Development of Low-Pollution Feeds for Sustainable Aquaculture

by

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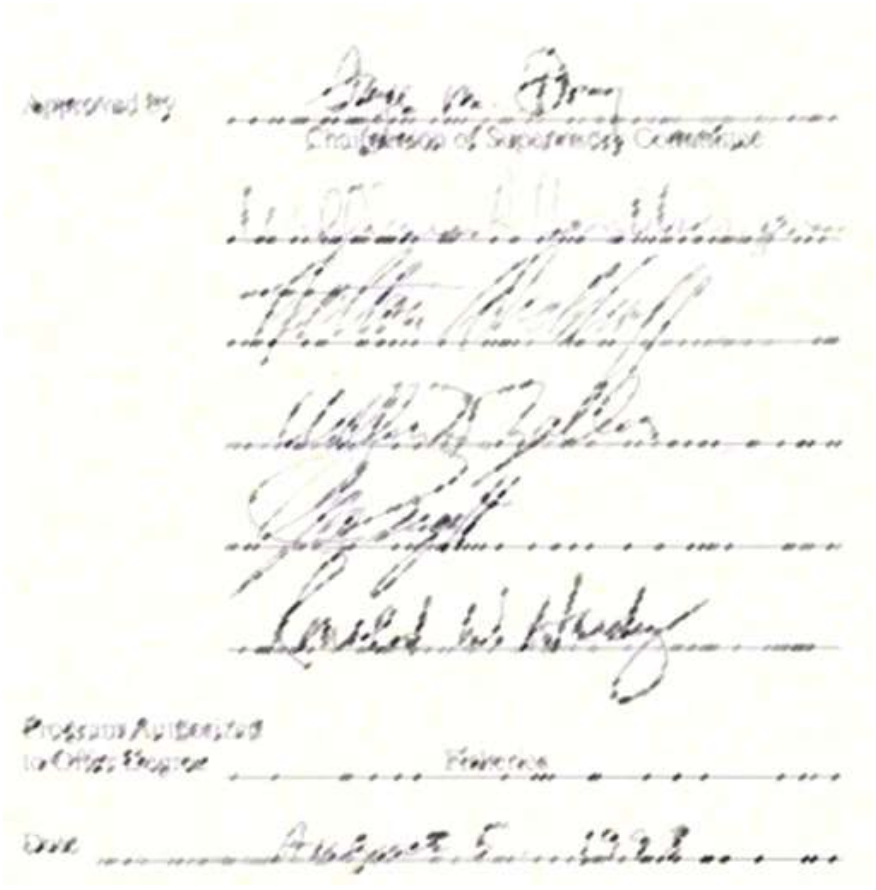

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Abstract

Development of Low-Pollution Feeds for Sustainable Aquaculture

by Shozo H. Sugiura

In order to cope with an ever-increasing world population, it is imperative to find a way to increase world food production and, at the same time, to reduce environmental pollution. The present research has a significant implication since it aimed to achieve both goals simultaneously by developing low-pollution feeds for the world's fastest growing food-producing sector, aquaculture. To determine the minimum dietary requirement of phosphorus for large fish, an original, sensitive and rapid method has been developed. This approach is based upon the urinary excretion of phosphorus of fish placed in a metabolic tank, and the phosphorus requirement can be determined within a few days. Other response parameters for dietary phosphorus were also studied, and were found to be less sensitive than urinary phosphorus concentrations; i.e., concentrations of glucose, glucose-6-phosphate, ATP, creatine phosphate, inorganic phosphorus, calcium, acetoacetate, total lipids, cholesterol, glycogen and alkaline phosphatase in blood, plasma, skeletal muscle, liver and feces. To select feed ingredients based on the nutrient digestibility (availability), the apparent digestibility (availability) of dry matter, protein, phosphorus, calcium, magnesium, sodium, potassium, iron, copper, manganese, zinc and strontium were determined for many feed ingredients, which provided substantial amount of new data currently unavailable in the literature. Many feed ingredients were found to have strong interactive properties with dietary trace elements that reduced their availabilities in complex feeds. The apparent availability of phosphorus in fish meal was found to be dose-dependent. Water-borne calcium had no measurable effect on the apparent absorption of dietary minerals. Low-phytate grains were found to be useful ingredients in low-ash feeds, but not in high-fish meal feeds. Citric acid was found to increase the availability of many minerals, including phosphorus, in fish meal-based diets. In addition, citric acid amplified (ca. 8 times) the effect of supplemental phytase in soybean meal-based diets. Citric acid supplementation (up to 10%/dry feed) did not affect the growth, feed efficiency or health of the fish in 35 days of satiation feeding. For phytase-supplemented feeds, a single feeding (per day) was found to be better than multiple feeding to reduce fecal excretion of phosphorus.

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INTRODUCTION

The primary difference between aquaculture and capture fisheries is that aquaculture is a practice to produce fish, whereas capture fisheries is a practice to harvest fish. The capture fishery harvest must, theoretically, reach a maximum in the future. Most estimates predict that this plateau will be about 100 million metric tons and this will remain static well into the next century (Ackefors et al., 1994). The annual increase of capture fisheries between 1985 and 1995 was 1.5 %, whereas that of aquaculture was 17.7 % (FAO, 1996, 1997; New, 1997). Accordingly, the future of fisheries will be increasingly dependent upon the supply from aquaculture. In fact, aquaculture already contributes about a quarter of the global food fish supply, almost double what it supplied a decade ago (New, 1997). There are, however, a number of problems or constraints associated with further expansion of world aquaculture. Currently, the following are considered the priority issues.

□ Priority issues for the development of aquaculture in the world (Pillay, 1992; Shell, 1993; Stickney, 1994; Hardy, 1995; Grover, 1996)

Environmental pollution due to aquaculture effluent. A rapid increase of world aquaculture production and concomitant increase of global environmental awareness has been increasing the priority of this issue.

Animal protein sources (feed ingredients). With continuous increase of world population, feeding fish (fish meal) to fish cannot be an affordable practice on any significant scale for aquaculture in the future.

Genetic and ecological effects of cultured fish on the wild strains. The possible adverse effects of aquaculture escapees, marine enhancement practice and the translocation of exotic species, including inadvertent distribution of non-indigenous pathogens, parasites, algae, shellfishes, to other areas have been generating increasing concern.

Medication of aquatic species. Large scale use of antibiotics and antibacterials in aquatic feeds and water in some culture systems, and the potential danger of resistant or virulent pathogens to the natural habitat and to humans has been increasing public concern. Also, the discharge of chemotherapeutants into the environment (water and sediment) has adverse impacts to the local ecosystem.

Techniques of artificial propagation and the supply of fingerling fish. For some important aquaculture species, the unsteady supply and availability of fingerling fish is a limiting factor for their production. This is due to the unsuccessful artificial spawning or larval breeding.

Mangrove destruction. In some parts of the world, destruction of mangrove and swamps, which are important spawning grounds and the essential habitat for indigenous fishes, has been causing increasing concern.

Quality issues in relation to aquaculture products. An increasing public consciousness for the quality and safety of seafoods has greatly increased the profile of this important issue.

Human factor. Aquaculture education, extension, technical transfer, research and development of appropriate technology for the development of local aquaculture program will be crucial for increasing world aquaculture production in the future, especially in developing countries.

Water resources. Due to excessive pumping of industrial water, construction of dams, deforestation, global desertification and drought, the volume of water available for aquaculture or indeed for many other human endeavors is decreasing. No water, no fish.

Location for aquaculture. In many countries, concerns for environmental impact and the pressures on coastal zone resources has meant that the installation of net pens, construction of ponds, use of public waters and lands have been subjected to increasing numbers of regulations and constraints.

Systematic research and public perception. This may apply to most of the above issues. Consistent scientific evidence as the rational basis for risk assessments is critical in decision- (policy-) making processes. This serves to structure a meaningful dialog with the public and the government, and helps to avoid unnecessary regulations and precautionary measures.

Clearly, environmental issues are not the only obstacles for the future development of aquaculture in the world. The priority will, however, largely depend on the region. In many industrialized countries, an increasing number of regulations for the discharge of wastes from aquaculture systems is leading to the need for immediate action to this issue. As is common in the fertilization of earthern ponds with organic manure or chemical fertilizers in extensive fish farming, phosphorus input to the system is essential for increasing the primary production of the food chain. Without phosphorus input, there will be only very limited growth of phytoplankton, plants and animal species. Fertilizing ponds or natural waters with organic or inorganic sources of phosphorus is thus essential to increase overall productivity. As an old Japanese proverb says ····"There are no fish in clean streams." Reducing phosphorus pollution (cleaning water) and increasing productivity of ecosystems (producing foods) are, therefore, quite opposite activities. The phosphorus problem for aquaculture appears to be at least partly attributable to the last issue in the above category "Systematic research and public perception." The critical problem of phosphorus pollution associated with commercial aquaculture is that the activity is so intensive and often in areas where water exchange rate is not high enough to disperse aquaculture wastes away from the site. Increasingly aquaculture production, particularly in the intensive sector, is being subjected to environmental regulations and waste discharge guidelines. Various regulations including limitations of production itself have already been set in many industrialized countries. In developing countries the problem is more often self-pollution and the destruction of local ecosystems. In the US, aquaculture effluent discharge is regulated by the National Pollutant Discharge Elimination System (NPDES) through permits issued by the Environmental Protection Agency (EPA) and various state agencies. The NPDES permit may require aquaculture facilities, as a point source, to

limit discharge of total suspended solids, settlable solids and total phosphorus to meet Total Maximum Daily Load or Waste Load Allocation given by the Clean Water Act (formally Federal Water Pollution Control Act). In Idaho, the largest trout producing state in the US, per-facility waste load allocations were developed in 1998 for phosphorus, with compliance levels requiring 20%-reduction over current levels in 1998 and 40%-reduction by the year 2002. This indicates that maintaining even current levels of aquaculture production will be difficult, not to mention increasing the production in the future.

There are three basic approaches to reduction of phosphorus discharge from aquaculture systems: (1) Improving quality of feeds by increasing availability and retention of dietary P by fish; (2) Collection of fish feces by settling or screening in annexed settling ponds or related facilities --- Soluble wastes are essentially uncollectable by this method; (3) Water filtration and recirculation, common in for example intensive eel farming greenhouses --- Such waste treatment systems, however, require considerable additional expense, technology and intensive management.

The ultimate source of wastes in an aquaculture system is feeds. Fish meal is a major ingredient in aquaculture feeds, containing high levels of phosphorus inherent to fish bone, which often elevates total phosphorus content in diets well above the requirement level of fish. Data from several studies with rainbow trout consistently indicate that approximately 80% of dietary phosphorus in typical commercial salmonid feeds is excreted into water as soluble and fecal forms (Ketola, 1982; Philips and Beveridge, 1986; Ackefors and Enell, 1990; Holby and Hall, 1991; Ketola and Harland, 1993). Phosphorus is an essential dietary nutrient for all animal species, necessary for adequate development and maintenance of the skeletal system, as well as playing a key role in many metabolic processes. However, currently available data for its specific dietary requirements are quite limited. In addition, the availability of P has been shown to be largely different depending on the form of the compound in feed ingredients. Ogino et al. (1979) reported availability of P in some feed ingredients and phosphate salts, using carp and rainbow trout, to range between 0-98%. To avoid potential phosphorus deficiency, normally manifested as growth depression, low feed efficiency and abnormal skeletal development (NRC, 1993), most commercial fish feeds contain dietary phosphorus at levels known to be sufficient for optimum growth. These of course could be well in excess of the minimum dietary requirements.

Consequently, reducing P load in aquaculture effluent can be most effectively implemented by increasing the retention (%/intake) of dietary phosphorus by fish. As mentioned above, the amount of unretained phosphorus is largely influenced by the amount of phosphorus in feeds and bioavailability of the compound. The following factors, therefore, should be considered critical to the development of low-polluting feeds: (1) minimizing the allowances of phosphorus in the feed; (2) selecting highly digestible feed ingredients for phosphorus; (3) processing feed ingredients with chemical, thermal or enzymatic measures to improve availability of inherent phosphate; and (4) integrating research findings and developing practical low-polluting feeds. In the present dissertation, each of these subjects was

allocated to a specific section, with results presented in its own chapter. Chapter 4 then provides a synthesis in which the preceding three chapters are integrated and discussed.

A brief description of each chapter follows. Chapter I. The minimum dietary phosphorus requirement for large fish should be accurately known because it is large fish that consume most of feeds in practical aquaculture feeding. Thus large fish are the major contributors of wastes into the effluent water. Phosphorus allowances used for commercial trout feeds are based upon data obtained with very small fish in a laboratory (Ogino and Takeda, 1978). Nutrient requirements, however, usually decrease as animal size increases. For example, the dietary phosphorus requirement of broiler chickens is 50 % higher for 0 to 3 wk-old birds as compared to market-size (6 to 8 wk-old) birds (NRC, 1984). Determining the minimum dietary phosphorus requirements for large rainbow trout will thus provide basic information for the formulation of low-pollution feeds for trout farming industries.

Chapter II. Many factors are known to influence dietary phosphorus utilization by fish, including the level and the form of phosphorus in the diet and its interaction with other dietary components. Phosphorus in salmon and trout feeds is contributed by fish bones in fish meal, phytate phosphorus in various plant protein sources, and phospholipids in fish and plant oils (Hardy, 1995). Some trout feeds contain added phosphorus supplements, such as dicalcium phosphate. Several investigators have researched the availability of phosphates from various sources to different species of fish (NRC, 1993). In spite of the increasing importance of this subject, however, currently available data are quite limited. Knowing precisely the available phosphorus content in common feed ingredients is essential to allow selection of feed ingredients based on the available phosphorus content, and to reduce available phosphorus in diets to the minimum required level for fish without the risk of potential deficiency problems.

Chapter III. Fish meal is one of the main ingredients in commercial feeds for salmon, trout and many other aquaculture species. The form of phosphorus in fish meal is mostly hydroxyapatite, which is not efficiently utilized by fish (Ogino et al., 1979; NRC, 1993). Several feed supplements appear to influence availability of phosphorus and other minerals in fish meal. There has been increasing attention paid to the replacement of fish meal in practical feeds with plant or grain by-product materials because of the decreasing supply / increasing price of fish meal in recent years, its relatively high phosphorus content, and the ethical issue of using fish meal in fish feeds (Tacon, 1994, 1997). Hardy (1995) suggested that the production of soybean meal continues to increase, and that it was the most promising alternate protein source for fish feeds in terms of future availability. Approximately two-thirds of the phosphorus in soybean meal and most other plant feed materials is present as a phytate form which is almost unavailable for fish and many other monogastric animals. Also, phytate forms indigestible complexes with protein, Ca, Mg and some essential trace minerals in complex feeds, and reduces their availabilities (NRC, 1993).

Processing plant-ingredients to convert phytate phosphorus into available phosphorus is, therefore, a procedure critical to the use of plant ingredients in low-pollution feeds.

Chapter IV. Fish farming is still largely empirical by nature, the application of many years of experience acquired through trials and errors is the fundamental technique and by far the most reliable source. A critical function of applied research if it is to change or to replace traditional techniques is, therefore, to show the efficacy of research findings at a practical level. Unfortunately, this critical element is often missing or ignored among scientists. Nothing therefore happens in the production sector even if potentially significant findings have been made in a "laboratory". This final chapter attempts to provide workable solutions based on the research findings of the project to help meet the environmental guidelines imposed on the fish farming industry by regulatory agencies.

The overall objective of this dissertation was to develop low-pollution (environmentally friendly) feeds, in order to reduce phosphorus discharge from aquaculture systems to the environment without compromising fish production, and thereby to contribute to the development of aquaculture for the future.

Chapter I: Determination of Dietary Phosphorus Requirement

ABSTRACT The minimum dietary requirement of phosphorus for large rainbow trout (initial mean body wt, 136–400g) was studied in a series of experiments using the concentrations of glucose-6-phosphate, ATP, creatine phosphate, inorganic phosphorus, calcium, glucose, total lipids, cholesterol, glycogen and alkaline phosphatase in whole blood, plasma, skeletal muscle, liver, feces and urine as response variables. Fish were fed egg albumin-based purified diets with incremental concentrations of supplemental phosphorus supplied as monopotassium phosphate for 5–24 days. The concentrations of glucose-6-phosphate, ATP, creatine phosphate, glucose, total lipids and cholesterol in blood and skeletal muscle were little affected by the 24 days of deprivation of dietary phosphorus. The concentration of inorganic phosphorus in blood, however, correlated significantly and positively with the concentration of ATP in the blood. Plasma and urinary inorganic phosphorus levels were significantly lower in fish fed phosphorus-deficient than phosphorus-supplemented diets. Urinary phosphorus levels responded very rapidly to dietary phosphorus levels, settling to a constant low level within a few days of feeding phosphorus-deficient diets. To determine the dietary requirement of phosphorus, the potency of the diet (N-gain of fish/diet), which differs from feed to feed, must be defined. Results showed that the requirements of available phosphorus (g-P per g-N gain) for rainbow trout having body wt 200g are 0.277 (based on non-fecal excretion), 0.237 (based on retention efficiency), 0.277 (based on balance plateau), 0.293 (based on 95%-body saturation) and 0.312 (based on 95%-plasma saturation). The requirement of available phosphorus for rainbow trout having body wt 400g is 0.284g per g-N gain based on the balance.

BACKGROUND

The dietary requirement of phosphorus (P) has been studied in many animal species including various fishes. Hart, McCollum and Fuller in 1909, using such response criteria as phosphorus concentrations in various hard and soft tissues, bone density and bone-breaking strength, studied the phosphorus deficiency of pigs and estimated the dietary requirement. The balance technique to estimate dietary phosphorus requirements has been used for more than a century (Forbes and Keith, 1914). In fish, similar response criteria, e.g., growth, feed efficiency, P retention, bone mineral accretion, tissue P concentrations and enzyme activities (NRC, 1993), have been studied to estimate dietary phosphorus requirements. These indicators, however, may not be sensitive enough to evaluate minimum P requirements, especially for large commercial-size fish which consume the major portion of feeds in aquaculture operations and thus contribute the most waste into the hatchery effluent. The P allowances used for commercial trout feeds are based upon data obtained with juvenile fish raised in laboratories. Nutrient requirements in most animal species, however, decrease as they become older or larger, because, as they grow larger, growth rate (retention or deposition of dietary nutrients) decreases and increasing

portions of dietary nutrients including P are used for maintenance (significant portions of which can be recycled). In order, therefore, to provide the necessary information for formulating low-pollution feeds for commercial aquaculture of large rainbow trout, it is necessary to determine the minimum dietary P requirements for large fish.

Since P plays a crucial part in the vital processes of all living cells, the depletion of this mineral should have definite pathological consequences. Studies in experimental animals and humans indicate that either deficiency of P or hypophosphatemia may result in various metabolic complications including erythrocyte, leukocyte and platelet dysfunction, glucose intolerance, rhabdomyolysis or acute necrosis of skeletal muscle, impaired myocardial function, central nervous system dysfunction, and osteomalacia (Day and McCollum, 1939; McCollum et al., 1939; Lotz et al., 1968; Knochel, 1977; Kreisberg, 1977; Berner and Shike, 1988; Hodgson and Hurley, 1993). Erythrocyte dysfunction, including a decline of 2,3-diphosphoglycerate (2,3-DPG) and a decline of ATP, is the most important biochemical abnormality associated with P deficiency. The decline of erythrocyte 2,3-DPG shifts the oxyhemoglobin dissociation curve to the left, resulting in the diminished release of oxygen at the cellular level and creates anoxia (Lichtman et al., 1971; Travis et al., 1971). This shift becomes especially important in tissues such as brain where oxygen is necessary for the oxidation of glucose (Glu) through the Krebs cycle and for the synthesis of ATP by oxidative phosphorylation. Hypophosphatemia leads to a reduction of serum and intracellular inorganic phosphorus (Pi), which in turn independently reduces the cell's capacity to produce ATP. Thus, an adequate supply of Pi is critical for repletion of ATP stores in every cell (Lotz et al., 1968; Kreusser et al., 1978; Knochel, 1981).

The decreases in serum and cellular concentrations of Pi are associated with a decrease in creatine phosphokinase, mitochondrial respiration, glycogen, glucose-6-phosphate (G6P) and phospholipid-P in myocardium, skeletal muscle or liver and with impaired fatty acid oxidation, marked hypercholesterolemia and slight hypertriglyceridemia (Horl et al., 1983; Brautbar and Massry, 1984; Brautbar et al., 1984; Kreusser et al., 1984). Although creatine phosphate (PCr) and ATP levels do not change in skeletal muscle, concentrations of both are significantly reduced (8-12 weeks) in myocardium, indicating phosphorus deficiency is associated with reduced mitochondrial capacity to produce ATP, impaired transport of energy via the creatine phosphate shuttle, and reduced myofibrillar ability to utilize ATP (Brautbar et al., 1982a, 1982b; Brautbar et al., 1983).

The effect of dietary P levels on energy metabolism has not been well researched in fishes; however, the accumulation of lipids in tissues by feeding fish with P-deficient feeds (Ogino and Takeda, 1976, 1978; Sakamoto and Yone, 1978, 1980; Eya and Lovell, 1997) or reduction of blood ATP under a hypoxic environment (Smit and Hattingh, 1981) suggests fish species might have essentially the same metabolic processes as laboratory animals and humans.

Under normal conditions, the gastrointestinal absorption of P is unregulated in that net absorption is directly proportional to the amount ingested. Thus, P homeostasis depends primarily on the mechanisms that govern renal excretion. Within 24 h of starting an essentially P-free diet, renal excretion of P is reduced to essentially zero (Moser et al., 1981). This remarkable linkage between intestinal absorption and renal excretion of P is clearly powerful, but the mechanisms involved are not fully understood (Lee et al., 1981; Dennis, 1992).

Intestinal and fecal alkaline phosphatase activities have been known to increase in association with decreased intake of dietary P (Pileggi et al., 1955; Davies et al., 1970; Kempson et al., 1979; Birge and Avioli, 1981). The response of alkaline phosphatase activities to a lower dietary P intake may precede the decrease of P reserve in the body pool (hard tissues) to prevent excessive loss and possible depletion of P.

This paper presents a series of experiments designed to determine the minimum dietary requirement of P for large rainbow trout based on accurate and sensitive response criteria. Experiments were conducted sequentially to improve new approaches: various physiological parameters were evaluated, and the urinary P was found to be the most sensitive indicator (expt.1); to reduce large fish-to-fish variation in urinary P concentrations, maturing fish were eliminated and the feed intake of individual fish was controlled (expt.2); to study the peak excretion of P after feeding, each fish was placed in a metabolic chamber and the urine was collected every 2 h using a catheter (expt.3); four different diets were used to study whether the pattern of P excretion was altered (expt.3); the P requirement for individual fish was estimated by the total collection of urine based on the balance (expt.3); urine samples were collected 8 h after feeding (excretion peak found in expt.3) from a large number of fish (expt.4); fish were placed in a metabolic tank, water-supply was withheld for 24 h, and the tank water was analyzed (expt.5); commercial feed was used and the requirement estimated by the balance (expt.6). The overall objective of this project was to reduce P levels in practical aquaculture feeds to the minimum requirement of fish; thereby to reduce environmental pollution associated with aquaculture effluent without compromising growth of the fish or expansion of world aquaculture production in the future.

MATERIALS AND METHODS

Rainbow trout, Oncorhynchus mykiss, Donaldson strain (obtained from the School of Fisheries' hatchery, University of Washington) was used in experiment 1, and House Creek strain (obtained from the trout hatchery of the College of Southern Idaho) was used in experiments 2, 3, 4, 5 and 6. The sizes of fish were shown in Table 1. A 12:12 h diurnal photoperiod was maintained in all experiments using incandescent (expt.1) or fluorescent (expt.2, 3, 4, 5 and 6) lighting. All fish were handled in accordance

with the guidelines approved by the Animal Care Committee of the University of Washington and the Animal Care and Use Committee of the University of Idaho.

