. It therefore appears that removing non-nutrients and ANF's causes a positive effect on feed intake and digestibility. The removal of ANF's from soybean meal would also have further benefits by possibly reducing the level of indigestible phosphorus in the diet, hence lowering the excretory waste of phosphorus from the fish into the water. The quality of soybean products is known to vary, not only as a result of the processing method but also growing conditions, cultivar and storage conditions (Snyder and Kwon, 1987). Therefore it is important not to generalise when using soybean products and to ensure that all aspects of soybean cultivation and production are considered when formulating diets using alternative protein sources from soybean.

Generally soybean protein concentrate has been reported in several studies to successfully replace fishmeal for rainbow trout by Medale *et al.*, (1998), Olli and Krogdahl, (1994), and Pfeffer and Henrichfreise, (1994), although reduction in growth has been noted for this species of fish by Kim *et al.*, (1998b), and Rumsey *et al.*, (1993). When compared to soy protein concentrate soy bean meal has often been judged as an inferior protein source and a poorer substitute for fish meal as suggested by Davies and Morris, (1997), and Pfeffer and Beckmann-Toussaint, (1991), although contradictory data has been produced by Oliva Teles *et al.*, (1994), and Sanz *et al.*, (1994). Results from this study show that the soy protein concentrate produces a higher digestibility when compared to the soy bean meals, particularly when the digestibility coefficients of the ingredients are compared individually. This indicates that SPC is a better substitute for fish meal in aquafeeds than the other soy bean meals which can possibly be attributed to a decrease in the content of ANF's in the SPC source.

Of the individual feedstuffs utilised in this trial the sunflower and maize gluten meal were found to be as readily digestible as or even more so when compared to the fishmeals. Whole diet digestibilities were 90.38% and 89.16% for sunflower and maize gluten respectively, where as the digestibility of the individual ingredients were 76.02% and 76.42%. These results are similar when compared to those published by Morales *et al.*, (1999), who calculated dietary apparent digestibility coefficients of 87.26% for protein when chromium was used as an inert marker and 90.71% when acid-insoluble ash was used in diets for rainbow trout. Likewise sunflower was found to be 92.68% digestible in diets for rainbow trout when replacing 40% of the fishmeal, however the digestibility of carbohydrates from this source were found to be very low in a study conducted by Sanz *et al.*, (1994). Sunflower meal was found to replace at least half of the dietary protein source without significant reduction in performance when fed to the European eel, (*Anguilla anguilla*) in research carried out by Garcia-Gallego *et al.*, (1998). Full replacement with

sunflower meal gave results that were not significantly different from the control fishmeal diet providing several essential amino acids were also provided in the diet (Garcia-Gallego *et al.*, 1998). Sunflower seed meal has also been found to give an ADC of 89.8% when fed to tilapia, (*Oreochromis niloticus*) by Sintayehu *et al.*, (1996), and could be used to constitute up to 20% of dietary protein for tilapia, (*Tilapia rendalli*) according to recent work by Olvera-Novoa *et al.*, (2002). Sunflower meal has also been suggested to be a potential major feed ingredient for poultry and pigs when compared to other alternative protein sources, particularly as it does not have the anti-nutritional factors such as those found in other plant proteins such as soybean (Senkoylu and Dale, 1999; Shelton *et al.*, 2001).

Findings for maize gluten meal digestibility by Allan *et al.*, (2000) for the Australian silver perch, (*Bidyanus bidyanus*) were higher at 99.8% than those from this study (89.16%). However ADC figures in excess of 100% were determined for other ingredients and dietary components so it is possible that there may have been to minor errors in measurement which were magnified in the calculations. Arndt *et al.*, (1999) also cited a high ADC value for maize gluten of 98.2% and which was used as the basal diet fed to Coho salmon, (*Oncorhynchus kisutch*), and Gomes *et al.*, (1995) calculated an ADC of 91.34% for a diet containing 15% maize gluten fed to rainbow trout. Wheat/maize gluten has also been shown by several other authors to be a successful substitute for a significant proportion of the fish meal in salmonid diets (Davies *et al.*, 1997; Pfeffer *et al.*, 1992; Rodehutscord *et al.*, 1994; Storebakken *et al.*, 2000), especially when supplemented with lysine (Rodehutscord *et al.*, 2000; Yamamoto *et al.*, 2001).

### **5.8.2 Protein digestibility from diets containing animal proteins**

The high apparent digestibility coefficient (102.84%) for protein observed in this study for spray dried blood meal as a feed ingredient is in agreement with figures reported by

Bureau *et al.*, (1999) and Cho *et al.*, (1982) who indicated that spray-dried blood meal was almost completely digestible. The blood meal ADC from this trial is subject to a small over-estimation error as the value obtained is higher than a total digestibility of 100%. However it should still be noted that blood meal is highly if not totally digested by rainbow trout and is an ideal alternative protein source to fish meal when substituted into diets for salmonid fish.

Apparent digestibility of protein for steam hydrolysed feather meal (74%) was higher than values published by Cho and Slinger, (1979), and Cho *et al.*, (1982) for rainbow trout, but similar to those of Hajen *et al.* (1993) who stated a 71% protein ADC for feather meal fed to chinook salmon (*O. tshawytscha*). However, the protein ADC for feather meal attained during this investigation is slightly lower than the recently stated values of 81 to 87% by Bureau (1999) and Sugiura *et al.*, (1998b) for rainbow trout fed steam hydrolysed feather meal. This may be attributed to an over-estimation by the latter two authors as they employed the Guelph system as their method of faecal collection. Conversely there might be an underestimation of protein digestion by use of the stripping method for faecal sample collection during this study. Although this method produced a protein ADC of 83% when feeding rainbow trout diets containing feather meal for Pfeffer *et al.*, (1995), yet this meal had been processed via acid-hydrolysis as opposed to steam hydrolysis. In general it appears that the protein digestibility of feather meals in fish diets has improved in recent years, it has been suggested that this increase in digestibility is associated with processing method and conditions used for hydrolysing feathers (Bureau *et al.*, 1999). Publications by Latshaw *et al.*, (1994) and Wang and Parsons., (1997) have illustrated that hydrolysis conditions can affect the digestibility and nutritive value of feather meal products for poultry, it is therefore probable that variable processing conditions could potentially affect the digestibility of feather meals for fish.

The ADC for protein observed for poultry meal by-product meal in this present study was 76% which is higher than fairly dated figures of 68% published by Cho and Slinger (1979) for rainbow trout (using the Guelph system). Furthermore, these results are comparable to the work of Dong *et al.*, (1993), who established protein digestibility coefficients for five poultry by-products ranging between 64 and 78%, and Pfeffer *et al* (1995) who observed a protein ADC from poultry by-product meal of 82%. Both authors employed the stripping technique for faecal collection. However, higher protein digestibility values for poultry by-product meal have been reported for salmonid fish as being 96% by Sugiura (1998) and ranging between 87-91% for Bureau *et al* (1999), although faecal samples in both these studies were collected by the Guelph system. Differences in digestibility coefficients appear to be affected by the technique used to collect the faecal sample. Research conducted by Hajen *et al* and Dong *et al* during 1993 provide support for this observation. In these studies, both researchers used the same batch of poultry by-product meal in their studies. Hajen used the Guelph system for faecal collection and obtained 85% protein digestibility for that product, compared with Dong who utilised a stripping method and produced a 78% digestibility coefficient. These results give credibility to the theory that digestibility values obtained when using faecal samples collected by the Guelph system are probably over-estimated and those produced by the stripping method are under-estimated, the true digestibility coefficient being possibly 'somewhere in the middle'. Incidentally, this discrepancy between methods additionally illustrates the need to describe the method used to collect faecal material when comparing apparent digestibility coefficients between studies. Once again it has been suggested that the digestibility of poultry by-product meal has improved in recent years, probably as a result of improved manufacturing and production procedures (Bureau *et al.*, 1999). Alternative protein sources in this study were substituted into the trout diets at a 38% inclusion level and proved to be successful with respect to feed acceptance and digestibility values. Although these diets were only fed to trout for a limited period of time further long term growth trials may have bearings on