Experiment-1 (Screening of response parameters for dietary P).

Egg albumin-based purified diets with incremental concentrations of P (0.10, 0.32, 0.52, 0.76, 1.07g P/100g diet, dry basis, Table 2) were prepared as cold-extruded pellets, air-dried, and stored at 0-4°C for no longer than 1 month before feeding. Thirty fish (Table 1) were stocked in five 80 L-glass aquaria (six fish/tank) receiving continuous, temperature-controlled (15 ± 1°C) water at 10 L/min from a common recirculating system to which de-chlorinated municipal water was constantly supplied at 5 L/min to prevent accumulation of nutrients and metabolic waste in the recirculating water. Levels of P and ammonia in the recirculating water were monitored daily during the experiment and were below 0.0325 mg/L for P and 0.47 mg/L for total ammonium nitrogen. Each fish within a tank was identified by clipping a tip of the adipose fin and ventral fins in different combinations. To acclimate fish to the purified diet, all fish were fed one of the test diets (containing 0.76g P/100g diet) once daily as much as they would consume for 7 days. Tanks were then randomly assigned to the test diets (1 tank/diet) and the fish were fed for 24 days.

Urine was collected ca. 24 h post feeding at day 0 (after feeding 0.76P diet for 7 days), and at day 2, 4, 10, 20, 21, 22, 23, and 24 by anesthetizing fish with tricaine methane sulfonate (MS-222), wiping the anus to avoid contamination of water, and applying slight pressure to the abdomen. Urine specimens were collected from individual fish into 1.5 ml-microcentrifuge tubes, stored at –20°C, and analyzed each day for concentrations of Pi according to Taussky and Shorr (1953).

After urine sampling, feces were collected at day 20, 21, 22, 23 and 24 by stripping. To avoid contaminating feces with urine (potential source of error for erroneously low availability of dietary P), feces were stripped into a beaker containing water, the water was decanted immediately thereafter, and the fecal pellets were collected as essentially urine-free feces. Fecal samples collected from the same fish over 5 days were pooled, ashed (550°C-12 h) and analyzed for total P content (Taussky and Shorr, 1953). Diet samples (low in Ca) were wet-ashed in Kjeldahl flasks to avoid loss of P at higher temperatures.

At the end of the feeding trial, specimens of blood, skeletal muscle and liver were collected 12 h after the last feeding. Fish were anesthetized without handling by introducing the anesthetic (MS-222) into the inflow water. Immediately, blood was collected from the caudal vessels using a syringe and, without stasis, ca. 0.5 ml of the blood was injected directly into a tared micro-centrifuge tube containing 1.6 ml of ice-cold perchloric acid (6%) and shaken vigorously. Ca. 1 g of white skeletal muscle was excised quickly from the post dorsal area (just below the adipose fin), directly homogenized with 3.25 ml of ice-cold perchloric acid (6%) using a loosely fitted Potter-Elvehjem tissue grinder, and weighed. Muscle samples were not freeze-clamped with liquid nitrogen because of the apparent stability of muscle

phosphagens (Thillart et al., 1980; Söderlund and Hultman, 1986; Harris and Hultman, 1992). Blood and muscle specimens were collected and treated as above before the onset of death. The acidified blood and muscle specimens were kept on ice, neutralized in 2 h, and assayed enzymatically for ATP, PCr, G6P and Glu within the day according to Passonneau and Lowry (1993), and assayed for Pi the following day according to Taussky and Shorr (1953). A portion of liver was excised and stored at 0-2°C for 1 day and homogenized with six times the volume of extraction media (sucrose 0.25 mol/L; Tris HCl 10 mmol/L; EDTA-2Na 0.5 mmol/L) using a motor-driven Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 1,500 × g for 5 min, and the supernatant was re-centrifuged at 15,000 × g for 10 min. The post-mitochondrial supernatant was assayed for Pi content by the above method. Portions of liver and plasma samples were stored at –20°C until analyzed for total lipid and cholesterol content according to Kates (1986).

Experiment-2 (Urine collection by stripping).

Fifty-four fish (Table 1) were stocked in nine 40 L-fiberglass tanks (six fish/tank) receiving untreated spring water at 5 L/min, which had a constant temperature (15 ± 0.5 °C), dissolved oxygen level (ca. 9.0 mg/L) and P and Ca concentrations ca. 0.011 mg/L and 28-36 mg/L, respectively (water from the same source was used for all the succeeding experiments). A basal diet was prepared with purified ingredients (Table 2) and incremental concentrations of P were added to the basal diet. The test diets were formulated as moist pellets and stored at 0-4°C for no more than two weeks. After a one week conditioning period during which all fish received a test diet containing 0.73g P/100g diet (dry basis), the diet was replaced with the test diets of varied P concentrations (0.04, 0.17, 0.27, 0.40, 0.50, 0.62, 0.73, 0.81, 0.90g P/100g diet, dry basis). During the conditioning and experimental period, each fish was force-fed, once daily, a constant amount of feed (0.9% of the body wt, dry basis) by the method of Post et al. (1965). Urine was collected at day -1, 0 (before switching to each test diet), day 1 (first day on the test diet), day 2, 3, 5 and 7 at the time of feeding (24 h after the previous feeding) by the same process described above. Daily urine samples from each fish were analyzed for Pi (Taussky and Shorr, 1953) and calcium (o-cresolphthalein complexone method, Sigma Diagnostics, Procedure No.587).

Experiment-3 (Urine collection by catheterization).

Ten fish (Table 1) were stocked in each of four 40 L-fiberglass tanks receiving continuous flow of spring water. Fish were fed one of 3 purified test diets (Table 2) or commercial trout feed at 1.0% body wt (dry basis) once daily for 5 days. On the 6th day, feces were collected from each fish by stripping, and test diets were fed at 1.0% body wt (dry basis) to each fish by the method of Post et al. (1965), after which the fish were stocked into the metabolism chambers. Although the original procedure (Smith, 1967 and 1971) used a rubber diaphragm and pure oxygen to bubble water in the head tank, neither procedure was used in this study; rather spring water was continuously supplied into the head tank. Urine samples were quantitatively collected using a catheter for 24 h. Urine collection

vials were changed every 2 h using an automatic feeding device (Falls, 1980). Collected urine samples were immediately diluted with 10% TCA and stored at −20°C before being analyzed for the concentrations of Pi (Taussky and Shorr, 1953), calcium (o-cresolphthalein complexone method) and ascorbic acid (dinitrophenylhydrazine method, Omaye et al., 1979). When urine collection was successful, the fish was allowed to stay in the same chamber as long as possible with continued collection of urine and the regular feeding provided every 24 h.

Experiment-4 (Urine collection by stripping; 8 h post feeding).

Two different sizes of fish (Table 1) were stocked into fiberglass tanks (6 to 20 fish/tank), and fed one of the test diets daily to apparent satiation. The test diets contained incremental concentrations of P in the basal diet with the same composition previously used in experiment 3 (Table 2) except for the source of vitamin C (ascorbic acid used in the previous trial was replaced with ascorbyl polyphosphate, trade name Stay-C®, in the present trial). After conditioning the fish by feeding the test diets for 5 days, urine was collected by stripping 8 h after feeding. The urine collection was repeated the following day. Collected urine specimens were immediately diluted with 2× volume of 10% TCA and stored at −20°C before being analyzed for Pi and ascorbic acid concentrations by the procedures described above. Since anorexic freshwater fish also excrete (dilute) urine to regulate osmotic pressure, samples from fish that did not eat were excluded by measuring urinary ascorbic acid levels, on the assumption that fasting fish excrete less ascorbic acid in urine than actively feeding fish (exclusion limit arbitrarily set at 5 mg ascorbic acid/L urine). Ascorbyl-polyphosphate was used because different dietary acidities caused by the different levels of KH_2PO_4 considerably affected the stability of ascorbic acid in diets during storage (data not given).

Experiment-5 (Urine collection in a metabolic tank; Dose-response).

Thirteen fish (Table 1) were stocked in each of 9 fiberglass tanks receiving spring water at ca.10 L/min. Fish were fed a constant weight. of commercial trout feed, once daily, for 2 weeks prior to feeding the experimental diets. The feeding level for the commercial feed and the experimental feeds was calculated from the estimated digestible energy level in diets, fish body wt and the water temperature (Smith, 1989). At “day 0”, fish were fed one of the 9 experimental diets that differed in P concentration (0.15, 0.32, 0.45, 0.59, 0.71, 0.85, 0.95, 1.08, 1.28g availableP/100g dry diet, Table 2) and after ca. 5 min fish were gently transferred to a metabolic tank (modified Hajen et al., 1993, Fig. 33). The system collected both fecal and non-fecal wastes separately. Fish remained in the metabolic tank containing clean spring water for a 24 h period after feeding (stocking) with aeration and recirculation but no filtration provided. Preliminary tests showed no loss of ammonia (NH_4Cl used) and P (KH_2PO_4 used) from the tank containing no fish. Water temperature was maintained at 16 ± 1°C and the oxygen levels were above 5 mg/L at all times. Feces and water samples were collected at day 0, 3, 6, 9 and 12.

Feed and fecal samples were dried (105°C-6 h) and analyzed for total N by a LECO FP-428 Nitrogen determinator (Leco Instruments, St. Joseph, Michigan) and for gross energy by a 1241 adiabatic oxygen bomb calorimeter (Parr Instrument Company, Moline, Illinois). Subsamples of dried feeds and feces were ashed (550°C-12 h), heated to boiling with perchloric acid-sulfuric acid mixture (1:1, v:v) and diluted to an appropriate concentration to measure concentrations of Cr (at 350 nm) and P (at 660 nm, on neutralized samples by Taussky and Shorr 1953). The fecal losses of P and N were estimated based on the apparent digestibility and the feed (P and N) intake instead of the total collection. Apparent digestibility of P, N and energy were determined based on both Cr and acid-insoluble ash (AIA) as indigestible markers. The digestibility values calculated based on Cr were used to determine fecal loss of N and P although very similar values were also obtained with AIA (see Results). The digestible energy (DE) values of feeds were determined by multiplying the gross energy of feeds by the apparent digestibility (%) of energy. Water samples were stored at 0-4°C after collection, and analyzed within 1 day for Kjeldahl-N (Golterman, 1970), with ammonium N in the distillate determined by the phenate method (APHA et al., 1989), and total Pi by the stannous chloride method (APHA et al., 1989). At the end of the feeding trial (12th day), feed was withheld for 24 h in a flow-through tank, and the blood, liver and fecal samples were collected from 5 randomly sampled fish/treatment (tank) using the anesthetic (MS-222) to tranquilize the fish before handling. Blood samples were collected into heparinized syringes and immediately chilled on ice. Plasma was separated within 1 h of the sampling, and assayed for Pi (Taussky and Shorr, 1953), calcium (o-cresolphthalein complexone method, Sigma Diagnostics # 587), total cholesterol (Assous and Girard, 1962), acetoacetate (Schilke and Johnson, 1965), glucose (Hyvarinen and Nikkila, 1962) and alkaline phosphatase (p-nitrophenol method, Sigma Diagnostics # 104, incubated at 15°C). The lower part of the liver was excised immediately after blood was collected, placed into a tared tube containing 30% KOH solution, screw-capped tightly, boiled immediately for 30 min, cooled to room temperature, weighed, and analyzed for glycogen content (Oser, 1965). All specimens were collected before the onset of death. Fecal samples collected by stripping were stored in capped glass vials at 0-4°C for 2 days, mixed thoroughly with an equal volume of distilled water using a flat-bottom glass rod, and centrifuged (1,000 × g-10 min). The supernatant was assayed for alkaline phosphatase activity (p-nitrophenol method, Sigma Diagnostics # 104) with a modified incubation temperature of 15°C. Available P in diets was calculated by subtracting obligatory loss; i.e., total fecal P of fish fed the basal diet (= sum of indigestible P and endogenous P) from the total P of each diet. N retention by fish was estimated by subtracting N loss in water and in feces from N fed. The body wt gain was calculated from N (protein) retained according to Shearer (1994).

Experiment 6 (Urine collection in a metabolic tank; Balance).

Two different sizes of fish (Table 1) were used in this trial. The large fish were carefully sorted in winter (reproductive season) by an experienced hatchery staff to exclude maturing fish. A total of 13

fish (small trout) or 7 fish (large trout) were stocked in a fiberglass tank with a flow-through water supply (no tank replication). The fish had been fed a commercial compressed trout feed for at least 3 months before starting this trial, and had been fed to apparent satiation once daily. Two weeks before starting the sample collection, fish were fed a constant amount of feed once daily, which was also near satiation level. During that period, fish received the same commercial diet except that it contained 0.5g SiO_2/100g dry diet (added by repelleting the diet). The diet had the following analytical composition (dry basis); ash, 9.27; N, 7.30; P, 1.65; Ca, 1.83g/100g diet; GE, 22.06; DE, 18.09 or 18.84 kJ/g (in large or small fish, respectively). All fish were placed in the metabolic tank 3 times (as replicates) every 3 days and fecal, nonfecal (water) samples were collected for 24 h. The amounts of P and N retained by the fish were calculated by the net balance, i.e., retained amount= intake−fecal loss−nonfecal loss. Sampling and analytical procedures were the same as described in experiment 5.

Statistical analysis.

Analytical data for all experiments were subjected to a single factor ANOVA followed by Newman-Keuls multiple comparison test, linear and polynomial regressions, or a sigmoidal dose response analysis using GraphPad Prism, version 2.01 (GraphPad Software, Inc., San Diego, CA). Treatment effects were considered significant at $P< 0.05$. All experiments were conducted in a laboratory under strictly controlled environmental and biological conditions; e.g., water quality parameters, light intensity, flow rate, fish size, strain and source. With those experimental procedures, tank replications were omitted assuming that tank effect should be small compared with dietary effect.

RESULTS

Experiment 1 (Screening of response parameters for dietary P).

Twenty-four days of deprivation of dietary P markedly reduced Pi levels in blood and skeletal muscle; however, the levels of G6P, ATP, PCr and glucose in the same tissues remained fairly stable (Table 3). Nevertheless, the blood ATP level was significantly correlated with the blood Pi level in either linear ($P< 0.01$), quadratic or cubic ($P< 0.001$) manners (Fig. 1). Urinary P concentration was by far the most sensitive indicator among other response criteria (Table 3). Fish responded to a different dietary P intake within 1 day by regulating urinary excretion, which was near zero within 5 days on low P diets (0.10 and 0.32g P/100g diet). In the groups of fish fed 0.52g P, 0.76g P or 1.07g P/100g diet, fish continually excreted a certain amount of P in the urine during the 24 day feeding period, and no trend was observed of either a consistent decrease or increase in urinary P levels over time (Fig. 2). Pi levels in the hepatic cytoplasm were little affected by dietary P levels; however, they were significantly correlated with blood P levels (linear effect, $P< 0.01$; quadratic or cubic effect, $P< 0.001$). Fecal P content was significantly higher in the group of fish fed the highest level of P in diets ($P< 0.05$ by ANOVA). Also, there were linear ($P< 0.05$), quadratic or cubic ($P< 0.01$) regressions of fecal P content on the dietary P

concentration, but not on the plasma P concentration (Table 3). Total lipids, cholesterol, cholesterol/lipid ratio and water levels in plasma and liver were not significantly changed by the dietary P levels (Table 3). Fish that received either the lowest (0.10g P/100g diet) or highest (1.07g P/100g diet) P in the diets reduced their feed intake compared with other groups of fish (data not given).

Experiment 2 (Urine collection by stripping).

Pi levels in urine collected by stripping (24 h post feeding) were lower in fish receiving lower levels of dietary P (Fig. 3); however, the response was not clear enough to estimate adequacy or requirement of dietary P even after 7 days of feeding with the test diets.

Experiment 3 (Urine collection by catheterization).

Urinary P excretion peaked approximately 6-12 h after feeding when the dietary P source was monopotassium phosphate (with or without calcium carbonate; Fig. 4). Conversely, urinary P levels were normally low (in most cases, lower than 100mg/L) and constant over time when the dietary source of P was fish bone or commercial fish meal-based diet. Urine volume was fairly constant over time, day and night. Urinary calcium levels were constant and independent of the dietary levels of calcium. Urinary ascorbic acid levels were initially high when fish were placed in a metabolism chamber; however, the levels progressively decreased to very low levels after 2 to 5 days in the chamber even though the fish had been receiving the same amount of diets daily. Fecal P levels were very low in fish fed the diet with KH_2PO_4; by adding $CaCO_3$ to this diet, fecal P levels were significantly increased and urinary excretion of P decreased (Fig. 5). Fish excreted most of the dietary P in the feces when the dietary source was fish bone or commercial feed, and urinary P levels became very low. Fecal calcium levels were directly proportional to the dietary levels (data not given). The dietary requirement of P for each fish estimated by this balance method, i.e., intake−urinary excretion−fecal excretion, ranged from 0.18 to 0.86g/100g dry diet (n=28 fish). Although the route of P excretion was largely different among dietary source of P and dietary calcium levels, retention of dietary P (g/100g dry diet) was not significantly different among treatments (ANOVA P= 0.27, pooled mean ± SEM, 0.494 ± 0.086, n=4 or 5 fish/treatment). Urine volume (% body wt/24 h) and urinary calcium (mg/L urine) levels also were not different (ANOVA P= 0.34 and 0.81, pooled mean ± SEM, 7.64 ± 1.08 and 90.9 ± 8.92, n=4 or 5 fish/treatment, respectively). Fecal calcium levels (g/100g dry feces) were directly proportional to the dietary levels (mean; P, 0.46; PCa, 6.02; FB, 5.00; C, 4.94; n=8 to 9 fish/treatment). Apparently the fish were stressed by being in the metabolism chamber and by being catheterized. Acclimation of fish to the metabolism chamber did not occur, and fish became exhausted and weakened over time.

Experiment 4 (Urine collection by stripping; 8 h post feeding).

Urinary P levels were generally low up to 0.40g P/100g dry diet with small and large fish (Fig. 6). At 0.65g P/100g diet, urinary P levels markedly increased. No clear break point was obtained which

would have allowed estimation of the minimum dietary requirement. Adjusting data using the ratio of P/ascorbic acid in the urine did not reduce the large deviation of values among fish fed the same diet.

Experiment 5 (Urine collection in a metabolic tank; Dose-response).

There was no appreciable increase of P in the tank water up to 0.61g P/100g dry diet. P excretion increased linearly beyond this level of dietary administration. This pattern was observed at the 3rd day on the test diets and the pattern remained constant for the following days (Fig. 7). Liver glycogen levels tended to be high in fish fed the low P diets; plasma Pi and cholesterol levels were significantly lower ($P < 0.05$ by ANOVA; $P < 0.001$ by linear, quadratic or cubic regressions) in fish fed the low P diets than in fish fed the high P diets; plasma calcium and alkaline phosphatase levels also tended to be lower in low P diet groups than high P diet groups; plasma glucose and fecal alkaline phosphatase were apparently unaffected by the dietary P levels (Table 4). Plasma acetoacetate was not detectable in any treatment (< 0.1mmol/L). Fecal P levels were higher in high P groups than low P groups (Table 4). The apparent availabilities (=net absorption, % of dietary intake) were similar regardless the dietary P level (0.32–1.28g P/100g dry diet); i.e., ranged 93.1–97.1% (n=32, based on Cr). In the group of fish fed diets of lowest P (0.15g P/100g diet, not supplemented with KH_2PO_4), the apparent P availability was the lowest; i.e., ranged 79.4-89.9% (n=4, based on Cr). The apparent availabilities of P determined based on Cr and those determined based on AIA closely agreed (two-tailed paired-t test; mean of differences 0.17%, $P > 0.05$, df=35). There was an increase of non-fecal N excretion in the lowest P group, while fecal N was unaffected by the dietary P level (data not given). Average N retention (lowest P group excluded) was 44.00±0.70% (mean±SEM, n=7 to 8) of dietary intake, or 2.47±0.04g per 100g dry diet consumed. The apparent digestibilities of N determined based on Cr (ranged 97.4–98.0%, n=9) and those determined based on AIA (ranged 97.2–97.9%, n=9) were in a good agreement (mean of differences by paired t-test, 0.094%, df=8); however, the difference was statistically significant ($P < 0.01$). The apparent digestibility of energy ranged from 81.5 to 84.5% (n=9), which was not correlated to the dietary P level. Determined digestible energy (DE) value of diets was 19.23 ± 0.075 kJ/g dry diet (mean ± SEM, n=9). The digestible protein/DE ratio of diets (mean) was 18.31 mg/DE kJ. Estimated weight gain per dry diet consumed (feed efficiency) was 92.4±1.40g/100g diet, except for the lowest P group which had a lower value. Estimates of requirements of available P based on the plasma or body saturation (95% level) were higher than those determined from either non-fecal (urinary) excretion, retention rate or balance plateau (Table 5). Retention rate (%) of dietary P was between 95.6-98.0% at low dietary P levels (up to ca. 0.6%; Fig. 8). Although ammonium-N accumulated in the tank water during the 24 h period (5.61 mg/L, max), this apparently was within the safety level for fish for a short time exposure (Piper, 1982) and no depression of feed intake or any adverse effect was observed.

Experiment 6 (Urine collection in a metabolic tank; Balance).

The mean apparent digestibilities (%) of P were 46.6 for large fish and 50.9 for small fish ($P< 0.05$), those of N were 89.2 for large fish and 91.7 for small fish ($P< 0.01$), and those for energy were 82.0 for large fish and 85.4 for small fish ($P< 0.01$). Determined digestible energy values (mean ± SEM, n=3) were 18.09 ± 0.063 kJ/g dry diet for large fish and 18.84 ± 0.025 kJ/g dry diet for small fish. P requirements per g N gain were not significantly different between large and small fish; however, the requirements in diets (g/100g dry diet) were significantly higher in small fish than large fish (Table 6). Large fish retained 33.5%N per N consumed, while small fish retained 39.6%N per N consumed. Grams of N retained per 100g dry feed consumed were 2.44g N (large fish) and 2.89g N (small fish). The estimated feed efficiencies (wt gain per dry feed consumed) were 91.3% and 107.5% ($P=0.093$) for large and small fish, respectively.

DISCUSSION

Experiment 1 (Screening of response parameters for dietary P).

Levels of G6P, ATP and PCr in blood and skeletal muscle did not appear to be sensitive indicators of either P status or adequacy of dietary P intake in rainbow trout. Presumably, structural tissues, e.g. bones and scales, served as a reservoir to maintain constant levels of phosphagen and high energy P compounds in the body since they are essential for intermediary metabolism and maintenance of life. Studies in experimental animals have shown that despite P-deficiency, adenine nucleotides and PCr in skeletal muscle did not show a significant change (Knochel et al., 1980; Brautbar et al., 1983; Brautbar and Massry, 1984). Hettleman et al. (1983), however, reported that ATP in muscle returns to resting levels more slowly in P-deficient than in the control animals during the recovery period following fatigue, indicating a reduced rate of ATP synthesis. In the present study with rainbow trout, the ATP level in blood was little affected by feeding a low-P diet for 24 days, although it has indeed been reported to decrease in P-deficiency in rats (Rapoport and Guest, 1938), chickens (Bishop and Williams, 1958) and humans (Lichtman et al., 1969, 1971; Travis et al., 1971). The blood P concentration, however, was positively correlated with blood ATP concentration ($P < 0.001$, quadratic or cubic effect) and with muscle ATP concentration ($P < 0.01$, linear, quadratic or cubic effect), suggesting that hypophosphatemic fish are less capable of generating ATP. In fact, Hardy et al. (1993) reported that rainbow trout fed P-deficient diets exhibited a transient lethargy that correlated with the extent of P-depletion in their bodies.

The anorexic effect of low-P diets might be due to the accumulation of reducing equivalent (Langhans et al., 1985) as a result of impaired oxidative phosphorylation in mitochondria or as a result of impaired glycolysis (impaired phosphorylation of G3P) due to lowered intracellular Pi concentrations (Travis et al., 1971). In the present study, fasting blood glucose levels were not significantly elevated with dietary P-restriction. If, however, there is an increased hyperglycemic response due to the reduced rate of glycolysis, and if this diabetogenic effect of P-deficiency stimulates insulin secretion, then

anabolic pathways, especially lipogenesis and glycogenesis could likely be stimulated. Studies in mammals indicate that P-deficiency or hypophosphatemia causes reduced glucose utilization (Rose et al., 1964; Davis et al., 1979; DeFronzo and Lang, 1980), increased insulin secretion in response to glucose (Marshall et al., 1978), or hyperinsulinemia accompanied with carbohydrate intolerance (Harter et al., 1976). Chronic hypophosphatemia (P-deficiency) thus elevates fasting blood glucose and insulin levels, increases lipogenesis, and reduces growth hormone (diabetogenic) secretion and protein synthesis, which appears to be similar to non insulin-dependent diabetes mellitus (NIDDM) and obesity (McCann et al., 1997). Feeding fish with P deficient diets for a prolonged period and determining clinical deficiency signs were beyond the scope of this study; however, the small differences of PCr, G6P and ATP levels among fish fed diets with different P levels suggest that energy metabolism could be affected fairly rapidly by dietary restriction of P based on the results of this study. In previous experiments with carp (unpublished data), the anorexic effect of P deficiency was not so pronounced as that observed in the present study with trout even though the growth of the fish was highly arrested. The excess energy absorbed from feeds then needs to be "effectively wasted" via energy dissipation systems which require no ATP; e.g., the proton leak by the uncoupling proteins in the mitochondrial oxidative phosphorylation to generate heat rather than ATP (Rolfe and Brown, 1997). Conversely, ATP-wasting processes to dissipate excess energy; e.g., increase of the resting metabolic rate induced by catecholamines (also induced by various stressors), increase of protein turnover, increase of futile cycles such as Cori cycle, may not be active due to the limited availability of ATP under P depletion. Changes in concentrations of metabolic intermediates appear to be too insensitive to assess requirement levels; however, the importance of elucidating the relationship between dietary P levels and the partitioning of dietary energy and nutrients, cannot be overestimated since it offers basic information for improving nutrient retention and utilization and for controlling final food fish quality.

Although Pi levels in blood (perchloric acid extract of whole fresh blood) responded well to the dietary P levels, urinary Pi appears to be a more sensitive and rapid indicator of dietary P intake and probably of P status in the body. The dietary P requirement estimated from urinary P concentrations (Table 3, Fig.2) is between 0.32 and 0.52g P/100g dry diet, which is lower than the requirement estimations determined previously with small rainbow trout (Ogino and Takeda, 1978; Ketola and Richmond, 1994; Rodehutscord, 1996).

Experiment 2 (Urine collection by stripping).

In experiment 1, the urinary Pi levels responded rapidly to dietary P levels. The concentration of P in urine, however, was found to be highly variable among individual fish. In the present study, smaller fish were used to minimize the potential effect of gametogenesis and reproductive hormones associated with large fish. In addition, each fish was fed a constant amount of feed (0.9% BW/d) to reduce variability in urinary level of P as a result of differences in feed intake. These changes, however,

did not effectively eliminate fish-to-fish variance, and thus no clear break point for the requirement level was obtained. This experiment, however, confirmed the results of the previous trial in that the urinary P levels respond quickly to the dietary levels, and the fish tended to excrete relatively low levels of P in urine up to ca. 0.4g P/100g dry diet.

Experiment 3 (Urine collection by catheterization).

Urinary excretion of dietary P was rapid when the dietary level of "available" P was high; however, no excretion peak was observed when the dietary P source was less available, and the route of excretion was mostly fecal (non-absorbed). This confirmed the earlier studies reporting that the absorption of P is via the stomach as well as the intestine (Phillips et al., 1964), and that dietary P from high-level P feed is recoverable in the 12 h after feeding ^{32}P, while low-level P feed does not cause this early excretion (Podoliak and Smigielski, 1971). The volume of urine collected from fish using the metabolism chamber and a catheter was highly variable among fish, and was approximately half of the value (ca. 15% BW/d) reported by Smith (1970). This might be due to the use of a diaphragm (in the original method), which damages a part of the skin and permits water (hypotonic) to enter into the body of fish as the consequence of osmotic pressure.

Increasing dietary calcium increased fecal excretion of P, resulting in a decrease of P availability in diets. This result also agrees with the earlier findings of Phillips et al. (1958, 1959) who suggested that the reduced utilization (of dietary P) was due to the chemical binding of P with calcium. This indicates that reducing dietary calcium might be an essential step to increase P availability in practical feeds. However, since P and calcium are normally present together as hydroxyapatite (bone) in fish meal-based feeds, removing calcium without removing P might be impractical. Using sequestrants such as citric acid in feeds will offer an opportunity to chemically "remove" calcium in practical feeds, thereby rendering P more available to fish (ref. Study 1, 2 and 3 in Ch. 3).

A rapid decline of urinary ascorbic acid concentrations suggests that fish were not under a normal metabolic status or, presumably, being stressed by the confinement and the catheterization in the metabolic chamber. In other words, the dietary requirements of ascorbic acid may be higher than the currently recommended if fish are kept in a stressful environment.

Experiment 4 (Urine collection by stripping; 8 h post feeding).

In experiment 3, the peak urinary excretion of P was found to be between 6 to 8 h after feeding for the majority of fish. In experiment 1 and 2, urine samples were collected by stripping 12-24 h after feeding. By that time, the urinary P levels had returned to baseline levels, so that the influence of dietary P level on urinary P had passed.

Collecting urine by stripping was still preferred to catheterization since it apparently is less stressful to fish. The amount of urine obtained by stripping was normally less than one drop or less, especially when fish were in the fed state. Collecting urine from fish that had not eaten, on the other

hand, was much easier because the volume was greater. Fish that have not eaten must, therefore, be excluded from sampling. Those fish providing dilute urine samples were excluded, firstly by visual observation of fish when collecting urine and secondly, by measuring another component in urine that corresponded to feed intake. The urinary level of ascorbic acid was chosen as the indicator of feed intake and the ratio (P/AA) was taken for the stripped urine, which, however, did not successfully reduce the large variation in urinary P level among fish. Another problem with stripping was the different excretion peaks among fish even though the peak normally appeared between 4 to 12 h after feeding. In summary, collecting urine by catheterization and stripping were both unsuccessful approaches to meeting the purpose of these studies.

Experiment 5 (Urine collection in a metabolic tank; Dose-response).

In this experiment (and also in experiment 6), a group of fish was placed in a metabolic tank (140 L), and water sampled from the tank. Thus, the fish were not stressed and there was no need to sample at the peak point of excretion after feeding. Also, the samples were the average of many fish, reducing variability associated with individual fish.

Liver glycogen levels tended to be higher in fish fed low P diets than high P diets although the difference was small, similar to the observation of McCartney (1969a, 1969b) who reported inconclusive results of liver glycogen levels with small brown trout fed low P diets for 20-22 weeks. Sakamoto and Yone (1978), however, reported lower liver glycogen levels in red sea bream fed low P diets compared to high P diets. The plasma alkaline phosphatase activity was higher in P-sufficient than P-deficient groups, which contradicted the finding with mammals (Kempson et al., 1979), but was in agreement with the finding with catfish (Eya and Lovell, 1997). Increased non-fecal N excretion in fish (both large and small groups) fed low P diets indicated reduced N retention or growth. Morris and Ray (1939) studying sheep and Kleiber et al. (1936) using beef heifers noted increased urinary N-excretion in animals fed low-P diets. Rudman et al. (1975) also noted in balance studies with hyperalimented human subjects that P removal from the hyperalimantation fluid interrupted retention of N, while weight gain continued due to repletion of adipose tissue. This effect of P-deficiency might be related to the reduced availability of ATP for protein synthesis since the energy (ATP) requirement for protein deposition is much more costly than for the accumulation of fat and glycogen (Hommes, 1980; Jobling, 1985; Mommsen, 1998).

Plasma (or serum) Pi concentrations could be another indicator (besides urinary P) to estimate adequacy or requirement of dietary intake. The plasma Pi concentration is the indicator used to estimate the requirement (EAR, Estimated Average Requirement) of dietary P in adult humans (IOM, 1997). The requirement level estimated from plasma Pi in this study (Table 5), however, showed higher values than those determined from urinary (non-fecal) excretion, retention efficiency or balance plateau. This is presumably due to the difference between “required” level and “saturation” level. The latter level should always be higher than the former, and for some nutrients, the saturation level even exceeds the

toxic level, demonstrating that tissue saturation may not be indicative of the minimum dietary requirement for animal species. Dietary Reference Intakes (IOM, 1997) also use the balance plateau to estimate the EAR and RDA (Recommended Dietary Allowances) for humans. Since for fish, dietary intake and composition can be precisely controlled over time, the balance technique should be better suited for fish and experimental animals than for humans. The dietary P requirement estimated in the present study, using the balance plateau, resulted in a close agreement with that estimated from non-fecal excretion (Table 5). This value, however, is a plateau value (Y); i.e., an excess level of dietary intake (X) is required to reach the plateau. To achieve 95% level of the plateau (Table 5), the diet still needs to supply P in an amount higher than that determined based on non-fecal or retention rate. The body saturation is an estimate for the dietary level which completes the maximum retention to the body. At near plateau level, the retention efficiency of dietary P will be very low while net retention still continues. The balance technique in terms of estimating the dietary requirement of P, therefore, may not be a method of choice since, at least theoretically, it overlooks the utilization efficiency of phosphorus at different dietary concentrations and also overlooks endogenous loss. The balance method, however, provides an estimate for the maximum retention (body saturation) because no living organisms can retain excessive amounts of any nutrients over extended periods, whereas the time required to reach the balance or equilibrium depends on the size of body pool which differs from nutrient-to-nutrient (Hegsted, 1976; Mertz, 1987).

Experiment 6 (Urine collection in a metabolic tank; Balance).

Small fish required higher levels of P in diets than larger fish. This difference is presumably due to differences in feed efficiency and/or nutrient expenditures for growth and maintenance requirements. When comparing the P requirement between large and small fish based on per N-gain, however, there was little difference. This indicates that growth (N-gain) governs the requirement of P. In other words, the body composition of fish will approximate the minimum essential requirement of P per N (protein) gain. This theoretical estimate of P requirement based on the P/N ratio in the body, however, may not be an approach of choice because 1) there is a considerable variability of N and P content in the body of fish (Papoutsoglou and Papaparaskeva-Papoutsoglou, 1978; Satoh et al., 1993a, 1993b), 2) endogenous (obligatory) loss and retention efficiency of dietary P are overlooked, 3) there could well be incidental losses of P from the body via e.g., loss of scales (high in P content), mucous, and bleeding caused by parasites or bacteria, agonistic behavior among fish and by handling stresses. For example, the P requirement of scaleless fishes such as catfish and eel is substantially lower than that of scaled fish (NRC, 1993), suggesting that the loss of scales may well elevate the dietary requirement of P. In the present study, fish were handled as gently as possible, yet aggressive behavior among fish could not be completely eliminated, which could in part be the reason for the higher requirement of P

determined in this study for the N-gain than the actual amount of P and N that can be recovered in the body of the fish.

Foy and Rosell (1991) reported that 10% of total phosphorus loading from a Northern Ireland fish farm was soluble (molybdate-) unreactive phosphorus. Also, Cowey (1995) noted that 85-95% of urinary P is in the form of soluble molybdate-reactive P. This indicates that in the balance trial, the retention of P in fish must be an overestimate since this soluble unreactive fraction of P was not quantified. In the balance trial, however, most P excreted from fish was via feces, and the excretion via urine was very small, suggesting that the quantity of soluble-unreactive P must be very small compared with total excreted P or retained P, and that this could unlikely be a significant source of error in the measurement. Another possible source of error is the leaching of water-soluble phosphorus from diets before they were ingested by the fish. Although the fish ingested experimental diets very rapidly (in a few seconds), there remained the possibility of leaching losses of highly soluble sources of nutrients, like potassium phosphate that was used as the P source in the diet. According to Murai and Andrews (1975) half of the pantothenic acid was lost in 10 sec and all was lost in 1 min in water from feed pellets having 1mm of diameter. Slinger et al. (1979) also noted that the leaching loss of ascorbic acid was the highest of all of the vitamins and that 15-67% was lost after 10 min in water from 1.18-2.36mm crumbled pellets (steam or extruded). Wilson (1989) also noted that a significant amount of the crystalline amino acids were rapidly leached from the amino acid test diets for channel catfish. Although the sizes of fish and feed pellets used in the present balance trial were much larger than those of the above-mentioned studies, leaching loss could also lead to an overestimation of P retention by fish since they were fed in a separate tank before being transferred to the metabolic tank. Neither the unreactive P, however, nor the rapid leaching would be likely to interfere with the value determined in the dose response trial (experiment 5) because complete collection of metabolic wastes in the dose response trial is not critical. The close agreement of the data obtained in the dose-response trial (experiment 5) and the balance trial (experiment 6) suggests that the two potential sources of error mentioned above were apparently minor.

CONCLUSION

Growth is a major determinant governing the dietary requirements of nutrients, especially for nutrients such as N, P and Ca, which are the major constituents of constructive tissues in the body. Nitrogen retention is tightly linked to protein synthesis and growth, while weight. gain involves a problem introduced by hypertrophy; i.e., fat-gorging adipocytes (Rudman et al., 1975; Mommsen, 1998). Dietary requirements of nutrients can, therefore, be most appropriately expressed on a per nitrogen retention (growth) basis rather than a per weight. gain basis. To determine dietary nutrient requirements, it is necessary to know the potency of the diet (N-gain of fish/diet). For example, when the potency of feed (≈ feed efficiency) is low due to low protein, low energy, amino acid imbalance, low digestibility or

availability of nutrients, a deficiency in some essential nutrients, etc., then the fish will not grow well per amount of diet consumed, and thus will require less P in the diet than do fast growing fish fed high performance feeds. Nutrient requirements in fish should, therefore, be considered on a growth basis rather than on a diet basis, energy basis or daily basis.

Since large fish already have substantial body mass (pool) and also the specific growth rate is low, studying nutrient requirements of large fish by means of conventional approaches, such as weight. gain, retention of specific nutrients, and analyses of soft or hard tissues, requires months of feeding to obtain meaningful data. Also, feeding large fish with purified diets for any extended period is economically impossible. Alteration of enzyme levels does not indicate that fish are “clinically” deficient; more likely it is simply an adaptive response of fish for a different level of intake. When fish receive nutrients in an amount more than they require (above the dietary requirements), the excess must be excreted via feces, urine or gills to avoid potential toxicity. The excretory route differs for different nutrients. Any excess P absorbed from feeds must be excreted (spill) in the urine. This renal response for dietary P is a far more sensitive and rapid indicator than any other response criteria for P. By closely monitoring P concentrations in urine, the requirement level for P (and possibly for some other nutrients) can be estimated within a few days. This rapidity and sensitivity will be particularly useful to research nutrient requirements for large fish at various physiological stages and under various environmental conditions.

Table 1. Materials and methods used in Experiment-1 through 5.

Expt No.	Fish size[1]	Tank	Feeding	Method	Period (days)	Response criteria	Urine sampling	Sampling time[2]	Results
1	258.1±16.0	flow through	fed to satiation	dose response	24	blood ATP etc.[3]	stripping	24 h	Table 3 Fig.1,2
2	390.6±6.5	flow through	force-fed a constant wt.	dose response	7	urinary Pi	stripping	24 h	Fig.3
3	310.6±5.0	metabolic chamber	force-fed a constant wt.	balance	6-11	(balance)	catheter	every 2 h	Fig.4,5
4	136.1±5.4 (S) 349.7±15.5 (L)	flow through	fed to satiation	dose response	6, 7	urinary Pi	stripping	8 h	Fig.6
5	203±0.65	metabolic tank	fed a constant wt.	dose response	12	urinary Pi etc.[4]	water	0-24 h	Table 4,5 Fig.7,8
6	203±0.65 (S) 400±2.88 (L)	metabolic tank	fed a constant wt.	balance	14	(balance)	water	0-24 h	Table 6

[1] Initial mean body wt. ± SEM. S: a group of small fish; L: a group of large fish.
[2] Collection of urine specimens; hours post feeding.
[3] ATP (blood, muscle), PCr (blood, muscle), G6P (blood, muscle), Glu (blood), Pi (blood, muscle, liver, urine, feces), total lipids (plasma, liver), total cholesterol (plasma, liver).
[4] Glycogen (liver), Pi (plasma), total P (feces), Ca (plasma), alkaline phosphatase (feces, plasma), Glu (plasma), total cholesterol (plasma), acetoacetate (plasma).

Table 2. Composition of the diet used in Experiment-1 through 5.

Experiment *No.*	1	2	3	3	3	3	4	5
DIET ID	Basal	Basal	P[1]	PCa[1]	FB[1]	C[1]	Basal	Basal
INGREDIENT	---------- g dry matter----------							
Egg white[2]	35	42	35	35	35	-----	35	10
Wheat gluten[3]	-----	-----	-----	-----	-----	-----	-----	30
Gelatin[4]	10	12	10	10	10	-----	10	-----
Dextrin[5]	14	16	14	14	14	-----	14	20
Fish oil[6]	23	8.5	20	20	20	-----	20	25
Vitamin mix.[7]	4	4.7	3	3	3	-----	3	3
Mineral mix.[8]	2	2.4	1	1	1	-----	1	2
Amino acid mix.[9]	2	2.4	2	2	2	-----	2	-----
α-cellulose[10]	10	12	14[17]	14[17]	14[17]	-----	14[17]	10[18]
KH_2PO_4[11]	*var*[19]	*var*[19]	3.51	3.51	-----	-----	*var*[20]	*var*[20]
$CaCO_3$[12]	-----	-----	-----	4.21	-----	-----	-----	-----
Fish bone (purified)[13]	-----	-----	-----	-----	7	-----	-----	-----
Trace mineral solution[14]	(30)	(30)	(30)	(30)	(30)	-----	(30)	(30)
Total dry matter	100	100	103.51	107.72	107	100	100+*var*	100+*var*
Analytical composition	---------- g/100g, dry basis ----------							
Crude protein	42.8	48.7					40.8	36.1
Crude ash		4.43	7.31	10.04	8.57	9.20		4.43
Acid insoluble ash		0.179	1.04	1.07	1.21	0.29		0.643
Total P	0.101	0.043	0.888	0.831	0.837	1.324	0.126	0.172
Total Ca	0.042	0.060	0.046	1.885	1.938	2.058	0.068	0.056
Ascorbic acid (μg/g dry diet)[15]			611	524	167	184		
DE (kJ/g dry diet)[16]	c 21.25	c 17.78	c 20.08	c 20.08	c 20.08		c 20.08	d 19.25

[1] Abbreviations: P (P added as KH_2PO_4); PCa (P and Ca added as KH_2PO_4 and $CaCO_3$, respectively); FB (P added as "fish bone"); C (Commercial feed).
[2] Egg white spray-dried (ICN Biomedicals, Aurora, OH); autoclaved (130°C-1 h); contained 1.06g P/kg.
[3] Wheat gluten (commercial grade), fortified with L-lysine 3g/100g wheat gluten; contained 2.84g P/kg.
[4] 50 bloom (technical grade, ICN Biomedicals); contained 0.590g P/kg.
[5] 80 % water-soluble, from corn (technical grade, ICN Biomedicals); contained 0.196g P/kg.
[6] Red #3 (fat-soluble) was added at 0.7g/kg dry diet in experiments 1,2,3 and 4, except the commercial diet; the fish oil contained 0.018g P/kg.
[7] Abernathy No.2 formula (Hoffmann-La Roche, Inc.), fortified with D-biotin 6 mg, ascorbic acid 2, inositol 2, choline chloride 5g/kg dry diet (ascorbic acid was replaced with L-ascorbyl-polyphosphate, 15%-active, in Experiments 4 and 5).
[8] KCl: NaCl: $MgSO_4 \cdot 7H_2O$, 2:1:1 ratio by weight.
[9] DL-Methionine: L-Lysine: L-Arginine: L-Threonine, 1:1:1:1 ratio by weight.
[10] Alpha-cellulose powder (Sigma Chemical Co., St. Louis, MO); contained 0.012g P/kg..
[11] Anhydrous, 99.0% min P-5379 (Sigma Chemical).
[12] Precipitated powder, U.S.P. (Merck & Co., Inc., Rahway, NJ).
[13] From whitefish, ground, purified by repeated washing.
[14] Supplied the following (mg/kg dry diet); KI, 1.9; $MnSO_4 \cdot H_2O$, 40; $ZnSO_4 \cdot H_2O$, 85; Na_2SeO_3, 0.9; $CoCl_3 \cdot 6H_2O$, 4; $CuSO_4 \cdot 5H_2O$, 12; $FeSO_4 \cdot 7H_2O$, 300.
[15] Determined on the remaining diets (after feeding trial),.
[16] Digestible energy (c: calculated values; d: determined values).
[17] Contained SiO_2 (100 mesh, Mallinckrodt Chemical Works, St. Louis, MO) at 1g/100g dry diet
[18] Contained SiO_2 at 0.5g and Cr_2O_3 (Fisher Scientific, Fair Lawn, NJ) at 0.5g/100g dry diet.
[19] Incremental levels of KH_2PO_4 (Sigma Chemical) were added to the basal diet replacing alpha-cellulose.
[20] Incremental levels of KH_2PO_4 were added to the basal diet but not replacing alpha-cellulose.

Table 3. Physiological parameters of rainbow trout fed various levels of phosphorus for 24 days (Experiment 1).[1]

		Dietary P[2]					Significance						
		0.10	0.32	0.52	0.76	1.07	ANOVA P[5]	Regression-1 P[6]			Regression-2 P[7]		
								L	Q	C	L	Q	C
G6P	µmol/g blood[3]	0.037	0.045	0.033	0.047	0.034							
	µmol/g muscle[3]	2.32[bc]	2.23[bc]	1.99[b]	2.70[ac]	1.92[b]	*			**		**	**
ATP	µmol/g blood[3]	1.77	2.13	2.04	2.10	2.11					**	***	***
	µmol/g muscle[3]	6.89[b]	7.71[bc]	8.15[ac]	7.88[bc]	7.69[bc]	*		**	**	**	**	**
PCr	µmol/g blood[3]	0.069	0.159	0.096	0.084	0.071							
	µmol/g muscle[3]	23.1	23.4	23.4	24.7	25.9		*	*	*			*
Glu	µmol/g blood[3]	4.15	3.37	3.92	3.12	3.41						**	***
Pi	µg/g blood[3]	28.4[a]	57.7[b]	71.8[b]	70.7[b]	67.9[b]	***	***	***	***	NA	NA	NA
	µg/g muscle[3]	1052[a]	1161[b]	1193[b]	1172[b]	1171[b]	**	*	***	***	**	**	***
	µg/g liver cytosol	607	684	718	671	656			*	*	**	***	***
	µg/g urine[4]	4.3[a]	14.4[a]	89.9[b]	100.1[b]	114.8[b]	**	***	***	***	**	**	**
	µg/g dry feces[4]	720[a]	1105[ab]	999[ab]	909[ab]	2084[b]	*	*	**	**			
TL	g/L plasma	21.5	19.0	18.3	18.4	16.0							
	g/100g liver	5.09	5.01	5.36	4.88	5.52							
TC	g/L plasma	6.47	5.61	5.91	5.89	4.93							
	g/100g liver	0.695	0.739	0.841	0.738	0.837				*			
TC/TL	plasma	30.2	30.6	32.4	32.3	31.5							
	liver	14.8	14.8	15.7	15.2	15.2							
Water	g/100g liver	79.1	78.2	79.1	79.7	80.1							

[1] Abbreviations; G6P, glucose-6-phosphate; ATP, adenosine triphosphate; PCr, creatine phosphate; Glu, glucose; Pi, inorganic phosphorus; TL, total lipids; TC, total cholesterol; TC/TL, percentages of total cholesterol/total lipids. All samples were collected 12 h after feeding. Values in rows with uncommon letters are significantly different (P< 0.05) by Newman-Keuls multiple comparison test, n=6 fish/treatment.