growth and feed efficiency and ultimately ADC values. Bureau *et al.*, (2000) demonstrated that the incorporation of up to 15% feather meal did not affect growth or feed efficiency. Also the inclusion of 24% meat and bone meal into trout diets did not affect growth but a small reduction in feed efficiency was noted after a growth trial of twenty weeks. Therefore it should be noted that although these protein sources appear highly valuable alternatives to fish meal inclusion level and the availability of micronutrients (such as amino acids) need to be assessed when optimising the use of these ingredients in fish feeds. The apparent digestibility coefficient gained for diets including krill meal as a protein source was fairly high at 72%. Unfortunately there are few published examples to compare this with, however shrimp meal, similar to krill has been utilised as a protein source for fish. Research data relating to shrimp meal diets directly to salmonid fish appears to be fairly dated with early papers quoting that use of this alternative protein source is somewhat limited. Due to its fairly low protein content and high levels of exoskeleton containing chitin and calcium carbonate which was thought to have detrimental effects on nutrition according to Meyers and Rutledge, (1971). However, several species of marine teleost fish have been found to naturally possess chitinase, the enzyme responsible for cleaving the glycosidic binds in the chitin polymer (Fange *et al.*, 1979; Lundblad *et al.*, 1979). Lindsay *et al.*, (1984) fed rainbow trout diets containing varying levels of purified chitin (4, 10 and 25%) their growth was significantly reduced when compared to casein/starch controls. All fish possessed relatively high levels of endogenous chitinase activity in the stomach and intestines irrespective of diets, but chitin even in relatively low dietary concentrations was not digested to any significant extent. No ready explanation was given for the inability of these fish possessing chitinase to digest chitin, although it was suggested that fish do not naturally ingest purified chitin. In crustacean exoskeletons the chitin is usually covalently bound to proteins, calcium salts and pigments, therefore it is possible that chitinase may bind differently when chitin is presented in a more natural form. Additionally chitinase activity is usually expressed at an

optimum of 37°C, the gastro-intestinal tract of a 'cold water' rainbow trout would be approximately 15°C where it is possible that the enzyme is at temperature too sub-optimal to function. In order to ascertain whether natural digestion of chitin occurs a more sensitive method is required potentially involving a radioactive chitin. Gastric chitinase was first purified and characterised in rainbow trout by Moe and Place, in 1998, and it has been proposed that chitinase maybe pathogenic in nature playing a role in the bodies natural defence mechanisms particularly as it has been located in the blood and lymphomyeloid tissues (Lindsay *et al.*, 1984).

Hughes *et al.*, (1992) fed diets containing 33.6% shrimp meal to juvenile striped bass, (*Morone saxatilis*). Although no digestibility coefficients were calculated it was established that the striped bass were unable to effectively utilise a diet containing shrimp meal as the major protein source (due to a high feed conversion ratio FCR and low protein efficiency ratio PER). Alternatively, more recent data has been published on shrimp meal as an alternative protein in feeds for other species of fish, which is more comparative with the fairly high apparent digestibility obtained from this investigation. In 1998, El-Sayed successfully fed Nile tilapia, (*Oreochromis niloticus*) diets containing 50% shrimp meal and concluded that growth rates and FCR, SGR and PER were not significantly different from those fed fish meal diets, therefore shrimp meal could totally replace fish meal in diets for Nile tilapia. Interestingly the fish fed the shrimp meal diets had significantly higher ash body contents reflecting the ash content of diet.

In poultry nutrition it has been suggested that efficiently processed shrimp meal can be used in relatively high levels in diets for laying hens without any effects on layer performance, but with a possible reduction in feed efficiency (Gernat, 2001). It is therefore possible that in more recent years an increase in the digestibility of shrimp/krill

meal could be attributed to more advanced processing techniques giving a potential use for this alternative protein source.

Icelandic low temperature (LT) herring fish meal was used as the protein source in the reference diets and was on average 70% digestible as an ingredient and 90% digestible as a whole diet. This protein ADC of the whole diet is directly comparable to 88.1% the coefficient stated by Johnson and Summerfelt, (2000) when feeding rainbow trout, (*O. mykiss*) a diet containing herring fishmeal, and 90.8% for common carp, (*Cyprinus carpio*) by Kim *et al.*, (1998a). Although slightly lower than the protein digestibility figure of approximately 96% for seabass, (*Dicentrarchus labrax*) was quoted by Gomes da Silva and Oliva-Teles, (1998) and Spyridakis *et al.*, (1989). However the fishmeal used for these investigations was not the LT variety. An LT herring fishmeal was fed to juvenile turbot, (*Scophthalmus maximus*) by Oliva-Teles *et al.*, (1999) producing a lower ADC of 77.8%. Although Hajen *et al.*, (1993b) established a protein digestibility for the LT herring meal ingredient of 83.6% for post-juvenile chinook salmon, (*O. tshawytscha*), which is substantially higher than the 70% obtained during this series of experiments. Hajen *et al.*, (1993b) stated in the discussion that data found for the aforementioned trial was generally higher than those reported by many other authors. Such findings are supported by Shearer *et al.*, (1992) suggesting that digestibility values for Atlantic salmon (*S. salar*) held in sea water are significantly greater than those obtained when the salmon were held in freshwater, a theory that has been presented and confirmed by other researchers. Aksnes and Opstvedt, (1998) established the differences in digestibility between various qualities of fishmeal fed to rainbow trout, LT herring fishmeal was named to be of high quality and possessed a protein digestibility coefficient of 89.3% which is in total agreement with the 90% obtained in this study.

There appears to be slight controversy relating to the effect of processing temperature on ADC of protein. It has been suggested that the ADC of protein of LT fishmeal was higher than that of fishmeal processed at normal temperatures for rainbow trout and Atlantic salmon (Pike *et al.*, 1990). However, no significant differences between protein ADC's were noted for diets with different processing temperatures for rainbow trout and chinook salmon by Clancy *et al.*, (1995) or turbot, (*Scophthalmus maximus*) by Oliva-Teles *et al.*, (1999). In 1995, Anderson *et al.*, established that the ADC of protein of LT fishmeal (Norse-LT94) was 87% which although slightly higher than the alternative herring meals (82.6 and 85.1%) was not significantly different.

This investigation yielded a protein digestibility figure of 75% for the Special 'G' a low ash fish protein hydrolysate feedstuff and when incorporated into a feed was 90.25% digestible. This latter value being only slightly lower than the 96.5% reported by Gomes da Silva and Oliva-Teles, (1998) for seabass, (*D. labrax*) but almost identical to the 89.63% quoted by Gomes *et al.*, (1995) for rainbow trout, similar results were also presented by Cho, (1993) again for the rainbow trout. Conversely a low 75.6% protein digestibility coefficient for diet containing 25% fish protein hydrolysate was obtained by Oliva-Teles *et al.*, (1999) when feeding juvenile turbot (*S. maximus*). Generally the digestibility of fish protein hydrolysate is similar to that of whole fishmeal.

When Norsesea white fish offal meal was incorporated into a diet the protein ADC was 89.41% which is similar to other fishmeal digestibility values in this study, however when this fishmeal is assessed as an individual feed ingredient a markedly lower level of digestible protein (63%) is found. The three fishmeals utilised in this study all possessed varying levels of ash; Special 'G' protein hydrolysate 5%, Icelandic LT herring meal 12%, with Norsesea offal meal the highest with 18%. The protein digestibility of these feedstuffs on an individual basis also follows an inverse pattern with the Norsesea fishmeal having the

lowest digestibility at 63.27% followed by Icelandic with 70% then Special 'G' at 75.23%. Such findings relating to the inverse relationship between fishmeal ash content and the apparent protein digestibility has been previously reported by Hajen *et al.*, 1993b for chinook salmon and for rainbow trout by Gulley, (1980) and Nose and Mamiya, (1963).

### ***5.8.3 Protein digestibility of diets containing mineral supplementation and non-mineral feed additives***

There is little published data relating protein digestibility to the mineral component of the diet available for comparative comment. The ADC of the diet containing di-calcium phosphate dihydrogen (DCPDH) was significantly reduced (87.99%) when compared to the reference diet (92.17%). A further significant reduction in protein digestibility was noted in the diet supplemented with di-calcium phosphate (DCP - 81.32%). Both of these minerals have a basic DCP structure  $\text{CaHPO}_4$  (the DCPDH obviously having attached water molecules). The other three mineral supplements have a more complicated structure with a higher water content or a magnesium component, contributing to different bonds. As bonding in these minerals is hydrostatic in addition to covalent bonds the compound is likely to disassociate in contact with water, or as soon as it enters the digestive system of the fish. It is possible that the mineral supplements that led to reduced protein digestibilities may have affected the pH of the gastrointestinal tract; one of the disassociated compounds could be phosphoric acid. This would lower the pH of the gut which would in turn decrease the activity of the protease enzymes present there leading to a reduction in protein digestibility. Additionally the disassociated calcium or magnesium ions may have the potential to adsorb/chelate onto protein molecules thus affecting their digestibility. Such protein molecules would be more difficult to cleave if they were larger in size and contain a mineral portion. It is also possible that the mineral may even distort the protease active site impeding the enzyme's action.

No obvious significant differences were noted in protein digestibility between diets containing feed additives, although the diet containing phenylalanine appeared to have a decreased apparent digestibility coefficient when compared to all other diets. This was to be expected as all of the dietary additives were formulated into the diets to ascertain if any affect was noted on the mineral not protein digestibility.

#### **5.8.4 Mineral digestibility of diets containing plant protein**

Apparent mineral digestibilities (usually reported as apparent absorption) for diets containing soybean proteins were similar to those published by Riche and Brown, (1999) and Medale *et al.*, (1998). Who quoted a values of 39.5% and 37.4% respectively for apparent phosphorus availability (APA) for soybean meal, as a feedstuff and when included in diets fed to rainbow trout. Gomes *et al.*, (1995) also quoted a similar ADC for phosphorus in a soybean diet fed to rainbow trout of 44.61%, likewise absorption values were presented by Satoh *et al.*, (2002) for rainbow trout, Gaylord and Gatlin, (1996), and Weerasinghe *et al.*, (2001) for red drum (*Sciaenops ocellatus*) and seabass (*Dicentrarchus labrax*) by Gomes da Silva and Oliva-Teles, (1998) for a range of feed ingredients including soybean. A higher digestibility figure of 67.4% was reported by Barrias and Oliva-Teles, (2000) for a diet containing 29.8% soybean fed to rainbow trout, however the faecal samples taken during this study were collected by settling column, this method often produces digestibility overestimates due to the minerals leaching into the water.

Mineral digestibility is a 'fairly' new area of research and so there are limited papers relating to the absorption of such minerals from a variety of feed ingredients, and commonly phosphorus is usually the only mineral specified. However, Kim *et al.*, (1998a) produced ADC's for both phosphorus (29.0%) and calcium (15.6%) for soybean diets fed to common carp (*Cyprinus carpio*) which are slightly lower than those presented in the current study. Additionally Yamamoto *et al.*, (1997) found apparent availabilities for

magnesium, phosphorus and zinc of 32.1%, 35.8% and 64.4% respectively for rainbow trout fed soybean diets, of which the zinc is relatively high when compared to zinc data from this study, the same author also produced similar values for corn gluten meal to those produced for maize gluten. Magnesium was particularly available from the maize gluten protein source and when this ingredient was considered on an individual basis; both magnesium and zinc were highly digestible, whereas calcium and phosphorus were poor. Sunflower meal by far produced the poorest mineral digestibility coefficients with only phosphorus being a positive value, indicating that calcium, magnesium and zinc were being absorbed from the surrounding water rather than from the feed.

Generally the soybean products yielded digestibility figures in accordance with other published research, but it is interesting to note that when the digestibility of the individual ingredients are considered the full fat soybean meal gives negative ADC's for calcium, magnesium and zinc. This suggests that this protein gives rise to the lowest mineral digestibilities of the three soybean products tested. Of the solvent extracted soybean and soy protein concentrate proteins the solvent extracted soybean has higher digestibility coefficients for phosphorus and zinc, unexpectedly suggesting a better mineral availability from this soy bean source.

#### **5.8.5 Mineral digestibility of diets containing animal protein**

The majority of published research relating to the digestibility of minerals as a result of feeding fish diets formulated from a range of animal proteins is usually limited to data for the mineral phosphorus only, with very limited data being available for minerals such as calcium, magnesium and zinc. Apparent digestibility coefficients established during this investigation for P for poultry meal of 45.49% are higher than those presented Gaylord and Gatlin, (1996) of 26.5% when fed to red drum (*Sciaenops ocellatus*), but similar to that of Satoh *et al*, (2002) for rainbow trout. When P digestibility was assessed from the poultry

meal alone the ADC was second to that of blood meal, but was higher than feather meal and the fish meal reference diet, suggesting this protein source as a good provider of minerals, in particular P and Mg. High values for P digestibility of 58-69% from poultry by product meal were found by an *in vitro* method conducted by Weerasinghe *et al.*, (2000) using rainbow trout. Feather meal produced fairly poor ADC's for P and Ca (27 and 8% respectively) when compared to Sugiura *et al.*, (2000) and Lall, (1991) who obtained values between 77-79% and 47%. Such differences maybe attributable to differences in the processing technique of the feather meal, or interactions with the basal diet as the test ingredients in this present study were substituted into a fish meal based diet as opposed to casein one. However high ADC's from feather meal were obtained for Mg (121%) and Zn (45%), which although higher are relative to those established by Sugiura *et al.*, (2000) of 71% and 24%.

Although not tested during this study various other authors have reported good P ADC's for meat and bone meal as an ingredient at 65.5 % for the red drum (*Sciaenops ocellatus*) by Gaylord and Gatlin, (1996), and 79.6% in seabass (*Dicentrarchus labrax*) by Gomes de Silva and Oliva-Teles (1998). When considering the mineral digestibility of the test ingredient only, blood meal appears to be the optimal protein source for mineral bioavailability with all minerals being the most digestible when compared to other animal protein sources, similar results were found by Sugiura *et al.*, (2000). Such a high ADC maybe due to a dilution effect as a result of the relatively low mineral levels in the ingredient, which increased the relative uptake by the fish, or perhaps the minerals are in a more available form. Alternatively Lall, (1991) established a slightly lower ADC for P of 81% from blood meal. These results indicate blood meal to be an ideal protein and mineral source to be utilised as a substitute for fish meal in salmonid diets, however current legislations ban the use of any animal by products in feeds for fish.



Apparent mineral digestibility coefficients calculated for the range of fish meals used during this investigation indicate 'Special G' fish meal to have the highest P, Mg and Zn availabilities, with 'Norsea' fish meal the poorest, generally Ca digestibility was poor for all fish meal sources used. The latter two fish meals contain ash contents of 5% and 18% respectively, indicating superior mineral digestibility from the diet containing lower ash content. Such findings have also been established by Rodehutschord *et al.*, (2000), Chaimongkol and Boonyaratpalin, (2001) and Sugiura *et al.*, (2000a) the latter author established that when rainbow trout were fed increasing incremental levels of fish bone (ash), the apparent availabilities of P, Ca and Mg decreased. In this study the highest dietary ash content was 18%, which led to a negative ADC for zinc supporting results from Hardy *et al.*, (1984), Satoh *et al.*, (1987a) and Shearer *et al.*, (1992). Shearer concisely reported that dietary ash levels in the region of at least 12-18% resulted in a reduction in zinc availability. Results from the present study further provide evidence illustrating the relationship between ash content of the fish meal to the level of mineral digestibility, which are discussed in more detail in Chapter 3.

#### ***5.8.6 Mineral digestibility of diets containing mineral supplementation and non-mineral feed additives.***

Apparent digestibility coefficients calculated from the reference diets for set of digestibility trials are in line with those published by Kim and Ahn, (1993), Storebakken *et al.*, (2000), Storebakken *et al.*, (1998b), and Sugiura *et al.*, (1998a) for standard fish meal diets.

In general all mineral digestibility coefficients were fairly similar, there was no optimal mineral supplement for providing phosphorus as mono-calcium phosphate (MCP), mono di-calcium phosphate (MCDP), di-calcium phosphate dihydrate (DCPDH) and magnesium phosphate (MP) all produced similar coefficients between 59-62%. However fish fed diets

containing di-calcium phosphate (DCP) had a significantly lower P digestibility of 50% which agree with results by Ogino *et al.*, (1979) and Nordrum *et al.*, (1997). These authors both stated that primary calcium phosphates gave higher P digestibilities when compared to those of secondary calcium phosphates for salmonid fish, which indicates that the MCP supplement is utilised more efficiently than the DCP. When fed diets containing a range of mineral supplements, phosphorus digestibility coefficients from channel catfish (*Ictalurus punctatus*) also produced similar results, with DCP having lower net absorption values when compared to MCP (Lovell, 1978). Diets formulated with DCP also led to the lowest Ca, Mg and Zn digestibility coefficients indicating this mineral salt supplement to have the lowest mineral bioavailability values, and hence would be the poorest choice when formulating a mineral supplement into a rainbow trout feed. The use of DCP in diets for salmonid fish has been found to reduce whole body zinc concentrations possibly as a result of poor Zn absorption (Sato *et al.*, 1993; Hardy and Shearer, 1985). Theories have been suggested by Sato *et al.*, (1993) that high levels of Ca and P and the ratio between them may interfere with the absorption of Zn possibly by chelation. From data presented in this study mono di-calcium phosphate (MDCP) appears to be the optimal choice for the mineral supplementation of rainbow trout diets, as good digestibility values were obtained for all minerals suggesting this minerals in this supplement to be highly available.