[2] Total phosphorus content g/100g dry diets (analytical values).

[3] Determined on the perchloric acid extract of whole blood or skeletal muscle.

[4] Each value for urine and feces is the average of 6 fish × 5 consecutive days (day 20-24).

[5] Single-factor ANOVA, * P< 0.05; ** P< 0.01; *** P< 0.001 (n=6 fish/treatment).

[6] Regression against dietary P levels (L; linear effect, Q; quadratic effect, C; cubic effect). * P< 0.05; ** P< 0.01; *** P< 0.001 (n=6 fish/treatment).

[7] Regression against blood P levels (L; linear effect, Q; quadratic effect, C; cubic effect). * P< 0.05; ** P< 0.01; *** P< 0.001 (n=30 fish).

Table 4. Physiological parameters of rainbow trout fed phosphorus gradient diets for 12 days (Experiment 5).[1]

		Dietary P[2]									Significance			
		0.15	0.32	0.45	0.59	0.71	0.85	0.95	1.08	1.28	ANOVA P[6]	Regression P[6]		
												L	Q	C
Gly	g/100g liver	9.78	6.11	7.27	8.78	4.54	4.44	5.19	5.37	6.14		*	*	**
Pi	mg/L plasma	91	131	143	167	169	182	192	187	177	***	***	***	***
Ca	mg/L plasma	101	98	104	109	104	109	109	109	111	*	***	***	***
ALP[3]	IU/g feces[4]	2.26	2.63	2.60	3.95	2.56	3.15	2.89	2.68	2.18				
ALP[3]	IU/L plasma	35.5	32.0	43.6	58.8	28.7	47.2	43.4	71.4	48.2	*	*	*	*
TC	mg/L plasma	2857	2694	3018	3487	3055	3211	3210	3674	4011	*	***	***	***
Glu	mmol/L plasma	5.50	4.70	4.60	4.92	4.62	4.84	5.90	5.08	6.33				**
AcAc	mmol/L plasma	tr.[5]	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.				
Total P	μg/g feces[4]	925	1078	1172	1294	1304	1421	2006	1634	2993		**	**	***

[1] All samples were collected 24 h after feeding. Each value represents the average of 5 randomly sampled fish except for the fecal P which was determined on a pooled sample of 13 fish/treatment. Abbreviations: Gly, glycogen; Pi, inorganic phosphorus; ALP, alkaline phosphatase; TC, total cholesterol; Glu, glucose; AcAc, acetoacetate.

[2] Available phosphorus (g/100g dry diet; analytical values).

[3] Incubation temperature 15°C.

[4] Dry basis.

[5] Not detected (< 0.1mmol/L).

[6] Single factor ANOVA, Regression against dietary P levels (L; linear effect, Q; quadratic effect, C; cubic effect). * P< 0.05; ** P< 0.01; *** P< 0.001.

Table 5. Requirement of available phosphorus (Experiment 5).

Criteria	g/ g-N gain	g/100g dry diet[1]	mg/ kJ-DE[2]
Non-fecal excretion[3]	0.277	0.686	0.357
Retention rate[4]	0.237	0.585	0.304
Balance plateau (Y)[5]	0.277	0.685	0.356
95%-plateau (X)[6]	0.293	0.724	0.376
Plasma saturation[7]	0.312	0.772	0.401

[1] The value should differ from feed to feed depending on the potency of the feed. With the present diet, fish retained 2.47±0.04 g N (mean±SEM, n=8) per 100g dry diet consumed except for those that received the lowest P diet, which had lower N retention. Estimated g wt gain of fish per 100g dry diet consumed (feed efficiency) was 92.4±1.40 except for the lowest P group that had a lower value.
[2] Digestible energy; determined from the gross energy content in feeds and feces.
[3] Based on the P concentration in the tank water. The value indicates the X-intercept of the linear regression of "floating" points (n=5). An average of day 3,6,9 and 12 (See also Fig. 7).
[4] The maximum efficiency of net retention (percentage of intake). The retention was estimated as Intake–Fecal loss–Nonfecal loss. An average of day 3,6,9 and 12 (See also Fig. 8).
[5] The maximum net retention (the plateau value, Y-axis). The plateau value was determined by sigmoidal dose-response curve on the assumption that there were a slope and a plateau in response to the dietary P concentration. The retention was estimated as Intake–Fecal loss–Nonfecal loss. An average of day 3,6,9 and 12.
[6] The concentrations of dietary P (X-axis) to achieve 95% of the maximum retention (the balance plateau). An average of day 3,6,9 and 12.
[7] The concentrations of dietary P to achieve 95% of the plasma saturation.

Table 6. Requirement of available phosphorus (Experiment 6).[1]

Size	g /g-N gain	g/100g dry diet[2]	mg/ kJ-DE[4]
Large fish (400g BW)	0.284	0.692	0.383
Small fish (200g BW)	0.278	0.793	0.421
P value[3]	0.830	0.022	0.051

[1] Estimated by balance (retention = intake–fecal loss–nonfecal loss).
[2] This value should differ from feed to feed depending on the potency of the feed. On the commercial diet fed in this experiment, fish retained 2.44g N (large fish) and 2.89g N (small fish) per 100g dry feed consumed; i.e., the feed efficiency (g wt gain of fish per 100g dry feed consumed) was 91.3 (large fish) and 107.5 (small fish).
[3] Probability values between large fish and small fish by two-tailed t-test, n=3 days.
[4] Digestible energy; determined from the gross energy content in feeds and feces.

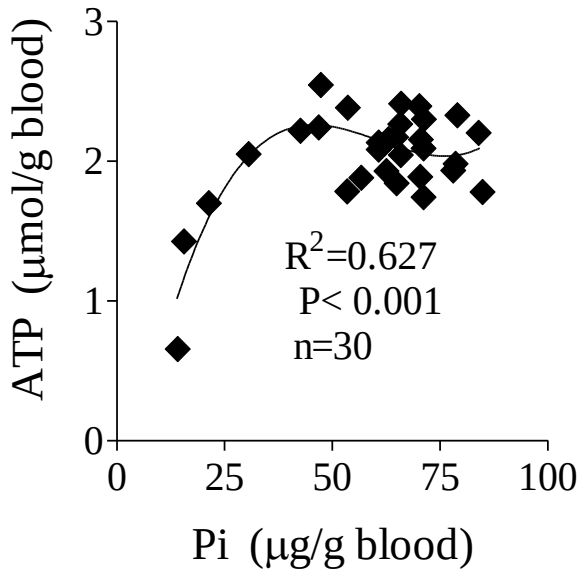


Figure 1. Correlation between inorganic phosphorus (Pi) and ATP levels in the blood of rainbow trout (Experiment 1). Fish were fed diets of varied P content for 24 days. The regression line was $Y=0.0000160X^3-0.00292X^2+0.166X-0.780$.

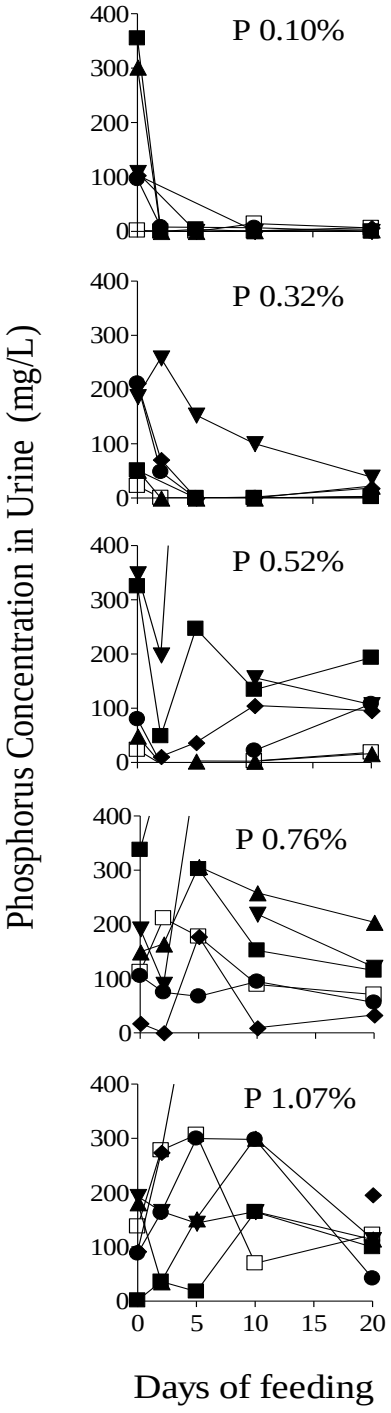


Figure 2. Change of urinary P levels over time (Experiment 1). Each line shows the change of urinary P concentrations of each fish over the 20 day feeding period with diets of varied P content. Fish were fed as much as they would consume once daily, and the urine samples were collected ca. 24 h post feeding by stripping.

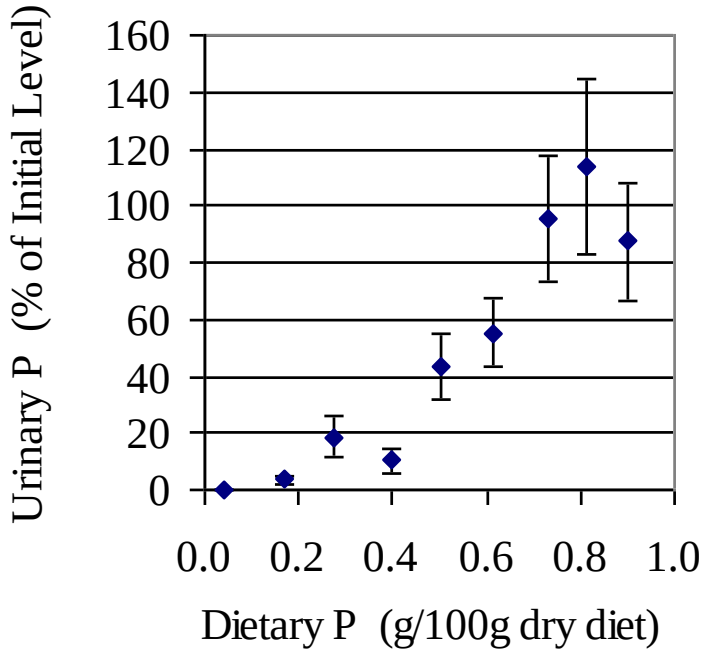


Figure 3. Urinary P levels at various dietary P intakes (Experiment 2). Each point represents the mean±SEM of 3-6 fish. Values were expressed as % of initial levels (urinary P mg/L at day 5 and 7 vs. at day "-1" and 0, determined for each fish). Each fish was force-fed a constant weight of feed (0.9g dry diet/100g BW) once daily, and the urine samples were collected 24 h post feeding by stripping.

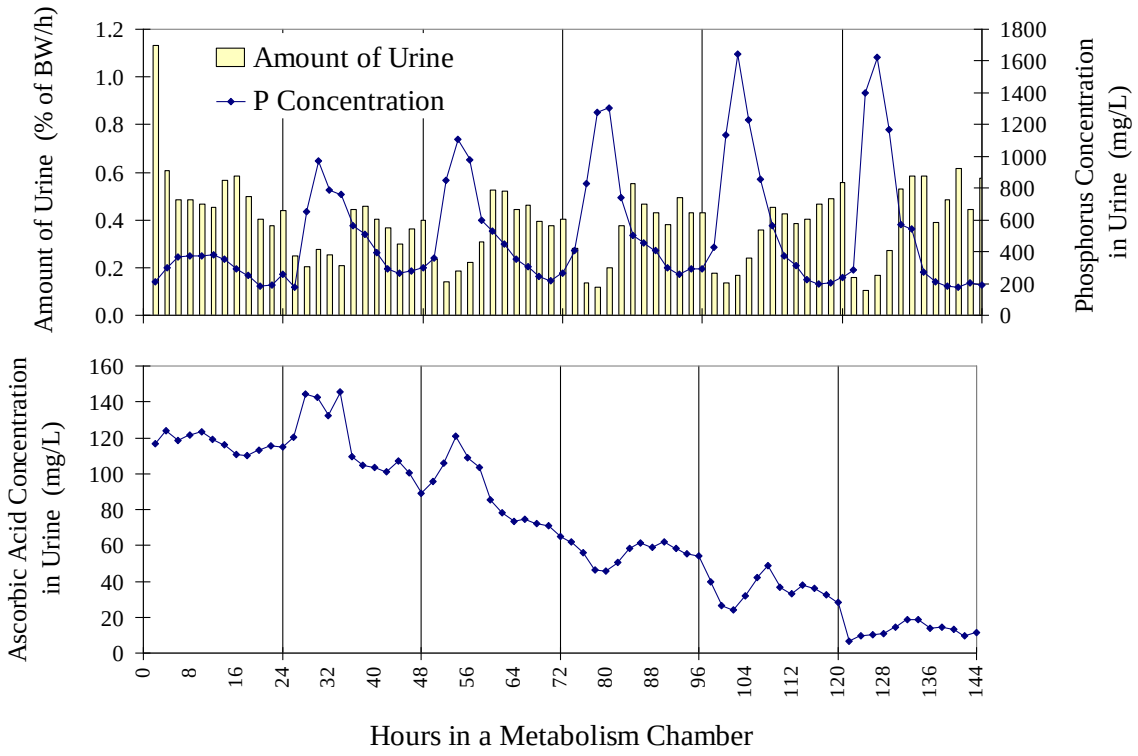


Figure 4. Typical pattern of urinary excretion of phosphorus and ascorbic acid in rainbow trout fed test diets in a metabolism chamber (Experiment 3). Fish (BW 366.0g) was force-fed and placed in the metabolism chamber at 0 h, and force-fed every 24 h thereafter (indicated by vertical lines). Dietary treatment was PCa (Table 2). Urinary concentrations of P peaked between 6 and 12 h post feeding in P and PCa groups (n=8 fish). There was no distinct peak (flat) in FB and C groups with much lower urinary P levels (n=10 fish; data not shown). Urinary ascorbic acid levels declined in 2-5 days to a base-line level in all fish studied (in all dietary treatments). See also Figures 45-50.

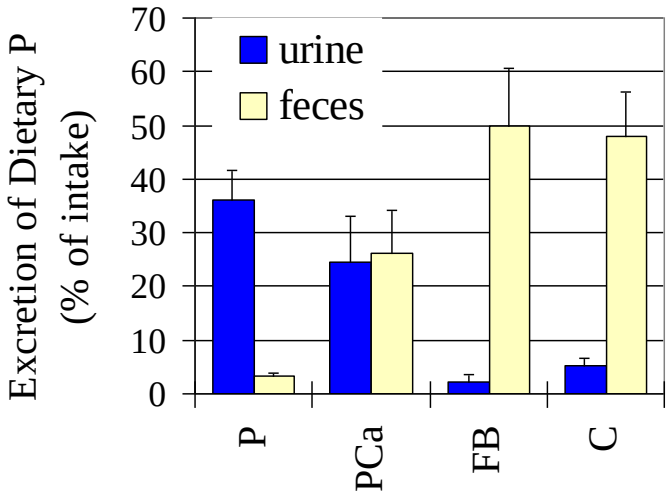


Figure 5. Excretion of dietary phosphorus into urine and feces (percentages of dietary intake; Experiment 3). Diet abbreviations (X axis), P: P (as KH_2PO_4) was added to the basal diet; PCa: P (as KH_2PO_4) and Ca (as $CaCO_3$) were added to the basal diet; FB: Fish bone was added to the basal diet; C: Commercial feed (see Table 2 for diet compositions). Each bar represents the mean±SEM (n=4-5 fish).

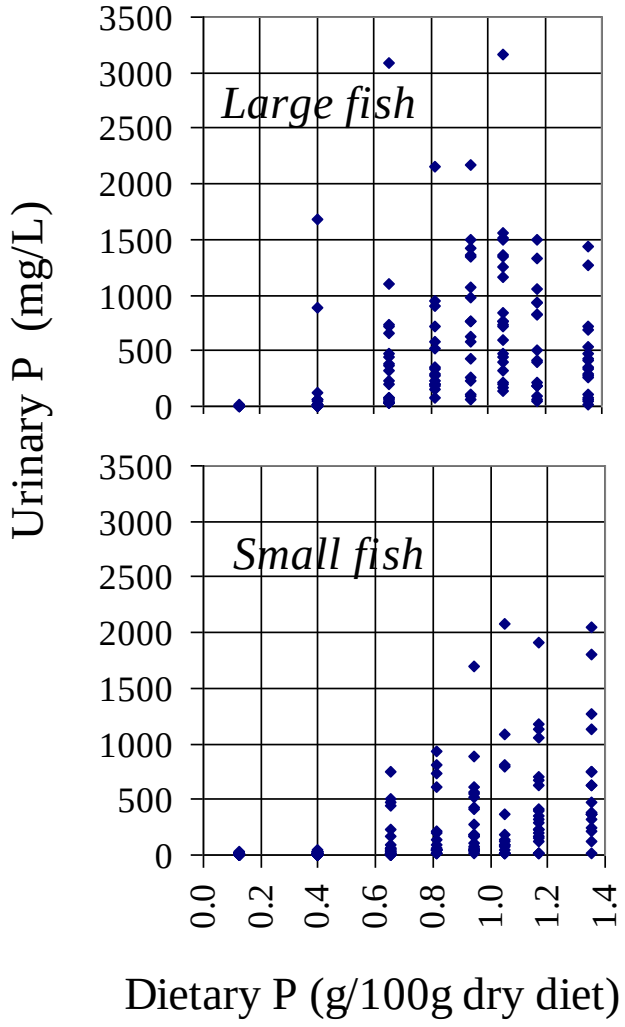


Figure 6. Urinary P levels in small and large trout (Experiment 4). Fish were fed as much as they would consume once daily. Urine samples were collected 8 h post feeding by stripping at day-7 and 8. Each point represents one sample (n=6-22/treatment for large fish; n=16-24/treatment for small fish; up to 2 samples/fish). Urine samples containing less than 5 mg/L ascorbic acid were excluded.

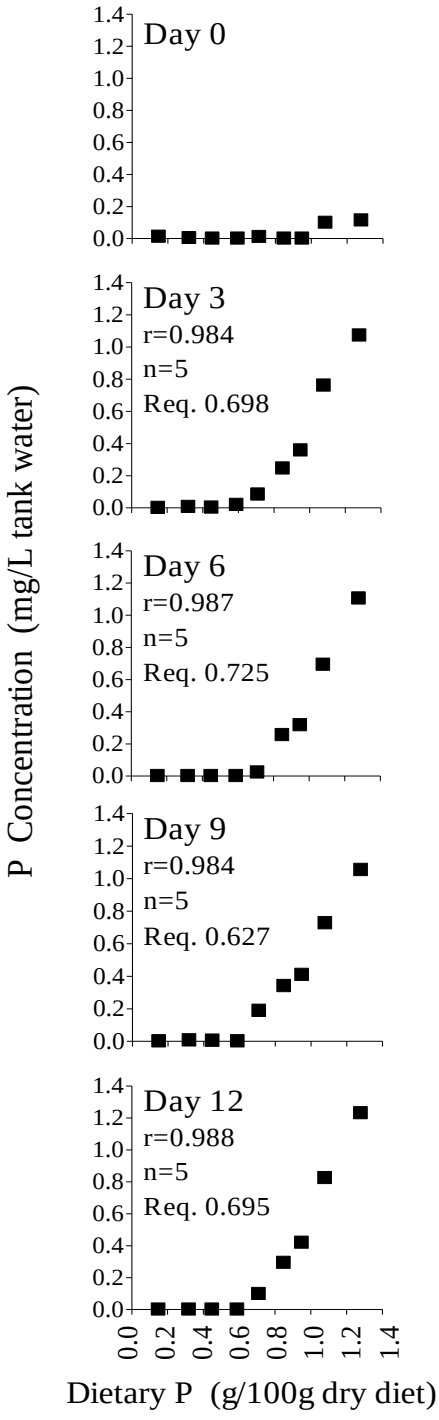


Figure 7. Non-fecal excretion of P in metabolic tanks (Experiment 5). Values (Y axis) were expressed as mg Pi/L water in the tank in which 13 fish were confined for 24 h without any external water supply. The linear regression lines, determined based on "floating" points, were Y=1.838X-1.283 (d3), Y=1.936X-1.403 (d6), Y=1.575X-0.987 (d9) and Y=2.071X-1.440 (d12).

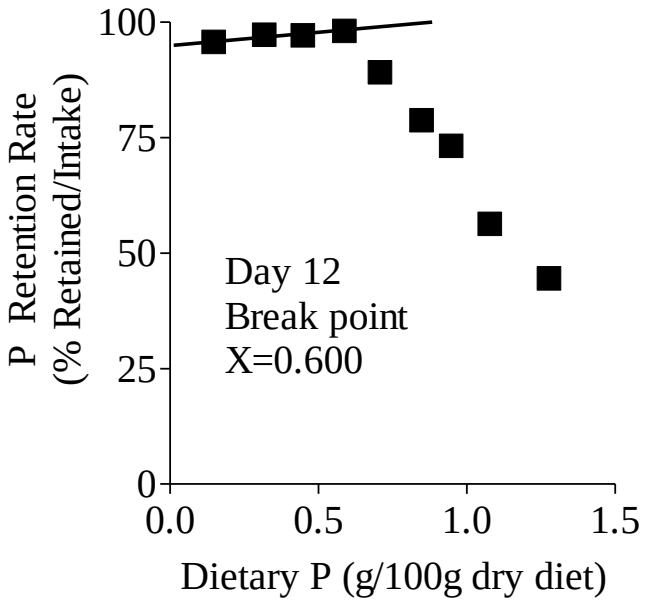


Figure 8. Retention of dietary P (percentages of intake) at various dietary P concentrations (Experiment 5). Retention rate (Y axis)= (P fed–nonfecal P–fecal P)×100/ P fed. Break points for the day 3, 6 and 9 were 0.541, 0.653 and 0.548, respectively. See also Figure 36.

Chapter II: Determination of Protein Digestibility and Mineral Availability

1. Availability of Nutrients in Common Ingredients in Salmonid Feeds

ABSTRACT Apparent digestibility of protein and availability of minerals (Ca, K, P, Mg, Na, Cu, Fe, Mn, Sr, Zn) in various feed ingredients were determined for coho salmon and rainbow trout using yttrium oxide (Y_2O_3) as the inert marker and passive feces collection tanks. The feed ingredients were herring meal, menhaden meal, anchovy meal, deboned whitefish meal, poultry by-product meal, feather meal, soybean meal, corn gluten meal, wheat gluten meal, wheat middling and wheat flour. Apparent digestibility (%) of protein and availability (%) of minerals were determined as a fractional net absorption of nutrients from diets. Apparent digestibility of protein and availability of K was high (> 80%) in all feed ingredients, whereas digestibility of dry matter and availability of Ca, Cu, Fe, Mg, Mn, Na, P, Sr and Zn were variable among ingredients. Apparent availability (%) of mineral elements was not significantly correlated to the amount of nutrient intake (μg nutrient/g BW/day) in any test diet. Net nutrient absorption (μg/g BW/day) was positively correlated ($P<0.05$) to the nutrient intake except for Mn, Fe and Ca. Fecal nutrient losses (μg/g BW/day) were positively correlated ($P<0.05$) to nutrient intake except for protein, Na, K and Zn.