The addition of phytase to the reference diet with the aim of treating the phytate content of the diet to improve mineral bioavailability, particularly P, resulted in a non significant effect on the apparent digestibility of Ca, P, Mg or Zn, when compared to the reference diet alone. However positive results relating to the increased digestibility of P when phytase has been added to soybean based diets has been found by Cain and Garling, (1995), Lanari *et al.*, (1998), Rodehutschord *et al.*, (1995), Rodehutschord and Pfeffer, (1995a), Spinelli *et al.*, (1983) Storebakken *et al.*, (1998b) and Sugiura *et al.*, (2001) for salmonid fish. For example a study conducted by Vielma *et al.*, (2000) reduced the P load

into the surrounding water from 8.5 to 4.6g P kg<sup>-1</sup> with the addition of phytase to diets fed to rainbow trout. Other species of fish have been the subject of trials utilising phytase in aquafeeds with a plant protein basis. Papatryphon *et al.*, (1999) and Papatryphon and Soares, (2001) fed high phytate diets supplemented with phytase to striped bass, (*Morone saxatilis*), establishing a 23% increase in P digestibility in addition to increased digestibility for Ca, Fe and Zn. Further positive results using phytase have been published by Oliva-Teles *et al.*, (1998) for seabass (*Dicentrarchus labrax*), Van Weerd *et al.*, (1999) for African catfish (*Clarias gariepinus*) and Yan *et al.*, (2002) for channel catfish (*Ictalurus punctatus*). The latter paper noted that significant increases in the Ca, P, Mg and Mn content of bone were seen when compared to fish fed an un-supplemented diet, faecal phytate levels were also seen to decrease with phytase supplementation. Schäfer *et al.*, (1995) also established improved utilisation of plant P and a 30% reduction in P excretion from diets containing phytase fed to common carp (*Cyprinus carpio*).

In addition to these effects of phytase on soybean based diets, other alternative plant protein sources have been the subject of investigation. The addition of dietary phytase was also found to produce a positive dose-response on dietary phytate and phosphorus availability when included at levels of 4500 U/kg in diets containing 41.58% canola protein concentrate fed to rainbow trout (Forster *et al.*, 1999). Other plant proteins used in conjunction with phytase include barley and corn (Sugiura *et al.*, 2000), wheat and wheat middlings (Cheng and Hardy, 2002). The inclusion of dietary phytase has also been found to be beneficial in improving P utilisation other monogastric animals such as poultry and swine (Simons *et al.*, 1990).

The results obtained for the supplementation of trout feeds with the enzyme phytase were at odds with the majority of published data, which can probably be wholly attributed to the method in which phytase was supplemented in the diet when compared to other techniques

employed by other researchers. The fundamental difference between this study and other investigations using phytase is in the preparation of the soybean meal, the majority of positive results when using phytase are probably as a result of pre-treating the soybean with phytase before final diet formulation. This method employs incubating the phytase with soybean in water at an optimal temperature for the working enzyme (approximately 36°C). Within 24 hours the soybean is cooled and dried ready for use in diet formulations (Storebakken *et al.*, 1998b). An alternative method used involves the inclusion of phytase within the diet which is then cold extruded and kept refrigerated as a moist pellet, very few experimental trials have coated the feed pellet with a phytase spray, the method employed during this investigation. It was noted that during feeding the phytase coating could easily be seen (as a white 'cloud') dissolving from the pellets on immediate entry into the water, thus giving a possible explanation to the results.

This section of the study highlights the importance of planning when preparing the diets, and indicates the need for phytase to either be incorporated into the diet with a sufficient gel coating or used to pre-treat the plant protein prior to formulation. Further trials utilising a range of phytase dosages (i.e. a range of IU) may also highlight the optimal usage of this dietary supplement, and its effectiveness at increasing phosphorus digestibility relative to a wide range of plant ingredients. Since the use of plant protein meal inclusion in fish feeds is increasing the use of phytase may be justified because of its effectiveness at releasing minerals such as P, Mg, Ca and Mn and hence reduces water pollution.

The addition of Vitamin D<sub>3</sub> (cholecalciferol) to the feeds used during this investigation appeared in general to have no significant effect on mineral availability, although zinc digestibility was doubled when compared to the control diet (14.25% vs. 28.01%). Implying that mineral absorption across the gastro-intestinal tract was not greatly

stimulated by the presence of the vitamin D metabolite cholecalciferol. The majority of published data relating to dietary cholecalciferol usually states any effect on whole body mineral concentration and fish growth. Lovell and Li, (1978) stated that channel catfish require a dietary source of cholecalciferol for normal growth and bone mineralisation. Skonberg *et al.*, (1996) fed rainbow trout two levels of cholecalciferol (2,000 and 200,000 IU/kg) and established that fish weight and mineral concentrations in the whole body, skin and vertebrae were not significantly affected by dietary cholecalciferol. Low dietary cholecalciferol levels of 100 IU/kg were found to significantly improve bone mineralisation by Vielma *et al.*, (1999), however no effect on urinary P was noted with either high (2600 IU/kg) or low levels of cholecalciferol. This study by Vielma also provides some evidence of cholecalciferol-dependent control of Mg, Zn and Mn homeostasis, which would support the increase in Zn digestibility found in this present study. Although only significant differences were noted for Zn digestibility during this investigation all mineral digestibilities for fish fed diets containing cholecalciferol were elevated when compared to the control. It may be possible that the cholecalciferol supplementation (400,000) in this trial was at an insufficient level, although Vielma *et al.*, (1998) states that high levels of cholecalciferol (250,000 and 2,500,000) has no beneficial effect on P utilisation. It has been documented that vitamin D<sub>3</sub> plays an integral role in the homeostasis of calcium, phosphorus and possibly other minerals in vertebrates. However it appears that there is a lack of information regarding the endocrinological control of bone mineralisation, gastro-intestinal mineral absorption and control of plasma phosphate in fish and the role that vitamin D plays in these physiological processes. Particularly when fish are fed under different dietary regimes and formulations, further investigation is needed into these areas involving the possible use of gut perfusions to ascertain the fundamental effects of vitamin D and its metabolites on mineral absorption.

The inclusion of dietary citric acid was found to have a significant effect on the digestibility of all minerals (calcium, magnesium, phosphorus and zinc) when compared to the control diets. In each case the apparent digestibility coefficients were significantly increased, similar findings to these were by Sugiura *et al.*, (1998) who fed diets containing 2 or 5% citric acid to rainbow trout over a period of 5 weeks. Sugiura found that adding citric acid to the diets greatly improved calcium and phosphorus availabilities and reduced faecal P levels by a half. This level of acidification in the diet was not found to adversely affect the feed intake or appetite of the fish, however pH faecal and urinary levels were decreased and urinary levels of P were increased. Vielma *et al.*, (1999) found dietary citric acid to improve whole-body ash content of rainbow trout but not affect the body P content. Citric acid has also been found to be beneficial in improving mineral digestibility in pigs by Radcliffe *et al.*, (1998). Citric acid is thought to act as a dietary acidifier, aiding in gastric digestion and chelating mineral ions (Ravindran and Kornegay, 1993). It is possible that the acidifying action of citric acid solubilised dietary minerals making absorption across the intestinal brush border easier and hence, mineral digestibility is improved. Additionally, a decrease in gastro-intestinal pH may also lead to increased enzyme activity, which in turn would alter nutrient digestibility. Such findings indicate the need for further investigation into the use of citric acid as a supplement in feeds for salmonid fish to aid in increasing the availability of P and other minerals.

The apparent digestibility coefficients reported in this present study for rainbow trout will complement other nutritional information available for this species. This information may be used to more precisely formulate rainbow trout diets on the basis of available nutrients in order to limit production costs as well as restrict waste production.

## **CHAPTER 6**

### **GENERAL DISCUSSION AND SUMMATION**

## CHAPTER 6

### GENERAL DISCUSSION

This project attempted to examine the complex physiological and biochemical processes related to macro-element and selected trace elements in salmonid nutrition. This was undertaken with the primary objective of addressing the environmental consequences of feeding high nutrient commercial diets rich in protein, energy and minerals using rainbow trout as the model fish. The strategy employed necessitated the dual approach of classical nutrition trials incorporating physiological and biochemical methods. The purpose being to validate established apparent digestibility coefficients for mainly Calcium (Ca) and Phosphorus (P) as the major elements of concern in this programme of research and also nitrogen (N), the latter elements being of environmental concern.

Central to the stated issues regarding environmental problems and legislation governing the discharge of P and N from intensive fish farming operations the project identified the current status and future direction for this area of concern. Particular attention was given to the situation in Scotland, Norway, Denmark and North America, and their relationship to European policy's regulating fish farm operations and their interaction with the environment.

The project reviewed the scientific literature to date with respect to nutrient requirements for salmon and trout according to NRC guidelines. The initial technical aspects of the research project were dependant on a comprehensive knowledge of the fundamental requirements of a selected range of minerals namely phosphorus, calcium, magnesium and zinc, as these are the important components of mineral trace premixes formulated into commercial aquafeeds. It was also important to review the physiological control mechanism underlying the homeostatic regulation of these minerals in fish and vertebrates in general, quantifying more accurately that



fraction of P excreted into the aqueous environment. The key tissues and organs involved in the assimilation of dietary and water borne sources of trace elements were highlighted. A crucial aspect of the research was to focus on the dietary source of the minerals in question in addition to the dietary protein intake. These factors were deemed to be essential in fulfilling the aims and objectives of relating modern aquaculture practice based on compound feeds and their environmental impact.

In the second chapter of this thesis the modern techniques, such as the use of the ICP-AES for mineral analysis were generally used for most of the studies are presented. These all conformed to international standards for the proximate analysis of diets, faecal material and fish carcasses with respect to N (crude protein), and obviously the key elements selected in all investigations with rainbow trout. It should be appreciated that the analytical methods for trace element analysis have been superseded in the last decade by advances such as the Inductively Coupled Plasma – Atomic Emission Spectrophotometer (ICP-AES). The use of traditional flame absorption spectroscopy is also important and is still used in most cases. However, ICP-AES instrumentation offers a more sensitive and greater potential for application where sample material is limited, often typical of research involving fish of different sizes and consequently body components and associated material. A further great benefit is the ability to simultaneously detect multiple elements in biological samples compared to the more traditional flame Atomic Absorption Spectrophotometer (AAS) systems with a single specific lamp for each element concerned. A vital consideration for this project is the fact that Ca is quite difficult to measure by flame absorption spectroscopy producing wide variation in many cases. Phosphorus cannot be determined by AAS and the only viable alternative is ICP-AES rather than colourimetric assays based on the classical molybdate phosphate interaction.

Sample preparation is recognised to be an important pre-requisite to the use of any detection approach. There are many different procedures quoted in the literature for the degradation and dissolution of biological material for mineral determination. In this current project wet acid digestion was carefully chosen as opposed to dry ashing prior to ICP-AES analysis in order to minimise the possibility of volatile losses. In more detail the merits of rapid microwave digestion offered the advantage of ease of handling, safety and the fast complete dissolution of the sample material in concentrated acid. A further point of consideration is the choice of drying method for organs and tissues and whole fish samples in addition to faecal matter derived from each one of the experimental investigations. The AOAC protocol for oven drying was generally used for whole fish with higher water content. Freeze-drying was more suited to samples of restricted size and mass, for example internal organs such as liver, kidney, spleen, intestine and muscle sections as well as the vertebral column, and was preferable in these cases.

In the majority of the studies, trials were conducted with fish of uniform initial weight within enclosed re-circulation systems with the full control of water temperature, chemistry and photoperiod. The significance of undertaking experiments under such defined conditions was carefully assessed, and this was especially viewed in the context of practical conditions where fish are typically cultured under ambient temperatures and natural photoperiodicity. However it was also appreciated that re-circulation systems carry an inherent risk of nutrient overload, biofilter collapse leading to major fluctuations in the water chemistry profile that would primarily include ammonia, nitrite, nitrate and also P and other minerals of concern. Prior to the studies dependant on the re-circulation technology as the life support unit for the rainbow trout in many experiments, the stability and background levels of the aforementioned were monitored. It is well known that some minerals and in particular Ca may be significantly

absorbed by fish from the aquatic surroundings. This issue was particularly important for P since this element was emphasised throughout this research programme. It should also be stated that the aquarium facilities were not a completely closed system, but were subject to routine 10% weekly water replenishment minimising the accumulation of nitrate and macro elements such as Ca.

In essence the systems were in equilibrium and at all times oxygen levels were held at saturation. Occasionally it was necessary to buffer the system with the addition of 'magnospheres' as source of carbonate to avoid a reduction in pH. The growth performance and health of fish appeared to conform to commercial expectations with the same type of feed thus giving credibility that data obtained from re-circulation systems is reliable and can be realistically used for application. Also it was essential that experimental treatments were replicated according to recommendations and protocols currently used in nutritional studies with fish as reported in the scientific literature. Consequently a main advantage of multiple tank units with similar water flow characteristics, exchange rate, dissolved oxygen levels, offer greater sensitivity. This is so crucial when subtle changes in dietary composition and fish performance etc may be expected in trials involving fish as in other animal nutrition investigations.

The first experimental chapter 3 describes the investigation of the retention and potential availability of four important selected minerals for the rainbow trout in a series of commercial diets. It is well known that fed ingredients vary considerably in quality and source, and it was the main consideration in this preliminary investigation to evaluate propriety diets that were either low or high in their ash content or varied with respect to energy level due to differences in fat content. The feeding trials were conducted both within the experimental facilities in

Plymouth and also in this particular group of studies in the field at the experimental facility based in Sparsholt College, Winchester and at a local experimental fish farm. The latter locations provided an appraisal of growth and feed performance under more realistic outside conditions. Aimed at representing the situation of a typical fish farm supplied with flow through water and natural photoperiod, the only restriction being the tanks fish were held in compared with ponds and raceways. It also enabled a comparison between the expected performance of trout fed according to chart feeding levels within an enclosed re-circulation system that would be the primary facility for all the remaining experimental studies in this programme of research.

The first study was a simple test of two diets with different fish meal sources. Fish meal being the primary protein source in fish feed formulation is also subject to much scrutiny due to considerable cost and varying quality. There is much interest in the use of low temperature (LT) fish meals for salmon and marine fish and LT fish meals are the preferred route if the digestibility and availability of amino acids for the critical profile of fish is to be met. However there is also demand for the use of slightly lower grades of fish meal in production stages that may be less demanding for certain fish. Even lower grades of fish meal (often classed as white fish meals) may be included in less expensive diets for larger fish or those entering into maintenance status or during over-wintering when temperatures and feed intake is low. For these reasons it was of interest to compare the use of two commercial formulations that differed in their fish meal source and quality and possibly phosphorous content and availability.

The results demonstrated the superiority of a good balanced diet formulation with a higher grade of fish meal on the mineral status of rainbow trout with respect to phosphorous and

calcium. These were reflected in the digestibility of these minerals and also the tissue levels. The distribution and levels of each of the minerals of interest were determined for all organs and tissues of metabolic and physiological importance. The experiment provided the first opportunity to develop the necessary techniques and methods that would allow qualitative and quantitative determinations for fish at the start and termination of the typical experimental duration that would be encountered in such trials with salmonids.

A related trial conducted in the spring water fed tank units at Sparsholt, were designed to evaluate the two further commercial diets that differed in the total phosphorous levels. The change in P level was deemed to be quite significant and this study has important relevance towards ascertaining the impact of P on both the environment and in terms of providing P for fish growth performance. The levels recorded in each respective diet were within the ranges reported for salmonid species and other farmed fish in general. Again all growth performance criteria and feed utilisation parameters were measured and the fish were analysed for mineral profiles in the key tissues and organs. The results of this trial supported the fact that the ash content of a feed could influence the mineral absorption characteristics of the diet and also manifest as quantifiable changes in digestibility coefficients and overall mineral status in the fish and component tissues. The trend was for a lower total mineral retention and reduced digestibility for the higher ash commercial diet. It was reasoned that a plausible explanation for these results was due to the form of mineral found in the diet such as the complex hydroxyapatite matrix in bone material principally found in fishmeal, whereas supplementary phosphorous as an inorganic source would be expected to have a higher digestibility due to its less complex structure.

The next series of tests were focussed on the effect of varying energy density on the mineral availability and status of rainbow trout. This investigation was conducted under a controlled fish farm environment and utilised commercial diets that ranged in oil level spanning the typical standard levels to much higher inclusions representing modern high energy dense feeds currently used in aquaculture. This was the first time that a non protein energy nutrient component was the target of research to determine if any possible influences on major elements and minerals might occur for trout. Additionally, two different size classes of rainbow trout were compared in the trial in order to ensure that energy density of feed, known to be important for juvenile fish, also affected larger fish in relation to their mineral requirements and efficacy of utilisation from the diet.

The results were most encouraging and showed that the lipid content of a diet was influential in the retention of minerals from diets for fish fed under intensive conditions. Basically, increasing the lipid and consequently the energy value of a feed enabled an increased uptake and retention of the main elements of interest, namely P, Ca, Mg and zinc. It was theorised that elevated dietary energy promoted faster growth and tissue accretion in fish and this increased the demand and capacity for mineral requirements to support growth. This was reflected in the higher mineral digestibility and tissue levels in rainbow trout fed the diets containing more oil.

Interestingly this was supported by the evidence that the trend was distinct for small trout with faster growth potential than for larger fish reaching maturity and market weight. The demand for energy is lower in these latter fish and the distribution of nutrients in the carcass is expected to favour lipid retention in the viscera and muscle rather than the utilization of energy for bone development, muscle growth and metabolism. Conversely these

physiological processes are at a higher rate for juvenile fish and hence, they demand an elevated mineral load.

Attention was directed to the performance of fish of similar weight class for experimental investigations conducted under laboratory conditions and those performed at each farm location. The main performance indicators such as growth rate (SGR) and feed conversion (FCR) indicated that no discernable differences in fish health or performance were noticeable. Indeed, rainbow trout held in the re-circulation systems under defined stocking density, and controlled temperature, photoperiod and feeding were able to demonstrate excellent feed intake as observed by appetite response, in addition to FCR values below unity ( $<1$ ) conforming to the expectations of the feed manufacturers and data reported in the literature for this species. As such, studies conducted in the laboratory based closed systems appeared to provide for reliable fish performance that was compatible with fish farm trials using commercial diets.