BACKGROUND

The ultimate source of waste in aquaculture systems is feed. Nutrients that are not retained by the fish are excreted into the water and discharged in effluents from the aquaculture system. Macronutrients, especially phosphate and nitrogen, stimulate eutrophication of the aquatic environment, whereas trace elements may be of concern because of trophic accumulation through food chains.

Ketola and Harland (1993) reported that the retention of phosphorus (P) from several experimental diets and one commercial salmon diet ranged from 14 to 22%, meaning that approximately 80% of dietary P may have been discharged into the water in soluble and fecal forms. It has been shown that bioavailability of nutrients differs markedly in different ingredients and in different diets (Lall, 1989). Consequently, management of aquaculture wastes can be approached through improvements in nutrient utilization and diet formulation. Although some investigators have studied the availability of P from specific feed sources using salmonid fishes (Ogino et al., 1979; Riche and Brown, 1996), currently available data for common feed ingredients are limited. Because of the increasing concern for the environmental effects of the expansion of aquaculture worldwide, further research in this area is essential.

Availability of many trace elements varies among different sources and ingredients (Hilton, 1989). To avoid potential deficiency problems, practical feed formulations are supplemented with inorganic minerals known to be available to fish; however, many compounds in feed ingredients further

reduce availability of those inorganic minerals by direct and indirect interactions. Mineral availability in common feed ingredients and the potential interactions of these ingredients with added inorganic mineral sources have not been well researched. To avoid excessive use of inorganic minerals in practical rations, availability of intrinsic minerals in feed ingredients and their potential antagonistic properties need to be understood.

The purpose of this research was to determine digestibility of protein and availability of specific minerals with particular attention to phosphorus in common feed ingredients for aquatic feeds. The use of this information in formulating feeds will help to minimize excess levels of available nutrients in diets without having a risk of clinical deficiency problems, and thereby to reduce the excretion of nutrients into aquaculture effluents.

MATERIALS AND METHODS

Diet preparation

Feed ingredients tested for apparent digestibility of protein and availability of minerals were herring meal (5-02-000); anchovy meal (5-01-985); menhaden meal (high quality, prime, 5-02-009); deboned whitefish meal; poultry by-product meal (5-03-798); feather meal (5-03-795); soybean meal (48% crude protein, dehulled, solvent extracted, 5-04-612); wheat gluten meal (5-05-221); corn gluten meal (5-28-242); wheat middling (4-05-205) and wheat flour (4-05-199). Test diets were prepared by mixing a basal diet (Table 7) and one of the test ingredients (Table 8) at the ratio of 7 to 3 on an as-is basis as described by Cho and Slinger (1979). This method provided several advantages in that the difference of nutrient content among feed ingredients would be reduced by the 70% inclusion rate of the basal diet for all test diets, which is helpful since biological adaptation to a different level of intake interferes with estimating actual availability of minerals. Also, the interaction (antagonistic or synergistic) property of test ingredients for the absorption of dietary minerals other than those contained in the ingredient could be determined. If calculated digestibility values are lower than 0 or higher than 100, the implicit assumption of this method; i.e., the digestibilities (absorption) of nutrients from the basal diet is constant or unaffected by the test ingredients, can not be justified, and the alternative hypothesis "interaction is present" has to be taken. The importance of knowing this interactive property of feed ingredients can not be over emphasized in practical feed formulations. The basal diet was formulated with purified ingredients rather than practical ingredients to minimize unknown variables, e.g. composition, contamination, property and quality factors. The basal diet also contained extrinsic inorganic mineral supplements since the diet should be nutritionally complete by itself. Yttrium oxide (Y_2O_3) was used as the inert marker at a concentration of 0.05% in dry diets. The use of yttrium in nutrient absorption studies, as an inert nonabsorbable dietary marker, has been studied previously in rats (Marcus and Lengemann, 1962), chickens (Hurwitz and Bar, 1965, 1966; Sklan et al., 1975) and fish (T.

Storebakken, personal communication, 1996). All diets were prepared as cold extrusion pellets containing approximately 30% moisture and stored at –20°C until fed.

Feeding and feces collection

Coho salmon (Oncorhynchus kisutch), with initial body weight 110.7 ± 3.5g (mean ± s.e.m., *n*=156), were stocked into 12 150-L tanks, each of which had a specifically designed feces collection system (modification of Hajen et al., 1993). The system permitted the collection of feces into a long narrow collection tube within a minute of evacuation by the fish. Even though soluble components in the feces might have leached into the unstirred water column (in the tube), they were separated from the tank water. Each tank was supplied with a continuous flow of dechlorinated municipal water at 3 L/min/tank. Water temperature ranged from 12.5 to 15.0°C during the six week experiment. Fish were fed once daily at 1800 h as much as they would consume. After seven days of acclimatization during which time the fish received the test diets, fecal samples were collected from the collection tube twice daily at 0800 and 1730 h for five consecutive days. The acclimation period to each test diet was minimized to reduce any adaptive response of fish. Fecal samples of each treatment were analyzed separately for each day of collection. The acclimation (week-1) and fecal collection (week-2) process was repeated three times to obtain triplicate measurements per treatment (diet). Experimental diets were randomly assigned to the fish tanks, and randomly re-assigned for each following round. The fish stayed in same tanks and were not rearranged during the experiment.

Small or large rainbow trout (Oncorhynchus mykiss), with respective initial body weight 42.5 ± 1.58g (mean ± s.e.m., *n*=348) or 170.3 ± 2.54g (mean ± s.e.m., *n*=60) were stocked into twelve 150-L aquaria used for the coho salmon trial. Each tank was supplied with biofiltered water at the rate of 3 L/min from a common recirculation unit with de-chlorinated municipal water added to the recirculation system at 3 L/min to maintain optimum water quality and to avoid accumulation of nutrients in the recirculating water. Water temperature was maintained at 17.0 ± 1.0°C during the 10 week-feeding period. Because significant effects of diet history were observed in the coho salmon trial, all fish in the rainbow trout trial were removed from experimental tanks, pooled, and randomly re-distributed to the tanks for subsequent rounds. Fecal samples were collected after a one week-acclimation period to each test diet, and the five days of fecal samples were pooled and analyzed by week. This process was repeated two times (for small trout) or three times (for large trout) to obtain duplicate and triplicate measurements per treatment, respectively. Other conditions were the same as those in the coho salmon trial described above. Fish were handled in accordance with the guidelines approved by the Animal Care Committee of the University of Washington.

Analysis and calculations

Fecal solution (column water containing feces) were dried in a convection oven at 105°C for 12 h. Dried feces and diets were weighed, finely ground using an electric grinder, and analyzed for crude protein by the standard micro Kjeldahl method (AOAC, 1990) using a semi-automated system (Buchi Kjeldahl/Nitrogen Analyzing System, Brinkmann Instruments, Inc., Westbury, NY). A small portion of dried, ground feces were ashed at 550°C for 12h. in a muffle furnace, dissolved in a concentrated acid mixture (nitric and hydrochloric acid, 1:1), diluted to an appropriate concentration, and analyzed for Ca, K, Mg, Na, P, Cu, Fe, Mn, Sr, Zn and Y by an inductively coupled plasma emission spectrophotometer (Jarrell-Ash Plasma Atom Comp., Waltham, MA).

Although ingredients were weighed and mixed with the basal diet on an as-is basis at a 3:7 ratio, all analyses and calculations were made on a dry basis. Because there were no significant differences in the measured apparent digestibility or availability between small rainbow trout and large trout, the data were pooled. Daily feeding rate at the apparent satiation level was estimated from the amount of feces that was collected nearly completely and from the change of indicator concentration in the diets and feces (dry matter digestibility). Net nutrient intake (μg nutrient intake/g BW/day) was calculated based on feeding rate and dietary concentration of the nutrient. Mean digestibility or availability value of each ingredient was subjected to a single factor ANOVA followed by Newman-Keuls multiple comparison test. Mean digestibility or availability value of each ingredient from different weeks were compared based on the data of five consecutive days per week by a single factor ANOVA to determine possible effect of previous diet history of fish (in coho salmon only). Linear correlations were determined between dietary concentrations of nutrients and the nutrient digestibility or availability of the diet. Diets containing test ingredients of animal origin and diets containing test ingredients of plant origin were compared separately. Net absorption of each nutrient was compared between coho salmon and rainbow trout by a paired t-test. Outliers were detected by Dixon's method and excluded at the 5% level (Snedecor and Cochran, 1980). Statistical analyses were performed using a computer program (GraphPad Prism, version 2.01, GraphPad Software, Inc., San Diego, CA). Treatment effects were considered significant at $P<0.05$.

Apparent digestibility (%) for protein and availability (%) for minerals in test and basal diets were expressed as a fractional net absorption of nutrients from diets. Then, apparent digestibility (%) for protein and apparent availability (%) for minerals in test ingredients were calculated using the following formula.

$$AD_{Ing} = (Nutr_{TD} \times AD_{TD} - 0.7 \times Nutr_{BD} \times AD_{BD}) / (0.3 \times Nutr_{Ing})$$

AD_{Ing}: Apparent digestibility (or availability) of nutrients in test ingredient
$Nutr_{TD}$: Nutrient concentration in test diet
AD_{TD}: Apparent digestibility (or availability) of nutrients in test diet
$Nutr_{BD}$: Nutrient concentration in the basal diet

AD_{BD}: Apparent digestibility (or availability) of nutrients in the basal diet

$Nutr_{Ing}$: Nutrient concentration in test ingredient

Note: $0.3 \times Nutr_{Ing}$ (in denominator) = $Nutr_{TD} - 0.7 \times Nutr_{BD.}$ The actual ratio of test ingredients to the basal diet deviates from 0.3:0.7 that is given in the formula because of the varied moisture content in each ingredient and the basal diet (Test ingredients and the basal diet were mixed on an as-is basis). See also p 59 and p 239.

RESULTS AND DISCUSSION

Apparent digestibility of dry matter and protein

Apparent digestibility of dry matter was highly variable among feed ingredients (Table 9). Among plant sources, apparent digestibility of dry matter was significantly correlated (r=0.99; P<0.01) with dietary protein levels, whereas the correlation was lower among animal sources (r=0.58). The high correlation of the plant sources means that fish cannot efficiently utilize non-protein components in plant feedstuffs, which are mainly starch and the fiber fraction. The diets and ingredients used in this study, however, were not heated during feed manufacturing and starch in plant ingredients was presumably "raw" and not highly digestible. In commercial extrusion pellets, this portion will be more digestible to fish and may increase dry matter digestibility of high-carbohydrate ingredients.

Apparent digestibility (%) of protein was high in all ingredients compared to dry matter and minerals (Table 10). Feather meal and menhaden meal had the lowest protein digestibilities. There was no significant correlation between dietary protein levels (%) or protein intake (% protein/BW/day) and the apparent digestibility (%) of protein. Consequently, protein intake (% protein/BW/day) was highly correlated to the net utilization of protein (% protein/BW/day) (Table 21).

Values for apparent digestibility of protein obtained in this study were higher than previously reported with rainbow trout (Cho and Slinger, 1979; Smith et al., 1980; Smith et al., 1995; Watanabe et al., 1996). Higher values obtained in this study may be attributed to the leaching of fecal soluble nitrogen compounds, which is always associated with the settling feces collection method, the extent depending on the efficiency of the collection method and nutrient solubility. Also, the consistency of feces collected in this study differed among different dietary treatments, which might alter the rate of leaching. The degree of errors associated with these variables remains to be studied. The collection of feces by stripping, however, encounters different problems such as collecting "incompletely" digested materials and contamination of feces with brownish intestinal fluid and urine which leads to erroneously low digestibility values. Higher digestibility values may also be attributed to the dietary marker used in this study (yttrium oxide), which is apparently less absorbable than chromium oxide by fish (unpublished data) and thus yields slightly higher values than the previously determined values based on chromium.

Apparent availability of calcium

Apparent availability (%) of calcium (Ca) was generally low and variable among ingredients. Both content and the apparent availability of Ca were generally lower in plant sources than in animal sources, except for wheat gluten meal (Table 11). Unlike many other minerals, increasing the intake of Ca (μg Ca/g BW/day) did not increase net absorption (μg Ca/g BW/day) of this element (Table 21), and fecal excretion of Ca was affected mostly by the amount of intake rather than by the availability, suggesting that absorption of Ca was regulated or that Ca precipitated as calcium phosphate in the intestinal lumen. This indicates that the apparent availability of Ca determined by the present balance technique may not be correctly estimating the actual biological availability of Ca in the test ingredients.

In terrestrial animals, as the body's demand for Ca increases, the process commonly termed adaptation is activated in which the synthesis of 1,25 $(OH)_2D_3$ from its precursor is increased, resulting in an increased rate of Ca absorption (Irving, 1973; Wasserman and Fullmer, 1989; Nemere and Norman, 1991). This mechanism is consistent with the present observation of a negative correlation between dietary Ca levels and the net absorption of Ca (%) among diets containing animal ingredient sources (r=-0.56 to -0.80). Calcium in plant sources, the level of which was much lower than that in animal sources, however, was apparently less absorbed, suggesting other effects such as endogenous loss (Carroll and Oh, 1989) or the presence of phytate, fiber, and other ligands in plant ingredients which reduce availability (Champagne, 1989). Mean availability values of Ca in the coho salmon trial were different at different weeks (ANOVA P<0.05) in five dietary treatments, which suggests that the diet history of fish may influence net absorption of Ca. Walker et al. (1948) noted that Ca balance became negative for several weeks when human subjects were put on a low Ca diet after having received a high Ca diet, indicating the significant effect of diet history and slow adaptation to different dietary levels.

The levels of Ca (also, of ash and P) in diets of animal sources showed inverse correlations with net absorption (%) of Ca, Fe, Mg, Mn, P, Sr and Zn, indicating possible antagonistic interactions of Ca with these minerals. Reducing the bone content of fish meals as studied by Babbitt et al. (1994) appears to be a rational procedure for increasing availability of dietary minerals in fish meal-based feeds.

Apparent availability of phosphorus

Apparent availability of phosphorus (P) ranged from 36.5 to 75.4% in animal ingredients and from 8.5 to 74.7% in plant ingredients (Table 12). Both coho salmon and rainbow trout had similar values for the apparent availability of P; however, they were not highly consistent with previously reported values with rainbow trout (Riche and Brown, 1996) or Atlantic salmon (Lall, 1991), falling between those reported values.

P availability values of ingredients from animal sources were inversely correlated with dietary levels of ash (r=-0.94; P<0.01, and r=-0.86; P<0.05 in coho salmon and rainbow trout, respectively), Ca (r=-0.98; P<0.01, and r=-0.94; P<0.01) and with P itself (r=-0.96; P<0.01, and r=-0.90; P<0.01). This indicates that high dietary ash, Ca or P levels in animal ingredients not only increase fecal excretion of P

proportionally, but also increase it further by reducing its fractional absorption. Also, the amount of P intake (μg P/g BW/day) was inversely correlated to the fractional net absorption or apparent availability of P (Table 21). Intestinal absorption of P is normally unregulated; *i.e.*, net absorption is directly proportional to the amount ingested, and homeostasis of P depends primarily on mechanisms that govern renal excretion (Podoliak and Smigielski, 1971; Wilkinson, 1976; Nakamura, 1982; Dennis, 1992). Reduced absorption of dietary P in diets containing animal sources is presumably due to antagonistic interactions between P and Ca or formation of precipitates of calcium phosphates in the intestinal lumen. Availability of P has been shown to decrease as the dietary level of Ca increases in rats (Hoek et al., 1988), pigs (Fox and Care, 1978a), chicks (Fox and Care, 1978b; Al-Masri, 1995), rainbow trout (Porn-Ngam et al., 1993), and in carp (Nakamura, 1982).

Apparent absorption of P at different weeks was significantly different in diets containing corn gluten meal and in the casein basal diet although the differences were small (71, 75 and 75% in the corn gluten diet, and 87, 92 and 94% in the casein basal diet for the average of week-2, -4 and -6, respectively), indicating a minor effect of diet history. Apparent availability of P and some trace elements in wheat middlings and wheat flour was high compared with that of soybean meal or corn gluten meal, which might not be expected had the diet been prepared as extrusion pellets and endogenous phytase inactivated. Although the differences were small, coho salmon absorbed significantly more P than rainbow trout ($P<0.01$) in most dietary treatments, suggesting a species difference in ability to utilize phosphates in feed ingredients.

Apparent availability of magnesium

Apparent availability of Mg in feed ingredients ranged between 0 and 100%, but most values were between 50 and 70% (95% CI) (Table 13). In both coho salmon and rainbow trout, apparent availability of Mg showed a strong inverse correlation with dietary levels of ash (r=-0.93 and -0.96; $P<0.01$), Ca (r=-0.99; $P<0.01$), and P (r=-0.90 and -0.96; $P<0.01$) among diets containing animal ingredients (n=7) and with P (r=-0.87; $P>0.05$ and r=-0.99; $P<0.01$) in diets containing plant ingredients (n=5). Studies with guinea pigs and rats (O'Dell et al., 1960; McAleese and Forbes, 1961; Toothill, 1963), chicks (Nugura and Edwards, 1963), and humans (Alcock and MacIntyre, 1962; Heaton et al., 1964) have shown that when dietary Ca or P levels or both were raised, dietary Mg absorption decreased. These results agree with the present observations of negative correlations between Mg absorption and dietary ash, Ca or P contents. Knox et al. (1981) did not find any increase in the Mg requirement in rainbow trout when the dietary levels of Ca or P were increased.

Absorption of Mg in mammals is influenced by the load presented to the intestinal mucosa. On a low-Mg diet (1-2 m mol/day) approximately 80% was absorbed, whereas it decreased to 25% on a high-Mg diet (25 m mol/day) (Aldor and Moore, 1970; Aikawa, 1976). Accordingly, intestinal absorption of Mg is controlled by factors that respond to dietary Mg content (Quamme and Dirks, 1994).

This agrees with the inverse correlation (r=-0.61 to -0.89) between dietary Mg levels and its apparent availability observed in this study. Also, the amount of intake (μg Mg/g BW/day) was inversely correlated with apparent availability (%) of Mg (Table 21).

Apparent availability of magnesium in wheat gluten and corn gluten meals had odd values. Both of these ingredients contained much less Mg than the other ingredients or the casein basal diet. When the concentration of the nutrient of interest in a test ingredient is low relative to that in the basal (reference) diet, calculated apparent availability values may have higher experimental error than when the test ingredient has a high level of the nutrient. Any variation of the values in test diets will result in a larger deviation of the calculated value of test ingredient, depending on the amount of the nutrient contributed by the test ingredient. Moreover, if the test ingredient contains compounds that reduce mineral availability or absorption, e.g., calcium and phytate, bioavailability of minerals from the basal diet portion of the test diet could be reduced and/or re-absorption of endogenous trace elements secreted into the gastrointestinal tract (primarily via bile) could also be reduced. This can lead to the calculated mineral availability of the test ingredient being falsely low, below the "theoretical minimum," or having negative apparent availability, a pattern that was more common with Ca, Cu, Fe, Mn, Na, Sr and Zn than with Mg. The negative availability values of test ingredients do not indicate negative balance of nutrients, but indicate the antagonistic property of test ingredients for the absorption of nutrients in diets. The present method provided the advantage of showing this antagonistic (or synergistic) property of test ingredients, which needs to be understood when formulating complex feeds.

Apparent availability of potassium

Apparent availability of potassium (K) was high in all ingredients (Table 14) and the net absorption was highly correlated to the amount of intake, which agrees with the observation of Dabrowski and Schwartz (1986) who noted that the intestinal absorption of K in carp increased up to 88.8% as the food passed through the gut. In humans, the portion of K excretion via the gut is normally less than 10% of total K excretion even in states of K excess, and the kidney has the major excretory role (Spencer, 1959). Dempsey et al. (1958) noted that fecal K tends to be quite constant and unaffected by the intake. The present observations agree with these previous reports in that variation in K intake (μg K/g BW/day) did not affect apparent availability (%) of K in any test diet, and the intake of K was correlated with net absorption (μg K/g BW/day) of K (Table 21).

Apparent availability of sodium

Apparent availability or absorption of sodium in feed ingredients was not as high as that observed with potassium (Table 15), and availability varied among feed ingredients. The amount of intake (μg Na/g BW/day) was correlated with net absorption (μg Na/g BW/day) (Table 21). Shaw et al. (1975) noted in Atlantic salmon that absorption of dietary Na was almost complete regardless of the NaCl levels in diets or in water. Dempsey et al. (1958) showed very low and constant levels of fecal Na

excretion regardless the level of intake. Dabrowski et al. (1986) noted that Na concentration of the digesta tends to increase towards the posterior intestine of rainbow trout in freshwater. Nakamura (1985) reported in carp that the degree of Na uptake from the intestinal lumen is dependent on the osmotic pressure of the digesta. The present study indicates that fecal Na and net absorption are variable among ingredients and are not related to dry matter digestibility or Na levels in the ingredients. Also, different absorption (%) of Na in some dietary treatments observed in different weeks indicates the possible effect of diet history. The concentration of Na in all plant ingredients was too low to estimate availabilities.

Apparent availability of iron

Apparent availability (absorption) of iron (Fe) in fish meals and plant ingredients was quite low, whereas that in wheat gluten meal was high but the amount of Fe in wheat gluten was very low. Feather meal contained a high level of Fe, and the availability or absorption (%) was also high, resulted in much higher levels of net absorption than in the other ingredients (Table 16). This indicates that Fe in feather meal is a highly available source; i.e., heme iron, and/or feather meal is devoid of antagonistic substances that interfere with Fe absorption. Net absorption of Fe (μg Fe/g BW/day) was significantly correlated with intake (μg Fe/g BW/day) in diets containing feather meal, poultry by-product meal, wheat gluten, wheat middling and wheat flour (r=0.94-0.98) but the overall correlation was low (Table 21). Negative values obtained in many ingredients indicate that the availability of Fe in those feed ingredients is low, and, in addition, unknown factors in the test ingredient are (1) inhibiting the absorption of Fe in the basal diet portion within the test diet, (2) inducing the endogenous excretion of Fe, and/or (3) the body is regulating the absorption of Fe or discharging the nutrient from previous intake. It has been shown that Fe homeostasis is maintained primarily by adjusting Fe absorption to bodily needs because of the limited capacity of the body to excrete Fe (Scott et al., 1982; Morris, 1987). There was, however, no significant inverse correlation between dietary intake of Fe and its apparent availability determined as net absorption ($-0.65<r<0.01$; $P>0.05$) in either coho salmon or rainbow trout.

Apparent availability (absorption) of Fe between weeks determined in coho salmon was significantly different in many dietary treatments, indicating the effect of previous diet history. High levels of phosphate have been shown to reduce Fe absorption in humans (Hegsted et al., 1949; Bour et al., 1984). In the present study, an inverse correlation (r=-0.81;$P<0.05$ for coho salmon, r=-0.85;$P<0.05$ for rainbow trout) was observed between dietary P (or Ca) levels and Fe availability among diets containing animal ingredients.