The next phase of the programme (Chapter 4) served to advance our understanding of the mineral requirements of trout in relation to diet formulation strategies where increasing emphasis is now made on the exploitation of plant proteins to replace fishmeal in diets for carnivorous fish species. This has serious repercussions in aquaculture and was considered to be an important point to explore in the research on phosphorous and other mineral. This section of work was also the threshold for further detailed physiological and biochemical studies to establish the optimum dietary levels of phosphorous that would meet the fundamental requirements of trout. It was also hoped to establish the upper dietary levels above which increasing metabolic losses and reduced digestibility may cause a noticeable reduction in the net retention of this element. For these reasons, the appraisal of laboratory

designed diets with varying fishmeal and soybean ratio was evaluated together with a series of diets where the standard fishmeal basal formulation was supplemented with an inorganic P source, in order to determine the effect of P level on key metabolic and physiological processes associated with growth and development.

It was envisaged that this chapter would provide additional information that would yield valuable data concerning the bioavailability of major elements such as phosphorous and calcium with respect to different protein concentrates and in particular soybean as an example of a plant protein. This work was expected to lead towards diet formulation and the need for supplemental P in compound feeds for fish in order to better meet known requirements. Consequently, rainbow trout were fed diets with varying levels of P in order to assess P status with respect to retention and overall efficiency of P utilisation.

The results demonstrated that the lowest P level in the un-supplemented feed was able to sustain the growth performance of trout and that additional increments of P were not able to increase growth and feed utilization beyond that of the control fish. It was inferred that P was not limiting under the conditions of this study and that trout are able to respond without adverse effects to higher P levels. Commercial diets often include P at levels somewhat higher than stated nutritional requirements, and the consequences were discussed in relation to losses to P nutrition and losses to the aqueous environment.

The whole body retention of Ca and P together with Mg and Zn was not affected by incremental supplementation of the diet with the inorganic P source and this was not related to the diet composition. It can be inferred that the regulation of P metabolism is such that no interaction occurred with other elements at the levels used in this study. Although an



antagonistic interaction between Ca and P is well established for many species, the diets were designed for rainbow trout to have a consistent Ca: P ratio. The relatively static concentrations for both Ca and P in the vertebrae of trout receiving these diets seemed to confirm the findings. Although the bones of fish are deemed to be an excellent tissue for the assessment of mineral status, this may only be reflective when a specific trace element is present at well below requirement levels in the diet and may not be a representative tissue when requirements are fully met or at high dietary levels. Little relationship between P or Ca and that for Mg and Zn were found for spleen and kidney tissues in trout. The consistent levels detected would appear to suggest that the status for these elements were within normal physiological limits and that no deficiency was induced due to the increasing P dietary supplementation.

The homeostatic mechanism for the regulation of P and possibly other related elements such as Ca appeared to be operational at the higher P levels in fishmeal based diets fed to rainbow trout. This was verified by the similar plasma P levels although the diet containing over 2% total P induced a significantly increased P level in blood. Since higher P dietary levels were not tested, thus it is not possible to speculate that P regulation may be impaired at dietary levels above 2% leading to elevated blood P concentrations.

In the studies that examined the addition of P to the diet, there were some interesting conclusions. Most noticeable was the decreased digestibility for P at each level of supplementation with the inorganic supplement. This would imply an overload on the P absorption from the intestine as dietary levels increased. This was clearly evident by the progressively higher P levels in the faeces for these fish. It should be noted that all digestibility values reported in this work are derived from the use of a marker in the feed and calculated as the apparent digestibility. This does not allow for the changes in endogenous

losses such as those within the bile fluids and direct secretion of gastro-intestinal fluids and enzyme secretions. The use of these values as a direct basis for comparison must therefore be treated with caution and it is suggested that future research must account for the endogenous loss of minerals from fish for the determination of 'true' bioavailability from the digesta. This can be achieved by the feeding of a diet containing a low protein or effectively zero protein mixture based on semi-purified ingredients.

It was considered that the mineral status of rainbow trout in this study could be better expressed by determining the net retention by relating whole body mineral retention relative to dietary intake during the growth study period. On this basis, the decline in P retention efficiency with increase in dietary level was clearly observed. This was due to a combination of a reduced apparent digestibility (elevated faecal loss) and a consistent absolute absorption of P from the intestine. The implications to the environmental problems associated with faecal P waste output are obvious and this will be addressed later.

A full comprehension of the sequential processes involved in mineral digestion and absorption from the gastrointestinal tract of fish and in this case salmonids such as the trout are a necessary prerequisite before practical interpretations can be realised. For these reasons, preliminary investigations presented in Chapter 5 centred on the determination of the major sites and locations for the relative absorption of the selected minerals and also protein within the gut regions of rainbow trout fed a commercial diet. Although these fish were fed continuously, it was stated as a matter of criticism, that this feeding regime may not fully represent the digestibility status of fish compared with feeding discrete meals or meals separated by a significant period of time. The use of diets that contained no marker meant that the sacrificial approach employed for the study relied on the absolute calculation of each

mineral in each segment of the gut based on total dry digesta and could only provide a relative 'apparent digestibility' value. However some useful information resulted from this initial study and this confirmed that most absorption of minerals occurred within the mid-gut region and that some significant mineral flux into digesta may occur in the hind gut area. This would suggest a quite complex profile for mineral concentrations in gastrointestinal material and caution against the reliance on sampling digesta from fish without attention to differential absorption characteristics and use of different digest and faecal collection strategies. A definitive approach in future should include controlled feeding in terms of ration size and meal frequency, and use of metabolic chambers for total faecal collection.

The final phase of the programme led towards a more detailed appraisal of digestibility of inorganic mineral supplements containing both calcium and phosphorous. In this respect chapter 5 focussed on a series of experiments that would be of practical value in diet formulation with the aim of providing data on mineral digestibility using natural feed ingredients commonly employed in the aquafeed industry. It was also the objective to use P sources that may provide higher digestibility coefficients to minimise the faecal loading and reduce pollution. Complete diets for fish are supplemented with a wide range of vitamin and mineral premixes and it is an area where considerable benefits may arise from improved formulations that would aid in reducing cost whilst providing an adequate balance of essential minerals and trace elements for fish production.

In the digestibility trials, it was important to conform to protocols that are acceptable for the determination of digestibility coefficients in fish with respect to fed ingredients and diets. It was proposed that the most suitable technique for salmonids was the stripping approach that involved removal of faeces by manual expression and subsequent processing. The relevance

of different markers was also considered and on balance the use of yttrium oxide as the standard inert dietary marker for protein and mineral digestibility was selected. The use of larger fish size classes was based on the realisation that such weight classes would provide more faecal material and would remain representative of rainbow trout performance for a significant period of their production cycle. A suitable acclimation period was also incorporated into all of these trials in order to secure the best possible results and consistency.

The selection of dietary materials and ingredients was made according to the advice of the project sponsors (Trouw Aquaculture UK) and these reflected the range of ingredients used in feed formulations for salmon and trout at the time. The proximate analysis and mineral profile for the main elements studied in the programme were determined and used as the foundation for the design of test diets. According to standard practice, a high grade fishmeal based diet served as the reference diet and also as the test diet for this type of fishmeal. A fixed substitution of this reference diet with different test ingredients was made and all diets fed to rainbow trout under defined conditions in the laboratory scale studies.

The results report considerable variations in mineral and protein digestibility coefficients between these dietary ingredients and some of these values are in accordance with the main stream scientific literature. This was particularly the case with crude protein and certain minerals although there were anomalies such as negative coefficients for calcium in ingredients with high fibre content in the plant protein categories. Digestibility coefficients are subject to fundamental errors if based on substandard approaches and it must be realised that such values may not be additive for ingredients that differ markedly in particle size, fibre and starch content and texture. Greater consistency was apparent in the animal protein concentrates evaluated, but relatively low values (approximately 5 to 20%) were found for

zinc in many sources. The negative values found for calcium would imply uptake from surrounding water and this demonstrates the futility of measuring DC values for trace elements and minerals under certain conditions. Nonetheless, the exercise did provide some data that could be of practical value and be used for the assessment of potential feeds destined for aquaculture.

A separate series of related experiments were applied to test the availability of calcium and phosphorous in specific industrial inorganic supplements. These were added to fishmeal based diets formulated for trout. The sources varied in their Ca:P ratio and also a pure magnesium phosphate supplement was also tested. The findings appeared to suggest a consistent P availability from all sources although Ca availability did vary considerably. The choice of inorganic supplement may therefore be an important consideration when designing mineral premixes for fish production. Due to the obvious concerns about increasing P absorption from the diet and thereby reducing wastage, an additional study tested the enhancement of the diet with vitamin D, Phenylalanine, and citric acid. There was a trend for an improvement for Ca and Zn absorption with these additives but the only significantly increased availability for all minerals (Ca, P, Mg and Zn) was for Citric acid, a potent chelation and acidification agent. There was an approximate two- fold increase in Ca absorption and a 50% improvement in P availability. The significance of these results for industrial application were deemed to be of paramount importance. This latter finding offers much scope for future investigations for developing a more refined approach to mineral premix formulation and possible *in vivo* effects on digestive function in fish.

The choice and sequence of experimental investigations using the rainbow trout in this programme of research reflected the need to ascertain the balance of mineral nutrients required

to provide levels for good growth and production with a view to possible overloads and detrimental effects on the surrounding aquatic environment. The main strategy has been to focus on the physiological and metabolic considerations associated with digestion, absorption and retention of the selected group of minerals used throughout the programme. One critical point relating to the theme outlined in the first chapter was the importance of nitrogen and phosphorous losses from fish and the impact on the environment. Detailed studies to evaluate these excretions would have met with considerable difficulties and time constraints given the range of diets employed, the differences in fish size and also the technical problems associated with the collection of samples of water reflecting the cumulative concentrations of N and P in both flow-through and recirculation systems. It would have been necessary to consider the use of metabolic chambers, with the separation of branchial metabolic products such as nitrogen as ammonia in restrained production size rainbow trout, with possible applications of invasive procedures such as the cannulation of the urinary duct for more accurate measurements *in vivo*. The physiological relevance of any data must be balanced against the stress incurred by such fish and the use of single fish specimens in sequence. Although these were all considered at the start of this research project, it was decided that the better strategy would be to concentrate on traditional nutritional experiments with an emphasis on growth and nutrient retention incorporating suitable digestibility trials where appropriate. In this manner, a model was proposed that would be developed from the integration of the various experiments to provide a reasonable budget for the expected losses of both protein-Nitrogen and Phosphorous arising from the intake of feed. The schematic outline is presented in Fig.6.1 for a typical rainbow trout. The partition of phosphorous within the fish is shown and the route of retention (represented by number 5) and losses (represented by numbers 2, 3 and 4).

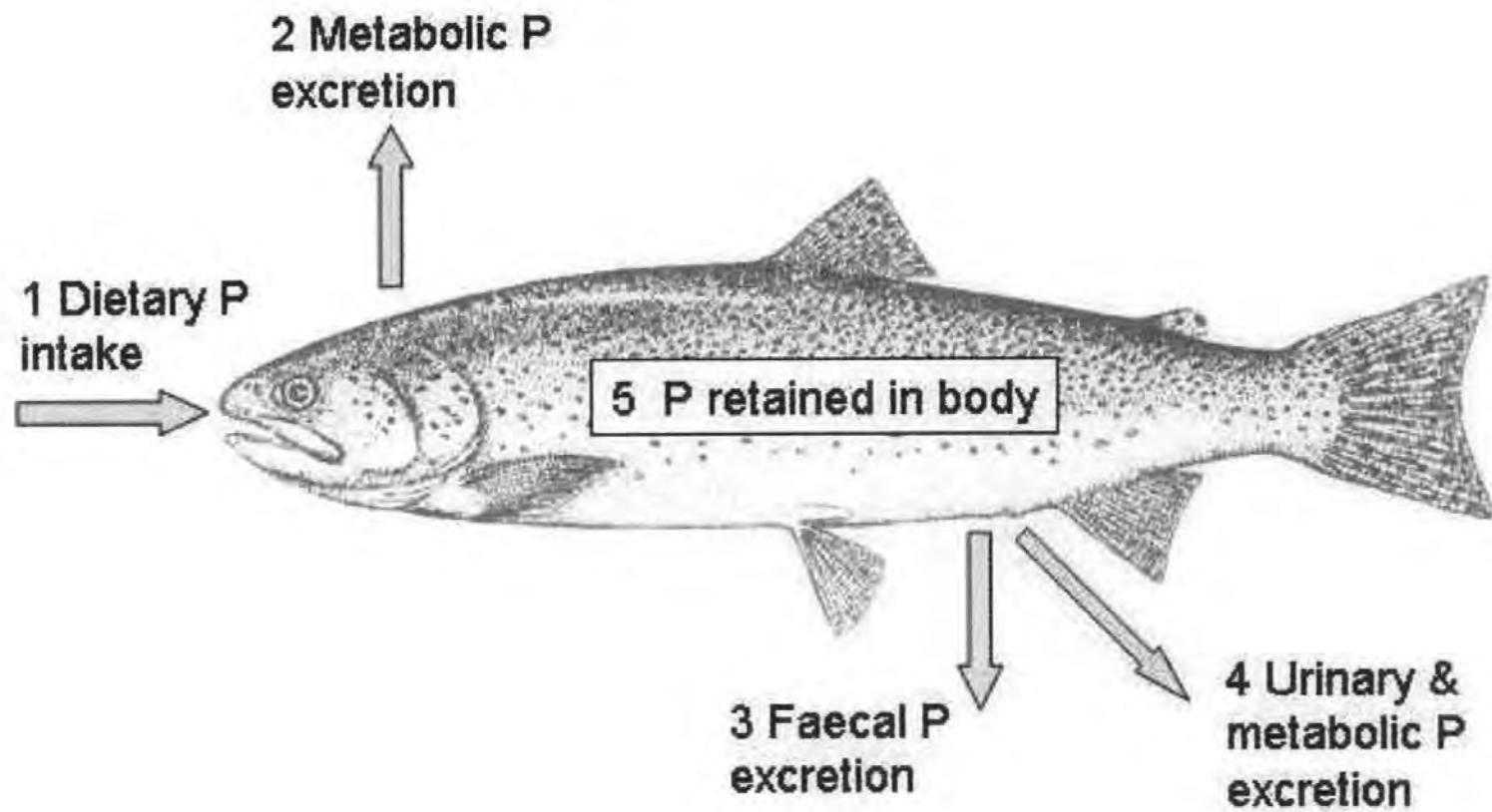


Figure 6.1  
Schematic outline of a typical rainbow trout (*Oncorhynchus mykiss*) displaying the fate of dietary phosphorus.

Excretory phosphorus rates can be calculated using typical data obtained during the course of this programme of research. Table 6.1 displays levels of phosphorus discharge based on a standing biomass of 1 tonne of rainbow trout over a range of feeding rates, calculated as a result of retention. It should be noted that this is an idealised model and assumes no feed losses. In practice feed losses would be quantified using smart feeder technology and specialised computer software to assess such losses.

Table 6.1

The fate of phosphorus for a standing biomass of 1 tonne of rainbow trout (P = g/tonne), over a range of feeding rates.

Feed - % BW/day	P ingested	P digested	P retained	P excreted	P lost via faecal route	P lost via urinary + metabolism
1.0	100	45.0	35.0	65.0	55.0	10
1.2	120	54.0	42.0	78.0	66.0	12
1.5	150	67.5	52.5	97.5	82.5	15

Rates were calculated based on a typical dietary phosphorus content of 1%, a standard P digestibility coefficient of 45% and a retention value of 35% (data obtained from chapter 4).

As can be seen from the table, total phosphorus excretion rates for 1 tonne of rainbow trout fed at a 1.5% is 97.5g of P per day, which equates to approximately 34 kg of excreted P per year.

Faecal losses account for approximately 85% of all P lost leaving the remainder of 15% lost through a combined route of metabolisable/endogenous plus urinary losses. It is possible that the urinary losses are actually higher than quoted, as it is often difficult to avoid the expulsion of urine when stripping the fish, hence the P lost through the faeces maybe slightly lower.



Although the urinary losses appear to be fairly low, the P from this source is dissolved as orthophosphate and is therefore immediately biologically available for use by other organisms and algae. Whereas P from a faecal source may settle on the substrate of the surrounding water body being broken down over time.

Data presented in Table 6.1 can be scaled and sequenced to match the size of the fish farm, information which is important when managing a fish farm, particularly as it is important to know P discharge rates. As the total P discharge is dependent on retention an increase in the digestibility leading to the possible increase in retention of 10% for example, would result in a reduction of the daily load of discharged P from 97.5g/tonne of biomass to 82.5.

Table 6.2  
Phosphorus discharge based on 1 tonne of rainbow trout production (P = kg/tonne), over a range of dietary P levels and retention values, based on an FCR of 1.