Apparent availability of copper

Apparent availability of copper (Cu) showed large fluctuations from week to week in both coho salmon and rainbow trout (Table 17). In coho salmon, large fluctuations from day to day were also noted, which could mask statistical difference between weeks. Net absorption of Cu (μg Cu/g BW/day)

was significantly correlated to intake (Table 21). Coho salmon apparently absorbed more Cu from most diets than did rainbow trout, which may reflect a difference between these two fishes in dietary requirements or ability to utilize dietary Cu. Davis and Mertz (1987) remarked that there was a large difference between true and apparent absorption of Cu because the intestines excrete as well as absorb Cu. Greger and Snedecor (1980) noted that urinary excretion of Cu in humans is relatively small compared to that from the biliary system. This suggests that the apparent absorption of Cu will be high when its dietary level is low, and vice versa. This pattern; i.e., the negative correlation between dietary Cu levels and its apparent availability, was observed only for coho salmon fed diets containing ingredients from animal sources (r=-0.92, P<0.01).

Apparent availability of manganese

Apparent availability (net absorption) of manganese (Mn) was found to be quite low in all ingredients except for wheat gluten meal and the basal diet (Table 18). Unlike many other elements, net absorption of Mn (μg Mn/g BW/day) was not increased by the amount of intake (Table 21). Many test ingredients showed negative availability or absorption values, meaning that the ingredients reduced absorption of inorganic Mn supplied from the basal diet portion within the test diet or inhibited re-absorption of endogenous Mn secreted into the gut (mainly via bile), yet overall balance of Mn remained positive in all dietary treatments. This result agrees with the observation of Dabrowski and Schwartz (1986) who noted that there was no significant apparent absorption of Mn in carp digestive tract. Sandstrom et al. (1986) reported negative values for net absorption of Mn, Zn and Fe in human subjects. Sandstrom (1992) reviewed the percentage absorption of Mn in human studies, which was most often below 10%.

The main excretory route of Mn is bile and the incomplete enterohepatic circulation (Papavasiliou et al., 1966). In higher animals, absorbed Mn is almost totally excreted via the duodenum and jejunum (Bertinchamps et al., 1966), and very little is excreted in the urine even when injected or added to the diet (Hurley and Keen, 1987). This indicates that fecal Mn includes a significant portion of endogenous Mn in addition to the unabsorbed dietary Mn, and apparent availability values may not approximate the true availability but instead may indicate the balance of this element.

Ca and P have been shown to interact with Mn to reduce its availability. Wilgus and Patton (1939) and Schaible and Bandemer (1942) reported that excessive Ca from bone meal or $Ca_3(PO_4)_2$ would precipitate $MnSO_4$ in the lower gut of the chicken, rendering the Mn unavailable to the chick and creating a perotic condition. Davidsson et al. (1991) noted in a human study that Mn absorption was significantly affected by dietary Ca. Excess dietary P may be more antagonistic to Mn than is excess Ca in chickens (Wedekind and Baker, 1990a,b). Dietary tri-calcium phosphate reduced availability of Mn and Zn in carp (Satoh et al., 1992). Satoh et al. (1991) also demonstrated the low availability of Mn in whitefish meal to rainbow trout. In the present study, net absorption of Mn in diets containing animal

sources were inversely correlated to dietary Ca and P but the correlation was not statistically significant. Among diets containing plant sources, net absorption of Mn was inversely correlated to dietary P (not significant). The negative availability of Mn in many feed ingredients could be due to some antagonistic substances other than Ca and P in animal and plant ingredients. Phytate has only a slight inhibitory effect on Mn absorption in humans (Bales et al., 1987). The present study indicates that feed ingredients could lower absorption of Mn by inhibiting absorption of available (inorganic) Mn and/or by inducing its endogenous loss. Net absorption of Mn (μg/g diet) was well below the dietary requirement of this element except for the basal diet and wheat gluten diet, which indicates not only the low availability of Mn in feed ingredients but their interference with absorption of inorganic Mn in the diets. The long term effect and the biological adaptation to different intake levels were beyond the scope of this study; however, the present results provide an evidence of lower (or negative) availability of Mn in feed ingredients relative to that of inorganic sources in the casein basal diet.

Apparent availability of strontium

Apparent availability of strontium (Sr) was higher in animal ingredients than in plant ingredients (Table 19). The contents of Sr in plant ingredients were, however, negligible. Net absorption of Sr determined with coho salmon showed large fluctuations among days (Table 19). In a study with humans, absorption of Sr was shown to fluctuate greatly between subjects as well as between weeks (Reynolds and Smith, 1994; Sips et al., 1995). Sr absorption via the intestine ranges from 5 to 25% in various mammalian species (Nielsen, 1986), which agrees with the observation in the present study with fish. Apparent availability of strontium in test ingredients was similar to that of calcium in both coho salmon and rainbow trout.

Apparent availability of zinc

Apparent availability of zinc (Zn) was significantly lower for rainbow trout than for coho salmon, especially that from fish meals (Table 20). This confirms the results of Satoh et al. (1987a) who reported growth depression, bone malformation and cataract incident in trout fed a whitefish meal-based diet. High bone content in diets has been shown to reduce Zn availability in rainbow trout (Ketola, 1979) and Atlantic salmon (Shearer et al., 1992). Hardy and Shearer (1985) and Satoh et al. (1987b, 1993) reported that the bioavailability of Zn was reduced by dietary tri-calcium phosphate in rainbow trout. Dietary P has been shown to decrease absorption of Zn in rats (Heth et al., 1966) and in rainbow trout (Porn-Ngam et al., 1993). In the present study, menhaden meal whose Ca and P levels were the highest among test ingredients showed the lowest Zn availability among animal ingredients.

In contrast to the animal ingredients, the availability of Zn from plant sources was generally lower than animal sources despite the lower content of Ca and P in those plant ingredients. Spinelli et al. (1983) and Richardson et al. (1985) demonstrated the detrimental effect of phytate on Zn absorption in rainbow trout and chinook salmon, respectively. In mammals, phytate and Ca have been shown to have

inhibitory effects on Zn absorption (Saltman et al., 1984; Smith and Rotruck, 1988; Gibson, 1994). Dietary phytate affects not only the bioavailability of dietary Zn but also the reabsorption of endogenous Zn and thus has a net effect on Zn homeostasis (Oberleas, 1983).

Body homeostasis of Zn is regulated by excretion rather than by absorption (Sandstrom, 1988). The main route of Zn excretion in man is via intestinal mucosal cells (Hambidge et al., 1986). Endogenous excretion increases as the dietary Zn intake is increased in mice (Cotzias et al., 1962), in rats (Kirchgessner and Weigand, 1983), and in humans (Jackson et al., 1984; Turnlund et al., 1986). Urinary Zn losses are not affected by dietary Zn in rats (Weigand and Kirchgessner, 1980). These observations in animal species suggest that the dietary level of Zn or the amount of intake should be inversely correlated to the net absorption (availability) of Zn. The present study indicated that dietary Zn levels have an insignificant but inverse correlation with net absorption (%) of Zn in both coho salmon and rainbow trout; however, the absolute amount of intake (μg Zn/g BW/day) showed insignificant but positive correlations with its availability (%) in most dietary treatments. Consequently, intake was highly correlated to the amount of absorption (Table 21), which is similar to the behavior of protein, electrolytes and major minerals but in contrast to Ca, Fe and Mn. Net absorption of Zn in most dietary treatments ranged between 60 and 100μg Zn per g diet, which is approximately 4 to 7 times higher than the dietary requirements of the fish. To maintain homeostasis for this element in the body, most of the (net) absorbed Zn must be excreted either via urine or gills or body surface. Hardy et al. (1987) demonstrated the role of gills in rainbow trout for the excretion of Zn, using radiolabelled ^{65}Zn administered via feeds. This suggests that excess Zn absorbed from the intestine is excreted via gills in fish rather than excreted back into the intestine as in terrestrial mammals.

Turnlund and King (1983) remarked on the significance of carry-over excretion of Zn from previous intake. Apparent availability of Zn in some test diets was significantly different between weeks (previous dietary regime) in the study with coho salmon. The difference was, however, primarily due to the small deviation within weeks. Negative values of apparent availability and large deviations were mainly due to the low content of Zn in the ingredients as discussed previously.

CONCLUSION

The net absorption of nutrients (P, Mg, K, Na, Cu, Fe, Zn) between coho salmon and rainbow trout fed the same test diets was significantly different. This might be attributed to differences in absorption of dietary nutrients or differences in nutrient requirements between these two species; however, since genetic-environmental interactions are known to influence fish performance and given that the rearing conditions were not identical between coho salmon and rainbow trout, differences in nutrient absorption could be the result of fish cultural conditions, e.g. frequency of feeding, composition of diets, water temperature and water quality.

Apparent availability (%) indicates fractional net absorption of nutrients from test diets or ingredients. The value does not account for any variation of the dietary intake of nutrients (μg/g BW/day) resulting from either different dietary concentrations or from different feeding rates. Fecal excretion of a nutrient is affected not only by nutrient digestibility plus the endogenous loss (apparent digestibility or availability) but also by nutrient intake. Nutrient intake is a function of nutrient concentrations in diets and the amount of feed ingested. The latter variable normally has less influence on nutrient absorption and retention since feed intake directly correlates to growth. Management of fecal excretion of nutrients must, therefore, be approached from two different ways: (1) selection of highly digestible or available sources; and (2) selection of feed sources of marginal nutrient content (not excess). Fecal excretion of Ca, Fe and Mn was affected more by intake than by availability; absorption of these elements was regulated. Unlike other elements, increasing the intake of Ca, Fe and Mn did not increase net absorption (μg/g BW) of these elements. Both intake and availability, however, appear to be important for protein, Cu, K, Mg, Na, P and Zn in terms of reducing their excretion via feces.

To determine the total amount of nutrient excretion into water, excretion of the nutrient via the urine and the gills, in addition to the amount of nutrient excreted in the feces, must be measured. Measurement of non-fecal excretion was beyond the scope of the present study; however, certain nutrients absorbed at levels higher than the requirement were evidently excreted via the urine and/or gills. Consequently, reducing dietary input of "available" nutrients to the minimum required level will be necessary to reduce total excretion (fecal and non-fecal) of nutrients into aquaculture effluents.

Table 7. Composition of the basal diet.

Ingredients	%
Casein[1]	44.8
Gelatin[2]	10.0
Dextrin[3]	12.0
Carboxymethyl cellulose	1.0
Alpha-cellulose	4.6
Mineral mixture[4]	3.3
Vitamin mixture[5]	2.0
Amino acid mixture[6]	4.1
Ascorbic acid[7]	0.2
Choline chloride (70%, liquid)	1.0
Herring oil	17.0
Trace mineral solution[8]	(40.0)

[1] Vitamin free (904520; ICN Biomedicals, Inc., Cleveland, OH).
[2] Type B from bovine skin, 225 bloom (G-9382; Sigma Chemical Co., St. Louis, MO).
[3] Type IV from potato (D-4894; Sigma).
[4] Supplied the following per kg dry diet: KCl, 15 g; $CaHPO_4$, 12 g; MgO, 3 g; NaCl, 3 g.
[5] Supplied the following per kg dry diet: thiamin mononitrate, 62 mg; riboflavin, 71 mg; niacin, 294 mg; calcium pantothenate, 153 mg; pyridoxine hydrochloride, 50 mg; folic acid, 22 mg; vitamin B_{12}, 0.08 mg; d-biotin, 0.8 mg; myoinositol, 176 mg; retinol acetate, 8818 IU; vitamin D_3, 588 mg; α-tocopherol acetate, 670 mg; menadione sodium bisulfite complex, 37 mg.
[6] Supplied the following per kg dry diet: L-methionine, 4 g; L-arginine, 10 g; L-histidine, 2 g; L-lysine, 10 g; L-phenylalanine, 5 g; L-threonine, 10 g.
[7] Type F-90, Coated ascorbic acid (Takeda U.S.A. INC., Orangeburg, NY).
[8] Supplied the following per kg dry diet: KI, 1.9 mg; $MnSO_4 \cdot H_2O$, 75.8 mg; $ZnSO_4 \cdot 7H_2O$, 132.0 mg; Na_2SeO_3, 0.88 mg; $CoCl_3 \cdot 6H_2O$, 4.0 mg; $CuSO_4 \cdot 5H_2O$, 11.8 mg; $FeSO_4 \cdot 7H_2O$, 298.5 mg.

Table 8. Composition of the test ingredients (dry basis).[1]

	CP[2]	Ash	K	Na	Ca	P	Mg	Cu	Fe	Mn	Zn	Sr
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(μg/g)	(μg/g)	(μg/g)	(μg/g)	(μg/g)
Herring meal	73.6	9.05	0.70	0.57	2.74	2.05	0.20	4.39	153	3.1	146	45.7
Anchovy meal	73.7	16.56	1.07	1.35	4.71	2.90	0.35	6.32	308	7.8	130	66.7
Menhaden meal	67.7	20.25	1.04	0.69	6.35	3.43	0.29	2.54	934	70.4	164	83.7
De-boned meal	78.2	9.12	0.52	0.57	2.93	1.69	0.22	1.79	219	10.8	130	157.0
Poultry-BP meal	81.0	12.04	1.81	0.82	2.54	2.17	0.22	3.68	198	tr.	119	56.8
Feather meal	77.3	4.81	0.25	0.53	1.29	0.75	0.08	19.10	768	74.8	252	50.5
Soybean meal	53.2	7.94	2.50	tr.	0.42	0.76	0.42	17.00	363	50.4	69	19.5
Wheat gluten	85.0	0.77	0.27	tr.	0.12	0.18	0.04	5.85	59	25.8	57	3.3
Corn gluten	72.3	0.68	0.24	tr.	tr.	0.55	0.06	11.70	174	tr.	24	tr.
Wheat middling	20.5	4.79	1.33	tr.	0.15	1.17	0.60	9.92	126	168.0	122	7.4
Wheat flour	15.5	1.00	0.48	tr.	0.03	0.32	0.14	2.50	27	21.6	13	1.4
Casein basal diet	58.6	4.00	0.74	0.41	0.50	0.74	0.23	4.93	98	34.9	123	3.4

[1] Values are the average of duplicate determinations.
[2] Crude protein (Kjeldahl N × 6.25).

Table 9. Apparent digestibility of dry matter in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon		Rainbow trout	
	Digest/diet[1]	Digest/Ing[2]	Digest/diet[1]	Digest/Ing[2]
	(%)	(%)	(%)	(%)
Herring meal	85.8 a	97.6 a	85.3 ab	89.2 abc
Anchovy meal	83.4 b*	89.4 b	84.9 ab	87.7 cb
Menhaden meal	81.4 c	82.6 c	82.3 c	78.6 d
Deboned meal	83.7 b	90.6 b	86.2 a	92.6 ab
Poultry-BP meal	85.1 a	95.5 a	86.0 a	91.6 ab
Feather meal	81.3 c	82.1 c	83.7 cb	83.8 c
Soybean meal	79.2 d	74.7 d	80.2 d	71.2 e
Wheat gluten	86.3 a	99.4 a	86.9 a	94.7 a
Corn gluten	83.0 b	88.0 b	84.9 ab	87.7 cb
Wheat middling	69.0 e	38.3 e	72.9 e	45.0 f
Wheat flour	68.8 e	37.5 e	72.3 e	43.0 f
Casein basal diet	81.0 c		83.7 cb	

[1] Apparent availability (%) in test diets (measured); *n*=3 in coho salmon, *n*=5 in rainbow trout. Values with asterisks are significantly different between weeks ($P<0.05$; in coho salmon study only).
[2] Apparent availability (%) in test ingredients (estimated). Values in each column having the same letter are not significantly different ($P>0.05$).

Table 10. Apparent digestibility of protein in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon		Rainbow trout	
	Digest/diet[1]	Digest/Ing[2]	Digest/diet[1]	Digest/Ing[2]
	(%)	(%)	(%)	(%)
Herring meal	96.6 c*	94.7 bc	96.6 bc	94.6 ab
Anchovy meal	95.5 e*	91.4 c	96.2 bc	93.7 ab
Menhaden meal	94.5 f	87.7 d	95.0 c	89.8 bc
Deboned meal	96.7 c*	95.1 b	97.2 ab	96.7 ab
Poultry-BP meal	96.4 cd*	94.2 bc	96.9 abc	95.9 ab
Feather meal	91.3 g*	79.7 e	93.4 d	85.9 c
Soybean meal	96.4 cd	93.0 bc	95.5 bc	90.1 bc
Wheat gluten	98.4 a*	99.6 a	98.6 a	100.0 a
Corn gluten	95.7 de	91.9 bc	97.4 ab	97.3 ab
Wheat middling	96.2 cd*	86.3 d	96.7 bc	90.7 bc
Wheat flour	97.7 b*	98.3 a	97.7 ab	100.0 a
Casein basal diet	97.6 b*		97.6 ab	

[1-2] See footnotes in Table 9.

Table 11. Apparent availability of calcium in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	2533	22.0 c	13.0 b	1492	13.9 cd	1.9 a
Anchovy meal	4038	23.7 c*	18.8 b	5452	31.8 ab	29.3 a
Menhaden meal	3699	17.1 c	12.1 b	4348	20.2 bc	16.0 a
Deboned meal	2077	17.5 c*	6.8 b	2879	23.9 bc	16.4 a
Poultry-BP meal	3684	34.2 b	30.1 b	3684	34.9 ab	31.8 a
Feather meal	2930	40.2 b	38.2 b	2506	35.1 ab	29.3 a
Soybean meal	744	15.7 c	0 (20.8)b	114	0.8 d	0 (1.1)a
Wheat gluten	2041	52.6 a	100 (48.1)a	1743	44.6 a	79.2 a
Corn gluten	631	18.1 c*	---- (19.4)	170	4.4 d	---- (6.0)
Wheat middling	162	4.0 d*	0 (4.5)c	142	4.9 d	0 (5.4)b
Wheat flour	1581	43.1 ab	74.0 ab	1242	32.7 ab	0 (33.5)b
Casein basal diet	2108	42.4 ab*		2063	41.3 a	

[1] Net absorption (μg/g test diets, dry basis) (measured); *n*=3 in coho salmon, *n*=5 in rainbow trout.
[2] Apparent availability (%) in test diets (measured); *n*=3 in coho salmon, *n*=5 in rainbow trout. Values with asterisks are significantly different between weeks ($P<0.05$; in coho salmon study only).
[3] Apparent availability (%) in test ingredients (estimated). Values in each column having the same letter are not significantly different ($P>0.05$). When apparent availability values of the test ingredients were negative or higher than 100%, apparent availability values of the basal diet portion within the test diets were re-calculated (shown in parentheses) assuming that the availability from the test ingredients were either 0 or 100%.

Table 12. Apparent availability of phosphorus in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	8212	73.1 d	57.3 bc	7026	63.1 d	44.4 bcd
Anchovy meal	8755	64.4 e*	47.4 cd	8675	63.7 d	50.4 bc
Menhaden meal	8764	58.2 f	40.4 d	8044	53.4 e	36.5 cd
Deboned meal	7462	73.9 d*	54.8 c	6779	66.7 d	46.8 bc
Poultry-BP meal	9011	78.5 c	67.7 ab	8377	73.2 c	63.5 ab
Feather meal	6423	86.5 b	75.4 a	5784	77.9 bc	61.7 abc
Soybean meal	5456	73.0 d	28.4 e	5026	66.5 d	22.0 de
Wheat gluten	5094	88.0 ab	56.9 bc	4848	83.7 a	74.7 a
Corn gluten	5066	73.9 d*	15.8 f	4631	67.2 d	8.5 e
Wheat middling	6199	72.1 d	41.0 d	6296	73.4 c	55.3 abc
Wheat flour	5313	85.1 b	50.1 cd	4957	79.1 b	47.0 bc
Casein basal diet	6748	91.0 a*		6268	84.5 a	

[1-3] See footnotes of Table 11.

Table 13. Apparent availability of magnesium in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	1877	85.4 c	66.7 c	1744	79.7 de	49.8 bc
Anchovy meal	2092	79.7 d*	59.5 c	1968	74.9 ef	49.5 bc
Menhaden meal	1846	75.3 e	42.0 d	1696	69.0 g	26.4 c
Deboned meal	1883	83.6 c	60.9 c	1878	83.3 cd	63.8 bc
Poultry-BP meal	1965	87.0 c	73.5 c	1861	82.8 cd	62.5 bc
Feather meal	1615	87.9 c	57.1 c	1589	86.7 bc	58.6 bc
Soybean meal	2384	84.5 c	73.8 c	2094	74.1 ef	51.3 bc
Wheat gluten	1644	95.7 a	100 (95.4)a	1642	95.7 a	100 (95.4)a
Corn gluten	1552	87.1 c	35.5 d	1323	74.0 ef	0 (81.5)d
Wheat middling	2489	75.5 e	59.0 c	2372	71.7 fg	53.0 bc
Wheat flour	1857	91.2 b	86.9 b	1798	88.5 b	79.2 b
Casein basal diet	2091	92.3 b*		2052	90.8 b	

[1-3] See footnotes of Table 11.

Table 14. Apparent availability of potassium in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	7071	96.8 ab	97.3 a	7226	99.0 abc	99.0 ab
Anchovy meal	8117	97.1 ab	97.9 a	8306	99.4 ab	99.9 a
Menhaden meal	7984	96.5 ab	96.2 a	8169	98.8 abc	98.3 ab
Deboned meal	6589	96.9 ab	97.9 a	6730	99.0 abc	99.0 ab
Poultry-BP meal	10242	97.9 a	99.2 a	10404	99.5 a	99.9 a
Feather meal	5760	95.9 bc	90.8 ab	5928	98.8 abc	96.9 b
Soybean meal	12033	97.2 ab	97.6 a	12251	99.0 abc	98.9 ab
Wheat gluten	5894	97.0 ab	100.0 (96.6)a	6005	98.9 abc	98.2 ab
Corn gluten	5753	95.9 bc	90.7 ab	5903	98.4 c	94.0 c
Wheat middling	8600	94.7 cd	92.1 a	8954	98.7 bc	98.2 ab
Wheat flour	6297	94.0 d	83.5 b	6598	98.5 c	96.5 b
Casein basal diet	7179	96.6 ab		7355	99.0 abc	

[1-3] See footnotes of Table 11.

Table 15. Apparent availability of sodium in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	3892	85.2 ab	92.8 a	3885	85.0 a	81.6 a
Anchovy meal	5965	87.7 ab*	92.9 a	6066	88.9 a	90.3 a
Menhaden meal	4046	82.3 bc*	84.4 a	3999	81.3 ab	72.9 a
Deboned meal	3642	80.0 bc	78.5 a	4137	90.8 a	98.0 a
Poultry-BP meal	4537	86.0 ab	92.4 a	4583	86.9 a	86.7 a
Feather meal	3340	75.1 cd	64.1 b	3503	78.9 ab	63.5 b
Soybean meal	1136	41.8 f*	---- (46.5)	1519	56.6 c	---- (60.1)
Wheat gluten	2279	91.5 a*	----	2285	91.8 a	----
Corn gluten	1093	46.6 ef	---- (57.4)	1246	53.3 c	---- (62.8)
Wheat middling	1168	51.4 e	---- (62.7)	1483	63.7 bc	---- (72.2)
Wheat flour	1734	69.3 d*	---- (74.1)	1873	74.9 ab	---- (78.8)
Casein basal diet	3331	80.9 bc*		3572	87.0 a	

[1-3] See footnotes of Table 11.