P retention (%) →	35%	40%	45%
Dietary P (%) ↓			
0.8	5.20	4.80	4.40
0.9	5.85	5.40	4.95
1.0	6.50	6.00	5.50
1.1	7.15	6.60	6.05

Phosphorus discharge rates based on 1 tonne production of fish are displayed in Table 6.2, this data was calculated based on the average daily intake of P being 150mg/kg of trout biomass and having a feed conversion ratio of 1. Retention data was based on results presented in Figure 4.6 (chapter 4), which shows phosphorus retention against the dietary phosphorus, extrapolating the graph would result in a 40 % P retention with a 1% dietary phosphorus level. Results in Table 6.2 are similar to those presented by the Warrer-Hansen, (2000) and a BIOMAR technical report (2002 unpublished). It can be seen that a reduction in dietary P and an increase in P retention will obviously lead to reduction in P discharge. Warrer-Hansen, (2000) showed that a reduction in FCR is also likely to lead to a reduction in P emission.

Optimal feed formulations are essential for lowering both the mineral content whilst utilising ingredients with a high mineral bioavailability and also reducing the FCR value through the use of highly digestible protein sources and good farm management. Ultimately the use of optimal ingredients will not only reduce the dietary P level but hopefully lead to increased mineral retention, lower FCR's which would both be economical to the farmer but also benefit the environment. A recent review by Cho and Bureau, (2001) discusses diet formulation strategies and feeding systems to reduce excretory and feed wastes, the research concludes that outlining the steps needed to lower the impacts of aquaculture, in order to produce less solid waste poorly digestible ingredients should be eliminated from diets. Ingredients should be carefully selected, that are highly digestible nitrogen waste can be reduced by reducing the digestible protein to digestible energy ratio of the diet. Again careful selection of feed ingredients are needed to reduce phosphorus loading, digestible P should be optimised and not in excess, additionally feeding practices and management should be refined to minimise feed waste.

## SUMMATION

In summary, the research programme presented in this thesis has reviewed the main minerals associated with their environmental impact and in relation to their importance from the nutritional perspective of fish. The apparent digestibility and retention of calcium and phosphorous were of particular interest and the latter was of central importance to the investigations that were described in the sequence of investigations for the rainbow trout.

The limitations of time, expense and also technical aspects when working with fish constrained many possibilities that would have led to more detailed experiments and conclusions. This included the duration of growth studies, the number of fish and replication of treatments that would have aided in resolving many of the conclusions that were made for the separate studies.

The concept of undertaking digestibility trials with trout was an important aspect of the work presented and this was a key strategy for providing data for the prediction of mineral absorption and consequently availability. Reliable data from such studies is vital for feed formulation purposes. Another matter of concern was the dependence of digestibility data based on selected markers in the diet that are known to be subject to varying degrees of sensitivity. There have been suggestions that certain markers such as chromic oxide and yttrium oxide provide different results and so other markers may have to be considered. It was particularly important to use a reliable dietary marker, when digestibility coefficients could be negative, as were found many minerals during some of the experiments. The contribution of selected mineral uptake from the surrounding water medium, and interactions within the digesta and associations with the marker warrants attention. Particularly for calcium and zinc where negative digestibility coefficients suggest that up to a third of the minerals excreted

could be from a water source. It may be possible to develop a gut perfusion system that could be used to simulate the conditions of both trout and salmon in order to test the bioavailability of phosphorous, calcium and other minerals from different sources. Such models could be used in parallel with *in vivo* experiments and prove very useful in providing relative absorption rates and confirming the sites of absorption for different regions of the fish gastrointestinal tract.

Restrictions in the use of certain weight classes of trout in the present work have suggested that future work should be applied to ascertaining the full mineral and nutrient digestibility profile for different ingredients and diets using fish of various sizes representing the full production range between 50-300g in weight. This also leads to the prospect of better understanding the influence of ration size, feeding frequency and temperature on the efficacy of digestibility and metabolism for trout fed different diets with varying mineral profiles. This is one of the most important husbandry related areas worth considering and likely to strongly affect the retention of minerals and losses to the environment under modern intensive aquaculture conditions. It would be of considerable merit to compare and contrast the methods of hand feeding with demand and automatic feeders to develop a more comprehensive model for the mineral intakes and outputs affected by the possibilities of over-feeding and other more efficient techniques of feeding management. One serious limitation encountered in the current studies was the lack of any comparisons with marine species such as salmon. It was initially mentioned that the main problems for mineral losses and consequent environmental impact would be that associated with the salmon industry.

Although the rainbow trout is closely related in many respects, it is not strictly comparable in size and especially in terms of the length of culture compared to the two sea years for salmon

resulting in fish of 2-3 kg. It would therefore have been necessary to have made studies with pre-smolt and post-smolt transferred fish over the whole production period under ideal conditions if time and resources had been permitting. This would have meant large scale sea cage experiments as well as indoor hatchery based trials, as well as digestibility evaluations of many feed ingredients and diets similar to those described for the rainbow trout experiments here.

In the experiments presented in the thesis, there were references made to current methods for determining the partition and fate of minerals such as phosphorous and calcium and protein (nitrogen) in diets and constituent ingredients for trout. In fact, much more work is needed based on metabolic type of experiments in which fish can be confined in purpose built chambers for accurate measurements of daily mineral intake and losses in water. There are many different approaches and some may be restrictive and not appropriate to large fish. The use of cannulated and catheterized fish to obtain urine and even blood samples *in situ* are possible and have been employed by many workers previously for studies on protein and energy metabolism in fish. However, future investigations must focus directly on the endogenous metabolic losses of the major elements as well as those relating to diet and specific dietary sources of these minerals. This would include collecting the branchial and urinary routes of loss and also the faecal output from confined fish such as salmon and trout. This would have given valuable cumulative profiles of absolute phosphorous and ammonia discharge in association with feeding and total metabolic losses. The advantages are obvious with respect to scale and replication since individual fish are involved and it would be possible to use flow-through or even re-circulation systems for maintaining fish in optimum conditions for considerable time periods. The clear disadvantages may be attributed to the distinct possibilities of the imposition of stress and the promotion of a non-physiological status to fish

under such restricted movement. It is also doubtful whether optimum feeding can be sustained and if fish are able to perform as well as under fish farm conditions.

More advanced biochemical methods to determine the fate of minerals in fish and in particular that of dietary phosphorous would be to use radio-labeled phosphate sources in the diet and this would have provided detailed measurements of the partitioning of the element within various body compartments. The distribution between various tissues and organs also providing much needed evidence to establish the route of elimination and rate kinetics of gastrointestinal absorption and clearance time. The ratio of unlabelled to labeled P could also identify the relative pool size of this element and its turnover rate in selected organs over time. These very useful techniques however require specialized equipment and laboratories and can only be really used for fish held in metabolic chambers as described previously. The experiments performed in the present research programme combined standard growth and digestibility approaches with a range of biochemical and physiological techniques. These also included an evaluation of the blood status of trout at a defined period after feeding. Although, the findings would indicate that homeostatic mechanisms for mineral balance in trout are fairly rapid, it would however be interesting to examine the rate kinetics of mineral absorption reflecting the post-prandial surge. This could be performed without the need for radio-labeling and would rely on sampling fish plasma over an extended period after the administration of either single or multiple meals. Such data could provide valuable information about the timing of maximum phosphorous elimination and how feeding management could aid in reducing any physiological overload.

In terms of feed formulation and developments in fish feed technology, several criteria are now deemed to be of major significance. These relate especially to the maximizing of mineral

retention and reducing the burden to the environment. Key research has focused on phosphorous due to its adverse effects resulting from high density fish culture operations. The interest of replacing costly fishmeal in fish feeds is now a primary issue and this has meant the increasing use of such plant proteins as soyabean meal. The problems of increasing the level of dietary phytate, thereby effectively limiting phosphorous availability were addressed in this research. This needs more investigation as attention is directed towards supplementing animal and fish diets with exogenous enzymes such as phytase to digest phytic acid in plant materials and assist in the release of phosphorous for absorption. A method of successfully incorporating phytase into feeds and/or pre-treating plant protein sources with this enzyme needs to be addressed in order to provide a future for this supplement in aquafeeds. Improvements to dietary formulations by including additives such as citrate will also serve to improve the availability of minerals and trace elements. The work presented in this thesis examined a range of mineral supplements with different calcium and phosphorous ratios and physical properties. The overall conclusions suggested that certain sources are more preferable and more useful in fish feed formulations. Indeed fish diet formulations are constantly evolving and therefore with the advent of nutrient dense, high oil feeds for salmon and trout and for other species too, it would seem prudent to re-evaluate the mineral requirements of fish destined for aquaculture.

The investigations constituting this research programme were able to demonstrate the complexity of this topic, and the interaction of nutrient components with respect to the physiological processes governing the utilization, retention and losses of the main macro-elements namely calcium, phosphorous, magnesium and zinc. It is imperative that the conclusions made in the course of this study lead to a more comprehensive knowledge derived from further experimentation in the areas suggested, in order to achieve optimum fish

production commensurate with excellent health, disease resistance whilst also minimizing the effect of such operations on the environment.

Donna Snellgrove, December, 2002



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