Table 16. Apparent availability of iron in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	31.0	27.2 c	8.7 bc	14.1	13.1 c	0 (21.5)b
Anchovy meal	26.7	16.9 de*	0 (38.3)bc	24.3	16.3 c	0 (36.8)b
Menhaden meal	19.4	5.8 g	0 (27.6)bc	15.3	5.2 c	0 (24.8)b
Deboned meal	12.5	9.5 fg	0 (17.8)c	13.2	10.2 c	0 (19.2)b
Poultry-BP meal	34.9	27.7 c*	13.4 b	26.2	21.1 c	0 (38.0)b
Feather meal	149.9	51.1 a	54.9 a	146.9	51.0 a	52.7 a
Soybean meal	39.2	22.7 cd	11.4 bc	29.2	16.8 c	0 (41.1)b
Wheat gluten	38.5	44.4 b	66.1 a	39.4	46.0 ab	48.4 a
Corn gluten	16.7	14.0 ef*	0 (23.9)bc	9.7	8.5 c	0 (14.5)b
Wheat middling	28.1	26.5 c*	1.5 bc	19.8	18.8 c	0 (28.3)b
Wheat flour	23.6	30.2 c*	0 (33.4)d	27.0	36.2 b	0 (40.1)b
Casein basal diet	38.3	39.1 b*		43.1	45.4 ab	

[1-3] See footnotes of Table 11.

Table 17. Apparent availability of copper in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	3.65	76.4 bcd	63.0 bc	3.09	64.7 bc	25.0 b
Anchovy meal	4.21	79.0 bcd*	74.4 abc	3.55	66.6 bc	42.1 ab
Menhaden meal	3.51	82.7 abc*	89.0 ab	2.36	55.5 c	0 (66.9)c
Deboned meal	3.11	76.7 bcd	43.7 c	2.23	55.7 c	0 (63.6)d
Poultry-BP meal	3.69	80.5 abcd	77.4 abc	3.00	65.3 bc	18.2 b
Feather meal	5.40	59.7 e*	46.0 c	5.33	58.9 c	46.1 ab
Soybean meal	7.49	90.0 a	96.4 ab	6.83	82.2 a	84.3 a
Wheat gluten	4.21	80.9 abcd*	79.8 abc	4.15	80.0 a	81.5 a
Corn gluten	5.07	73.8 cd	65.8 bc	5.03	72.9 ab	66.1 ab
Wheat middling	4.63	73.1 d	62.5 bc	4.01	61.8 bc	39.5 ab
Wheat flour	3.64	85.6 ab	100 (82.7)a	3.27	76.9 a	64.6 ab
Casein basal diet	4.01	81.4 abcd		3.89	79.3 a	

[1-3] See footnotes of Table 11.

Table 18. Apparent availability of manganese in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	1.9	7.7 d	0 (7.9)	0.8	4.2 d	0 (4.3)
Anchovy meal	2.7	9.9 cd*	0 (10.8)	4.5	16.5 d	0 (18.0)
Menhaden meal	2.4	5.3 d	0 (9.6)	2.6	5.9 d	0 (10.6)
Deboned meal	1.9	6.6 d	0 (7.4)	2.8	10.2 d	0 (11.5)
Poultry-BP meal	3.3	13.4 cd	---- (14.5)	1.6	8.5 d	---- (9.7)
Feather meal	9.4	20.2 cd	0 (37.9)	8.7	21.1 d	0 (39.6)
Soybean meal	8.8	22.2 cd	0 (34.8)	6.4	16.5 d	0 (25.8)
Wheat gluten	17.0	52.6 a*	95.2 ± 12.6	21.7	67.7 a	100 (58.3)
Corn gluten	2.5	9.9 cd	---- (10.1)	2.6	10.4 d	---- (10.5)
Wheat middling	3.6	5.1 d	0 (14.5)	8.1	11.4 d	0 (32.9)
Wheat flour	7.8	25.0 c	0 (31.0)	11.4	37.7 c	0 (46.8)
Casein basal diet	13.9	39.9 b		18.3	53.7 b	

[1-3] See footnotes of Table 11.

Table 19. Apparent availability of strontium in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	1.97	12.5 c	10.8 bc	1.83	12.7 e	7.8 c
Anchovy meal	3.22	15.0 c	14.1 bc	5.78	26.6 bcde	24.9 bc
Menhaden meal	3.29	12.5 c	11.5 bc	4.28	16.4 de	14.0 bc
Deboned meal	8.74	18.6 c	18.5 bc	17.61	37.2 abc	37.0 bc
Poultry-BP meal	5.10	27.4 bc	28.3 bc	6.39	35.3 abcd	34.6 bc
Feather meal	6.50	38.0 ab	40.6 b	7.88	47.1 ab	48.2 ab
Soybean meal	-0.34	-4.2 d	0 (-13.9)c	-0.68	-11.0 f	0 (-36.0)d
Wheat gluten	1.43	42.9 a	96.2 a	1.65	49.0 a	70.7 a
Corn gluten	-0.67	-27.6 e	---- (-27.5)	-0.92	-39.4 g	---- (-39.3)
Wheat middling	-0.91	-20.4 e	0 (-37.9)d	-0.48	-10.2 f	0 (-18.9)e
Wheat flour	0.57	20.0 c	8.2 bc	0.68	23.2 cde	0 (26.9)e
Casein basal diet	0.77	21.9 c		1.37	40.5 abc	

[1-3] See footnotes of Table 11.

Table 20. Apparent availability of zinc in feed ingredients determined with coho salmon and rainbow trout.

	Coho salmon			Rainbow trout		
	Abs[1]	Avail/diet[2]	Avail/Ing[3]	Abs[1]	Avail/diet[2]	Avail/Ing[3]
	(μg/g)	(%)	(%)	(μg/g)	(%)	(%)
Herring meal	96.6	74.4 b*	62.0 b	69.3	54.4 bc	3.0 b
Anchovy meal	91.2	72.9 b*	55.1 bc	76.1	61.0 b	17.2 b
Menhaden meal	83.1	61.6 c	26.0 bc	61.4	46.2 c	0 (70.6)b
Deboned meal	91.3	73.0 b*	54.9 bc	66.5	53.3 bc	0 (77.5)b
Poultry-BP meal	94.1	77.2 b	68.8 b	74.3	61.8 b	15.7 b
Feather meal	100.8	62.8 c	41.7 bc	95.7	60.4 b	37.7 b
Soybean meal	64.6	60.0 c	0 (73.1)d	66.9	63.8 b	0 (77.8)b
Wheat gluten	92.8	89.3 a*	100 (87.2)a	85.8	82.8 a	100.0 a
Corn gluten	61.2	64.6 c*	0 (69.6)e	50.3	52.6 bc	0 (56.7)c
Wheat middling	76.6	62.5 c*	15.7 c	61.3	50.0 c	0 (69.3)b
Wheat flour	70.7	76.7 b*	0 (79.8)	70.4	77.1 a	16.5 b
Casein basal diet	99.0	80.5 b*		97.3	79.6 a	

[1-3] See footnotes of Table 11.

Table 21. Overall correlation between intake, availability, absorption and excretion of dietary nutrients.[1]

	CP	Ca	P	Mg	K	Na	Cu	Fe	Mn	Zn
Intake[2] vs Avail.[3]	-0.21*	-0.12	-0.52*	-0.37*	0.18*	0.49*	-0.22*	-0.16*	-0.08	0.13
Intake vs Abs.[4]	1.00*	0.66*	0.94*	0.97*	1.00*	0.98*	0.89*	0.45*	0.24*	0.91*
Intake vs Exc.[5]	0.67*	0.96*	0.87*	0.79*	0.38*	0.40*	0.75*	0.92*	0.91*	0.66*
Avail. vs Exc.	-0.84*	-0.32*	-0.81*	-0.83*	-0.79*	-0.50*	-0.77*	-0.47*	-0.42*	-0.63*

[1] Coho salmon study only. Intake range of each nutrient (μg/g BW/day, n=168-174); Ca (13.2-337.8), P (19.7-269.3), Mg (5.8-53.1), K (20.7-178.9), Na (8.5-134.7), Cu (0.02-0.12), Fe (0.3-4.2), Mn (0.11-1.16), Zn (0.35-2.47) and CP or crude protein (0.22-1.25% of BW/day). Values with asterisks are statistically significant ($P<0.05$).

[2] Dietary intake (% of BW/day for protein and μg/g BW/day for minerals).

[3] Apparent availability (%).

[4] Net absorption (% of BW/day for protein and μg/g BW/day for minerals).

[5] Fecal excretion (% of BW/day for protein and μg/g BW/day for minerals).

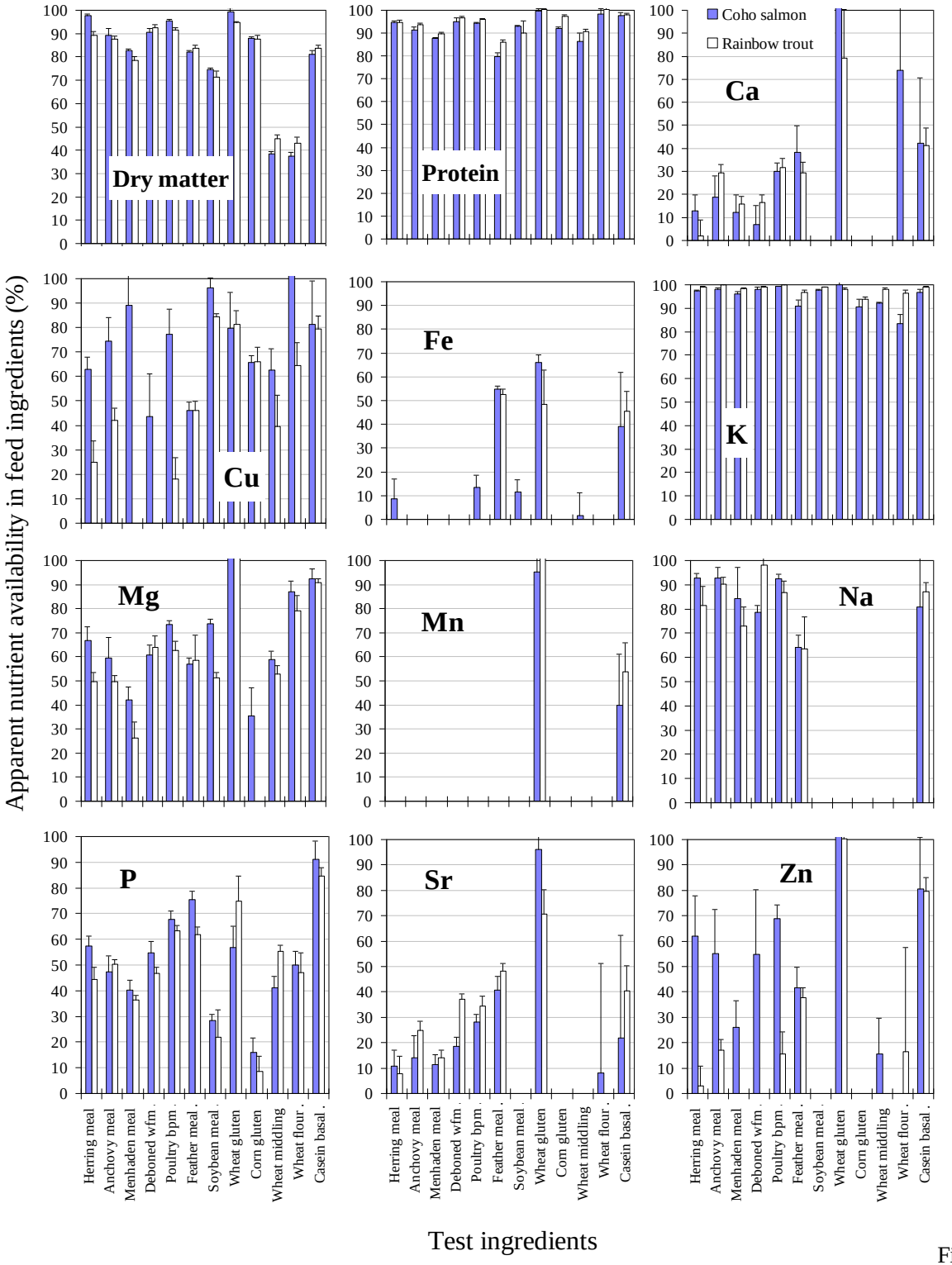


Figure 9. Apparent digestibility (%) of protein and availability (%) of minerals in feed ingredients determined with coho salmon and rainbow trout. Error bars are the s.e.m. (n=3 in coho salmon, n=5 in rainbow trout).

2. Availability of Nutrients in Animal By-products

BACKGROUND

Aquaculture of many carnivorous fishes including salmonids and most seawater fishes relies heavily on the supply of fishmeal, which composes the major portion of the feed for those species. As a result of the rapid growth of world aquaculture production in the last few decades, there has been a concomitant increase of demand for fishmeal. The increasing demand, however, cannot be met due to the limited production of fishmeal from capture fisheries which is approaching the plateau or maximum sustainable limit (FAO, 1997). Also, there has been an increasing contention and skepticism among the general public in regard to the aquaculture of carnivorous fishes since there is a substantial loss of animal protein in this process. Considering the ever increasing world population, feeding animal protein sources (fishmeal) to fish on any significant scale will not be an affordable or a sustainable practice. Consequently, replacing fishmeal with other ingredients that are not directly usable for human consumption should receive high priority in formulating aquaculture feeds in the future. The issue is particularly important in developing countries where the use of fishmeal in aquaculture feeds is often economically prohibitive (Tacon, 1996).

There are numbers of ingredients that may replace a significant portion of the fishmeal in aquaculture feeds. The availability of alternate protein sources (fishmeal-replacers) is largely dependent upon the region or country (Tacon, 1994). There are, however, some ingredients that are commonly available in many parts of the world, such as animal-, poultry- and fish-byproduct meals.

In the present study, the digestibility and availability of nutrients in some byproduct meals were studied and the potential use of those alternate ingredients in low-pollution feeds was discussed. Because the quality of animal byproducts is known to be largely dependent upon the processing conditions, e.g., availability of processing plant and equipment, quality of raw materials, it must be necessary to reevaluate the quality of locally available byproducts before being used as a feed ingredient (New et al., 1995). The purpose of this study was to present a general picture of the byproduct materials (fishmeal replacers) in terms of the available nutrient contents with particular emphasis on phosphorus, and their potential use in low-pollution feeds.

MATERIALS AND METHODS

Ten rainbow trout (initial mean body weight 106 ± 1.2g, sem) were stocked in each digestibility tank receiving continuous flow of dechlorinated municipal water (16 ± 0.5°C). Fish were fed as much as they would consume once daily. Feed intake ranged between 0.94-2.08 (as % fed, dry basis, per body weight of fish). The apparent digestibility of protein and availability of minerals were examined in the following ingredients; menhaden fishmeal, Peruvian fishmeal, meat &bone meal, feather meal, blood

meal (ring-dried), whitefish meal (deboned), whitefish meal (whole) and whitefish meal (skin & bone). The digestibility and availability values of protein and minerals were determined as previously described (ref. Study 1 in Ch. 2). Briefly, each of the test ingredients was mixed in with the casein-gelatin semi-purified diet at 3:7 ratio (dry basis) to formulate test diets. Fish were fed the test diet for 12 days and the fecal samples were subsequently collected over six consecutive days by settling, using digestibility tanks. The fecal samples collected over the period of six days were pooled by treatment, dried, ashed and analyzed for minerals and protein contents by the inductively coupled plasma emission spectrophotometer (Jarrell-Ash Plasma Atom Comp., Waltham, MA) and semi-automated micro-Kjeldahl method (Buchi Kjeldahl/Nitrogen Analyzing System, Brinkmann Instruments, Inc., Westbury, NY)., respectively.

In an additional experiment, the apparent digestibility of dry matter, protein and the apparent availability of phosphorus were studied for poultry byproduct meal, poultry byproduct meal (deboned), poultry byproduct meal (low ash), meat meal, meat meal (low ash), meat & bone meal, meat & bone meal (low ash), feather meal, blood meal (spray-dried) and blood meal (ring-dried) using chromium oxide as the inert indicator. The test ingredients were mixed in with the casein-gelatin semi-purified diet at approximately 3:7 ratio (as-is basis). The precise ratio (dry basis) of test ingredients to the basal diet was determined based on the chromium content in the basal diet and that in the test diets. Diet and fecal samples were digested in Kjeldahl flasks using sulfuric and nitric acids, oxidized with perchloric acid, diluted to an appropriate concentration to determine dichromate content at 350nm and phosphorus content at 660nm according to Taussky and Shorr (1953) using a spectrophotometer (Spectronic® Genesis™ 5, Spectronic Instruments, Inc., Rochester, NY). The subsamples of diets and feces were used for the determination of crude protein using a nitrogen determinator (LECO FP-428, Leco Instruments, St. Joseph, Michigan, USA).

RESULTS AND DISCUSSION

Dry matter and Protein

Apparent digestibility of dry matter was highly variable among feed ingredients ranging from 50.5 to 95.8% (Table 22; Fig. 10). The low-ash ingredients such as blood meal and deboned whitefish meal showed higher digestibility than the other ingredients. The dry matter digestibility of feather meal was not high even though the ash content was low, which could be related to the processing conditions of the ingredient. The apparent digestibility of protein was relatively similar among ingredients ranging from 76.1 to 94.8% (Table 22; Fig. 10). Blood meal and deboned whitefish meal again showed the two highest digestibility values among the ingredients examined.

Phosphorus

The apparent availability of phosphorus in feed ingredients, like that of ash and calcium, was highly variable (Table 23; Fig. 10). High-ash ingredients such as meat meal and skin&bone meal had very low values for the apparent availability of phosphorus, whereas low-ash ingredients such as blood meal and feather meal had over 100% of the calculated availability value. The calculated apparent availability coefficients which have a value over 100% indicate that the ingredient had a synergistic effect, enhancing availability of phosphorus in the casein basal diet portion within the complex diet (ingredient 30%, casein basal diet 70%) or reduced endogenous excretion of phosphorus in the gastrointestinal tract. In the case of blood meal, however, it is most likely due to the dilution effect; i.e., the concentration of phosphorus in blood meal was so low that the blood meal-casein basal complex diet had low phosphorus content, which resulted in an increased uptake (% of intake) of phosphorus by the fish. Apparently, fish regulate the uptake (absorption) of phosphorus from the intestine and, regardless of the phosphorus content in diets, fish absorb dietary phosphorus up to an amount of the requirement level, and excrete (not absorb) any excess. This excretory route is mostly fecal when the source of phosphorus is insoluble salts (e.g., hydroxyapatite or tri-calcium phosphate), while the urinary route predominates when the source is soluble salts (e.g., potassium or sodium phosphate). When the dietary concentration is similar, phosphorus in whitefish meals appears to be less available compared to that in menhaden or Peruvian meals, which agrees with Ogino's findings who reported higher availability values of phosphorus with brown fishmeals than with whitefish meals (Ogino et al., 1979). This difference, although not examined in the present study, may be attributed to differences of bone particle size (due to different fish size), bone density, processing conditions of the fishmeal or possibly to the different concentration of histamine and gizzerosine, potent stimulants for gastric acid secretion.

Ash

Apparent digestibility of ash was largely different among ingredients (Table 24; Fig. 11). Some animal byproduct meals contained a high percentage of ash, which corresponded to an increase of ash in the feces. The ash portion of most ingredients is largely composed of calcium and phosphorus. Reducing ash content in feed ingredients is, therefore, imperative to reducing phosphorus excretion in feces.

Calcium

Apparent availability of calcium was relatively low and highly variable among feed ingredients (Table 24; Fig. 11). This appears to be related to the calcium content in the ingredient, and the availability (expressed as the fractional net absorption) was reduced at high calcium intake. The absorption of calcium from the gastrointestinal tract may therefore be saturable, and the excess calcium in feeds will be either undigested or precipitated in the intestine (with co-precipitation of trace minerals) and will be excreted into feces.

Copper

The concentration of copper in some ingredients (whitefish meals) was much lower than that in the casein basal diet (Table 25). In that case, the calculated apparent availability values for those ingredients may involve high experimental error (Table 23) because small differences of the measured availability values (of the whole diet) will be responsible to the small amount of minerals contributed from test ingredients (for detail, see p 43). In this case, however, the absorption of nutrient (copper) from the entire diet will provide additional information; i.e., interaction of the ingredient within the complex diet. Since the absorption (apparent availability) of copper is not largely different among test diets (ignore ingredient-ADC here), the absorption from whole diets is relatively unaffected regardless of the source (ingredient) or the amount present in diets (Fig. 12). Feather meal had a relatively high copper content, however, the absorption of copper apparently was not regulated. As a consequence, fish absorbed copper in an amount about 3 times higher from the diet containing feather meal than from the other diets.

Iron

Except for feather meal and blood meal, byproduct meals did not contain appreciable levels of available iron and further they reduced the absorption of iron supplied from the non-byproduct portion of the diet or increased endogenous excretion (Table 25; Fig. 11 and 12). In three high-ash ingredients, the iron balance was even negative. The blood meal contained very high levels of iron, in addition, the fractional absorption (% of intake) was the highest of all of the ingredients examined, indicating that fish fed diets containing blood meal absorbed substantial amounts of iron. In fact, the fish absorbed 8.7 times more amount of iron than the dietary requirement. Since iron absorption is regulated at the gastrointestinal level, this level of net absorption can not persist in order for fish to maintain homeostasis of this element in the body and to avoid overdose toxicity. Long-term feeding will eventually reduce net absorption of this element, bringing it towards a more balanced level, which may be close to the requirement level of this element. Measuring nutrient availability at this stage, however, cannot be justified since fish are strongly regulating the absorption of this element. For this reason, the acclimation period to the test diet should be minimized when measuring availability of minerals; the collection of fecal samples should be completed before fish start regulating (or increasing, when the level of intake is sub-optimal) the absorption of dietary minerals. Although the acclimation period to the test diet needs to be minimized, there should be a sufficiently long conditioning period preceding the feeding of test diets. This period will be critical to neutralize any carry-over effect of previous dietary regimes, to replenish body pools for each of the minerals and vitamins, and to normalize growth rate and feed intake. The present series of studies used the 30/70 ratio to determine mineral availability coefficients, which would “buffer” the direct effect of the fish’s regulatory system by standardizing (calibrating) the availability coefficients by the casein basal diet which was fed at the same time to the same group of fish having the same nutritional and physiological background. Nevertheless, it must be essential to use

physiologically and nutritionally "normal" fish and therefore there should be a certain conditioning period followed by a very short test period.

Potassium

In contrast to sodium, the absorption of potassium is almost complete regardless of the source (ingredient) and the amount (Table 26; Fig. 11). This also indicates that the absorption of potassium is not regulated at the gastrointestinal level, or possibly the level of potassium fed was less than the optimum dietary level for fish.

Sodium

Apparent availability of sodium ranged between 37.2 and 94.2% (Table 26; Fig. 11). The concentration of sodium in Peruvian fishmeal was high and the absorption (availability) also was high, indicating that the absorption of sodium is not regulated at the gastrointestinal level. Two high-ash ingredients, skin & bone meal and meat & bone meal, showed somewhat lower values of sodium absorption (availability), which is presumably related to the lower digestibilities of dry matter in these ingredients and the larger fecal mass.

Magnesium

Apparent availability of magnesium in byproduct materials ranged between 0 and 100% (Table 27; Fig. 11). The calculated apparent availability value showing over 100% is due to the nutrient interaction or dilution effects as explained above. The negative apparent availability values indicate that the nutrient (magnesium in this case) in the test ingredient is almost unavailable (availability 0%), in addition, certain compounds in the test ingredient are reducing the absorption of magnesium in the casein basal diet portion (Fig. 12; antagonistic interaction) or increasing the endogenous loss from the gastrointestinal tract by, for instance, interfering with the enterohepatic circulation of bile.

Manganese

The apparent absorption (availability) of manganese was low in most byproduct meals (Table 27; Fig. 11), which is in accord with the observation in the digestibility study-1. Except for the blood meal diet and the casein basal diet, the amount of manganese that was absorbed by fish was less than the requirement level (NRC, 1993), indicating long-term feeding with these ingredients could cause clinical deficiency of manganese unless fish can increase the net absorption of this element over time by a certain adaptive mechanism. In contrast to many ingredients examined, blood meal (and casein basal diet) did not interfere with the net absorption of manganese (Fig. 12). This indicates blood meal and the casein diet contain only minor levels of the substance which reduce the absorption of manganese.

Strontium

The concentration of strontium, unlike copper, was high in whitefish meals, especially in skin & bone meals, compared with other ingredients (Table 28; Fig. 11). However, fish apparently regulated

the uptake of strontium by reducing its fractional absorption from the diet, which ranged from 6.3 to 76.3%. Despite this regulatory system, fish actually absorbed higher amounts of strontium from high-strontium diets or ingredients.

Zinc

Apparent availability of zinc was highly variable among ingredients examined (Table 28; Fig. 11); three high-ash ingredients did not contain any appreciable amount of zinc and further they reduced the absorption of zinc from the non-byproduct portion (casein-basal diet) of the diet or increased endogenous excretion in the intestine (Fig. 12). However, with the diet containing skin & bone meal, whose zinc availability was the lowest among the ingredients examined, fish still absorbed 35 mg of Zn from the diet. Since this is above the dietary requirement for fish, long-term feeding will unlikely be detrimental to the fish. In fact, the basal diet supplied 2.8 times higher amount of zinc than the requirement level for fish. This indicates that fish could have been deficient in zinc if the diet had contained only a requirement level of inorganic zinc supplement even if the high-ash ingredients indeed contributed zinc at a level much higher than the dietary requirement.

CONCLUSION

The phosphorus content in blood meal was low and the digestibility of protein and the availability of many minerals in it were shown to be high. The use of blood meal in practical feeds, however, should be limited because of its amino acid imbalance (low BV, NPU or PER) which could likely reduce retention of dietary protein and increase nitrogen (ammonia) excretion. Because of the low phosphorus content, feather meal and deboned fishmeal may be the two most feasible fishmeal replacers. Since other byproduct meals have a high phosphorus content, the maximum inclusion level in low-pollution feeds would need to be substantially reduced, lest the total dietary phosphorus level exceeds the requirement level of fish. As shown in the fishbone study (p 71), the absorption of phosphorus increases as the dietary concentration decreases (in terms of % absorption per intake), which indicates that the availability of phosphorus in high-ash (high-phosphorus) ingredients will be substantially increased by simply reducing their dietary inclusion level or preferably by reducing their bone (phosphorus) content per se. In summary, the limiting factor in the use of animal byproduct meals in low-pollution feeds appears to be their relatively high phosphorus content. Reducing phosphorus content in those ingredients by mechanically removing the bone fraction in high-ash fishmeals as suggested by Babbitt et al. (1994) could be a single most effective procedure to utilize animal or fish byproduct materials in low-pollution aquaculture feeds and to reduce phosphorus excretion into the effluent.

Table 22. Apparent digestibility of dry matter and crude protein in animal byproducts determined with rainbow trout.[1]

	Dry Matter		Crude Protein				
	Digestibility		Content			Digestibility	
	Diet	Ingr.	Diet	Ingr.	Feces	Diet	Ingr.
	%	%	%	%	%	%	%
Blood meal, ring-dried A	88.8	93.5	70.8	93.0	21.3	96.6	94.1
Blood meal, ring-dried B*	85.8	95.8	68.9	103.4	24.6	94.9	94.8
Blood meal, spray-dried*	83.7	88.3	66.8	94.9	26.8	93.5	91.2
Feather meal A	84.1	77.7	65.6	75.6	28.5	93.1	83.3
Feather meal B*	82.2	83.2	67.2	102.3	35.8	90.5	83.3
Meat & bone meal A	77.5	55.9	60.5	58.5	19.1	92.9	79.8
Meat & bone meal B*	75.7	61.6	56.4	58.9	23.3	90.0	79.0
Meat & bone meal (low ash)*	74.3	56.5	56.7	59.8	22.5	89.8	78.3
Meat meal*	75.1	59.5	57.6	63.0	22.3	90.4	80.9
Meat meal (low ash)*	76.9	66.0	59.1	67.3	25.6	90.0	80.9
Poultry byproduct meal*	81.7	81.4	59.4	68.2	26.8	91.7	85.8
Poultry byproduct meal (deboned)*	79.4	73.7	59.7	70.2	26.9	90.7	82.7
Poultry byproduct meal (low ash)*	82.7	84.6	60.5	72.7	24.2	93.0	89.5
Menhaden fishmeal	81.8	70.3	62.9	66.6	20.9	94.0	84.8
Peruvian fishmeal	84.4	79.0	61.5	61.7	22.0	94.4	85.6
Whitefish meal (deboned)	86.6	86.2	64.4	71.5	15.6	96.7	93.7
Whitefish meal (skin & bone)	75.9	50.5	57.0	46.9	17.0	92.8	76.1
Whitefish meal (whole)	83.0	74.0	64.5	71.7	19.1	95.0	88.4
Casein basal diet A	86.8	----	61.3	----	8.1	98.3	----
Casein basal diet B*	81.8	----	55.3	----	15.2	95.0	----

[1] Fecal samples were collected either by settling (no asterisk) or by stripping (with asterisk). Contents of ash and calcium (%, dry basis) in the ingredients affixed with asterisk were (by the order given in the table); 1.92, 0.06 (BM-RD); 6.23, 0.10 (BM-SD); 1.75, 0.29 (FM); 21.4, 4.75 (MBM); 21.2, 4.05 (MBM-LA); 23.4, 5.31 (MM); 19.4, 4.25 (MM-LA); 13.6, 3.42 (PBM); 13.4, 3.48 (PBM-DB); 10.0, 2.03 (PBM-LA). Content of calcium in the casein basal diet B was 0.63% (dry basis). Data for the other ingredients were included in the following tables.

Table 23. Apparent availability of phosphorus in animal byproducts determined with rainbow trout.[1]

	P				
	Content			Availability	
	Diet	Ingr.	Feces	Diet	Ingr.
	%	%	%	%	%
Blood meal, ring-dried A	0.55	0.12	0.79	83.8	107.4
Blood meal, ring-dried B*	0.63	0.08	1.35	69.4	118.4
Blood meal, spray-dried*	0.81	0.72	1.15	76.8	103.5
Feather meal A	0.89	1.26	1.07	81.0	79.4
Feather meal B*	0.64	tr.[3]	0.64	82.1	----
Meat & bone meal A	2.19	5.59	5.86	39.8	26.9
Meat & bone meal B*	1.39	2.68	3.39	41.2	21.8
Meat & bone meal (low ash)*	1.33	2.49	2.62	49.5	35.0
Meat meal*	1.42	2.76	4.02	29.6	2.5
Meat meal (low ash)*	1.29	2.28	2.52	55.0	44.7
Poultry byproduct meal*	1.36	2.50	3.26	56.2	47.7
Poultry byproduct meal (deboned)*	1.21	2.09	2.52	57.3	47.4
Poultry byproduct meal (low ash)*	1.09	1.65	2.50	60.0	50.8
Menhaden fishmeal	1.60	3.61	4.38	50.2	35.0
Peruvian fishmeal	1.39	2.92	3.75	58.0	43.9
Whitefish meal (deboned)	0.98	1.57	2.93	60.1	36.0
Whitefish meal (skin & bone)	2.74	7.41	8.51	25.0	11.8
Whitefish meal (whole)	1.56	3.50	5.65	38.5	17.2
Casein basal diet A[2]	0.73	----	0.99	82.1	----
Casein basal diet B[2]*	0.85	----	1.51	67.6	----

[1] See footnote 1 in Table 22.
[2] Approximately 37.3% of total P in the casein basal diet was supplied from dicalcium phosphate ($CaHPO_4$).
[3] Trace amount (< 0.05 %).

Table 24. Apparent digestibility of ash and availability of calcium in animal byproducts determined with rainbow trout.[1]

	Ash					Ca				
	Content			Digestibility		Content			Availability	
	Diet	Ingr.	Feces	Diet	Ingr.	Diet	Ingr.	Feces	Diet	Ingr.
	%	%	%	%	%	%	%	%	%	%
Menhaden fishmeal	9.0	20.4	28.1	43.3	26.2	2.35	6.67	10.54	18.4	14.5
Peruvian fishmeal	7.7	15.9	21.7	56.1	42.0	1.80	4.84	8.53	26.2	22.8
Meat & bone meal	12.2	31.1	34.9	35.9	22.4	3.74	11.32	14.57	12.4	9.5
Feather meal	5.1	7.2	9.7	69.5	56.6	1.03	2.29	3.53	45.4	47.7
Blood meal	3.5	2.0	6.4	79.3	79.9	0.36	0.04	1.91	40.0	12.4
Whitefish meal (deboned)	5.7	9.3	17.6	58.6	37.3	1.15	2.67	6.77	20.8	12.1
Whitefish meal (whole)	8.5	18.5	32.7	34.1	10.6	2.47	7.08	13.67	5.7	-0.1
Whitefish meal (skin & bone)	14.5	38.5	46.1	23.1	9.0	5.05	15.67	19.39	7.3	4.9
Casein basal diet[2]	4.1	----	6.5	79.2	----	0.49	----	2.20	40.9	----

[1] Fecal samples were collected by settling.
[2] Approximately 71.8% of total Ca in the casein basal diet was supplied as $CaHPO_4$.

Table 25. Apparent availability of copper and iron in animal byproducts determined with rainbow trout.[1]

	Cu						Fe					
	Content			Availability			Content			Availability		
	Diet	Ingr.	Feces	Diet	Ingr.	Basal	Diet	Ingr.	Feces	Diet	Ingr.	Basal
	ppm	ppm	ppm	%	%	%	ppm	ppm	ppm	%	%	%
Menhaden fishmeal	4.79	3.72	7.70	70.8	47.6	*NA*	359	966	1949	1.4	-5.7	7.1
Peruvian fishmeal	5.20	5.11	9.94	70.3	52.3	*NA*	160	303	908	11.8	-2.8	27.3
Meat & bone meal	5.07	4.68	9.47	58.0	6.2	*NA*	205	455	963	-5.2	-23.5	-15.6
Feather meal	13.37	32.33	28.67	65.8	61.3	*NA*	230	539	1150	20.6	16.2	*NA*
Blood meal	5.12	4.84	8.87	80.6	87.5	*NA*	967	2993	3951	54.2	56.0	*NA*
Whitefish meal (deboned)	4.28	2.03	11.04	65.4	-9.4	76.3	130	205	883	9.4	-14.9	17.7
Whitefish meal (whole)	4.05	1.26	10.99	53.7	-180.9	59.2	123	182	927	-27.6	-101.4	-49.5
Whitefish meal (skin & bone)	4.09	1.40	6.34	62.6	-70.3	69.8	129	201	584	-8.7	-54.0	-16.3
Casein basal diet[2]	5.24	----	8.79	77.8	----	77.8	98	----	514	31.0	----	31.0

[1] See footnote 1 of Table 24.

[2] Approximately 57.2% of total Cu and 60.8% of total Fe in the casein basal diet were supplied as $CuSO_4$ and $FeSO_4$, respectively.

Table 26. Apparent availability of potassium and sodium in animal byproducts determined with rainbow trout.[1]

	K					Na				
	Content			Availability		Content			Availability	
	Diet	Ingr.	Feces	Diet	Ingr.	Diet	Ingr.	Feces	Diet	Ingr.
	ppm	ppm	ppm	%	%	ppm	ppm	ppm	%	%
Menhaden fishmeal	7948	8893	922.9	97.9	95.0	4596	6544	4867	80.8	81.3
Peruvian fishmeal	7809	8430	543.7	98.9	98.0	6417	12614	4746	88.5	94.2
Meat & bone meal	7215	6450	494.9	98.5	96.1	5414	9271	8153	66.1	52.7
Feather meal	6031	2503	525.3	98.6	93.5	4084	4838	5333	79.2	77.0
Blood meal	5281	tr.	635.7	98.6	----	4626	6644	6002	85.4	92.2
Whitefish meal (deboned)	7097	6056	603.2	98.9	97.5	4249	5388	6170	80.5	80.8
Whitefish meal (whole)	6548	4226	697.8	98.2	93.4	4289	5521	4541	82.0	84.5
Whitefish meal (skin & bone)	6392	3706	476.4	98.2	92.8	4285	5508	6445	63.7	37.2
Casein basal diet[2]	7543	----	377.7	99.3	----	3761	----	5582	80.4	----

[1] See footnote 1 of Table 24.

[2] Approximately 100% of total K and 31.4% of total Na in the casein basal diet were supplied as KCl and NaCl, respectively.

Table 27. Apparent availability of magnesium and manganese in animal byproducts determined with rainbow trout.[1]

	Mg						Mn					
	Content			Availability			Content			Availability		
	Diet	Ingr.	Feces	Diet	Ingr.	Basal	Diet	Ingr.	Feces	Diet	Ingr.	Basal
	ppm	ppm	ppm	%	%	%	ppm	ppm	ppm	%	%	%
Menhaden fishmeal	2410	2713	3743	71.8	37.0	NA	41.8	61.8	211.1	8.3	-54.7	14.9
Peruvian fishmeal	2752	3853	4208	76.2	57.9	*NA*	28.0	15.6	156.6	12.9	-213.7	15.5
Meat & bone meal	2322	2420	4233	59.0	-8.1	85.9	27.9	15.5	109.7	11.7	-223.1	14.0
Feather meal	1789	643	1398	87.5	71.1	NA	25.9	8.9	134.9	17.1	-345.0	19.0
Blood meal	1682	287	1302	91.3	124.5	*NA*	22.4	tr.	68.9	65.5	----	63.0
Whitefish meal (deboned)	2318	2407	2760	84.0	71.9	*NA*	27.5	14.2	181.3	11.8	-243.3	13.9
Whitefish meal (whole)	2268	2240	3924	70.5	25.3	*NA*	31.4	27.2	193.6	-5.0	-185.9	-6.7
Whitefish meal (skin & bone)	2454	2860	4539	55.4	-8.1	85.2	43.7	68.0	171.7	5.2	-55.6	9.8
Casein basal diet[2]	2280	----	1805	89.5	----	89.5	33.3	----	104.5	58.4	----	58.0

[1] See footnote 1 of Table 24.

[2] Approximately 79.4% of total Mg and 74.1% of total Mn in the casein basal diet were supplied as MgO and $MnSO_4$, respectively.

Table 28. Apparent availability of strontium and zinc in animal byproducts determined with rainbow trout.[1]

	Sr					Zn					
	Content			Availability		Content			Availability		
	Diet	Ingr.	Feces	Diet	Ingr.	Diet	Ingr.	Feces	Diet	Ingr.	Basal
	ppm	ppm	ppm	%	%	ppm	ppm	ppm	%	%	%
Menhaden fishmeal	28.0	85.8	125.2	18.9	16.3	155	236	477	44.1	2.9	NA
Peruvian fishmeal	29.7	91.4	138.3	27.6	25.9	131	155	364	56.7	16.9	*NA*
Meat & bone meal	24.8	75.1	95.0	14.0	10.6	133	162	338	42.8	-19.4	67.5
Feather meal	9.5	24.0	32.2	45.8	45.2	147	209	412	55.4	24.1	NA
Blood meal	3.3	3.4	12.8	56.6	76.3	98	45	188	78.5	76.9	*NA*
Whitefish meal (deboned)	45.5	144.2	227.2	33.1	32.4	127	141	413	56.3	11.9	*NA*
Whitefish meal (whole)	107.8	351.7	587.2	7.1	6.3	125	133	485	33.6	-61.5	49.5
Whitefish meal (skin & bone)	234.0	772.4	890.8	8.2	7.8	133	164	410	26.1	-63.6	41.5
Casein basal diet[2]	3.3	----	12.8	47.8	----	120	----	194	78.7	----	78.7

[1] See footnote 1 of Table 24.

[2] Approximately 24.9% of Zn in the casein basal diet was supplied as $ZnSO_4$.

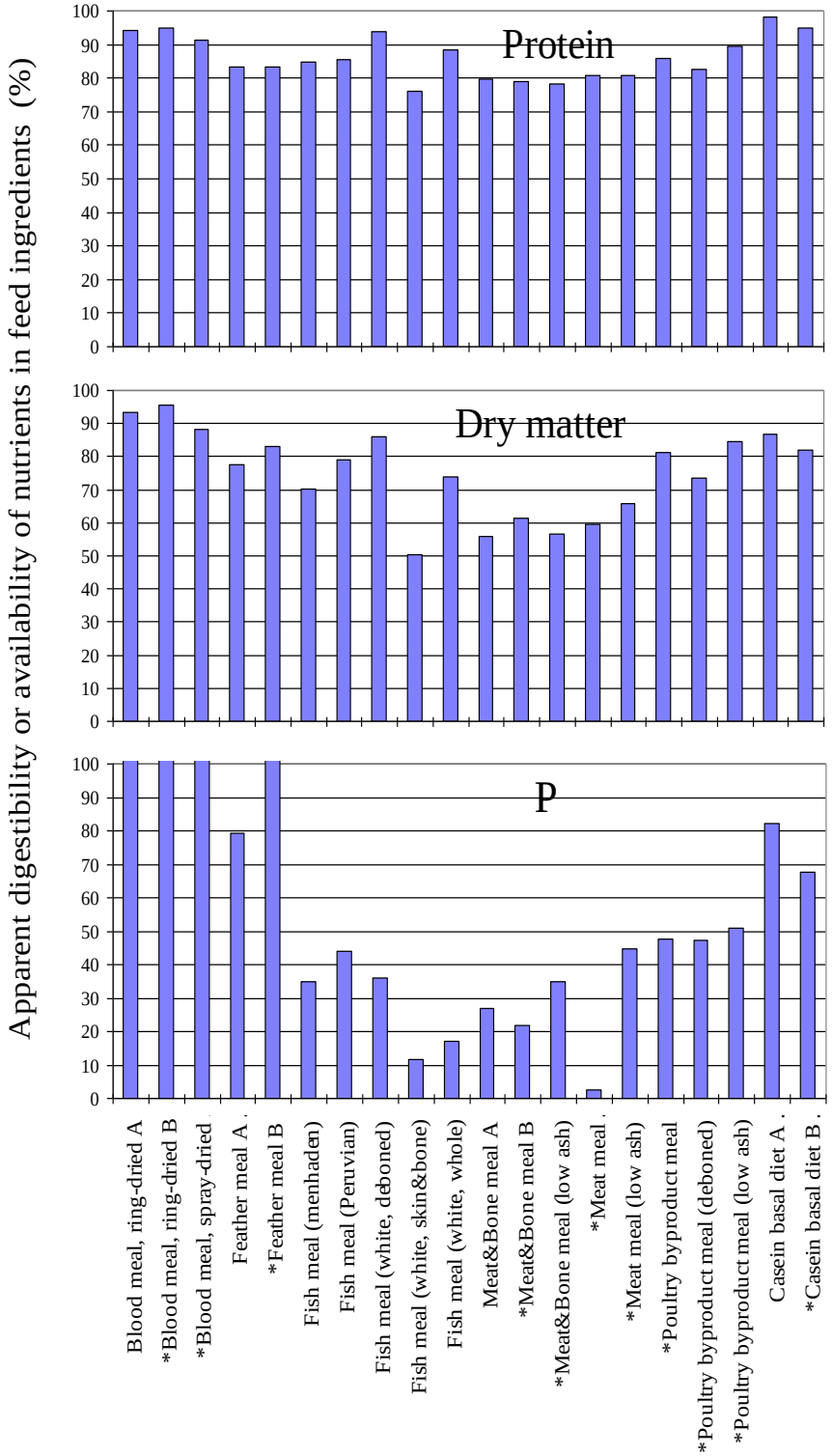


Figure 10. Apparent digestibility of protein, dry matter and availability of phosphorus in various animal byproducts. Fecal samples were collected either by settling (test ingredients without asterisk) or by stripping (with asterisk).

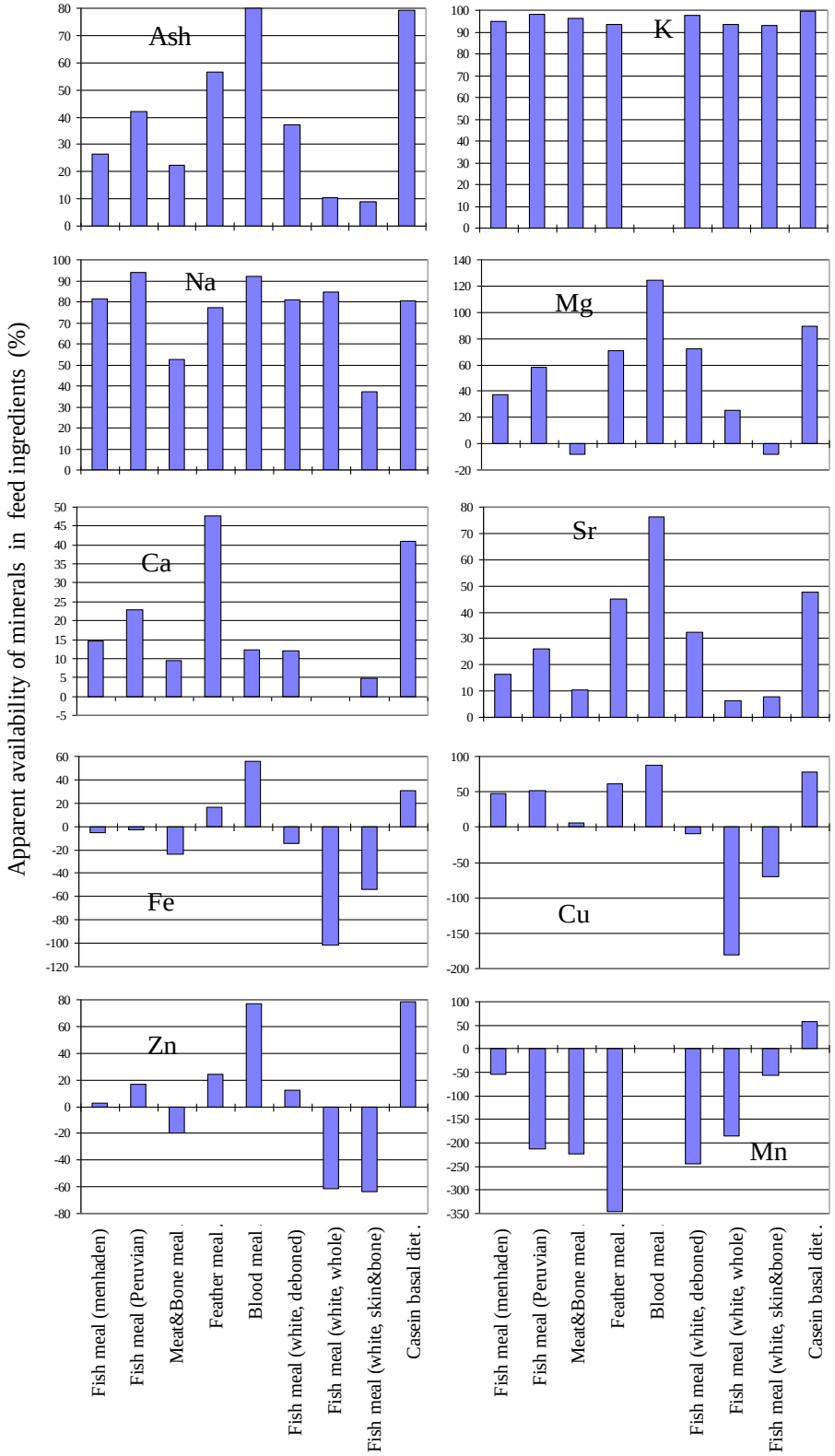


Figure 11. Apparent availability of minerals in animal byproducts.

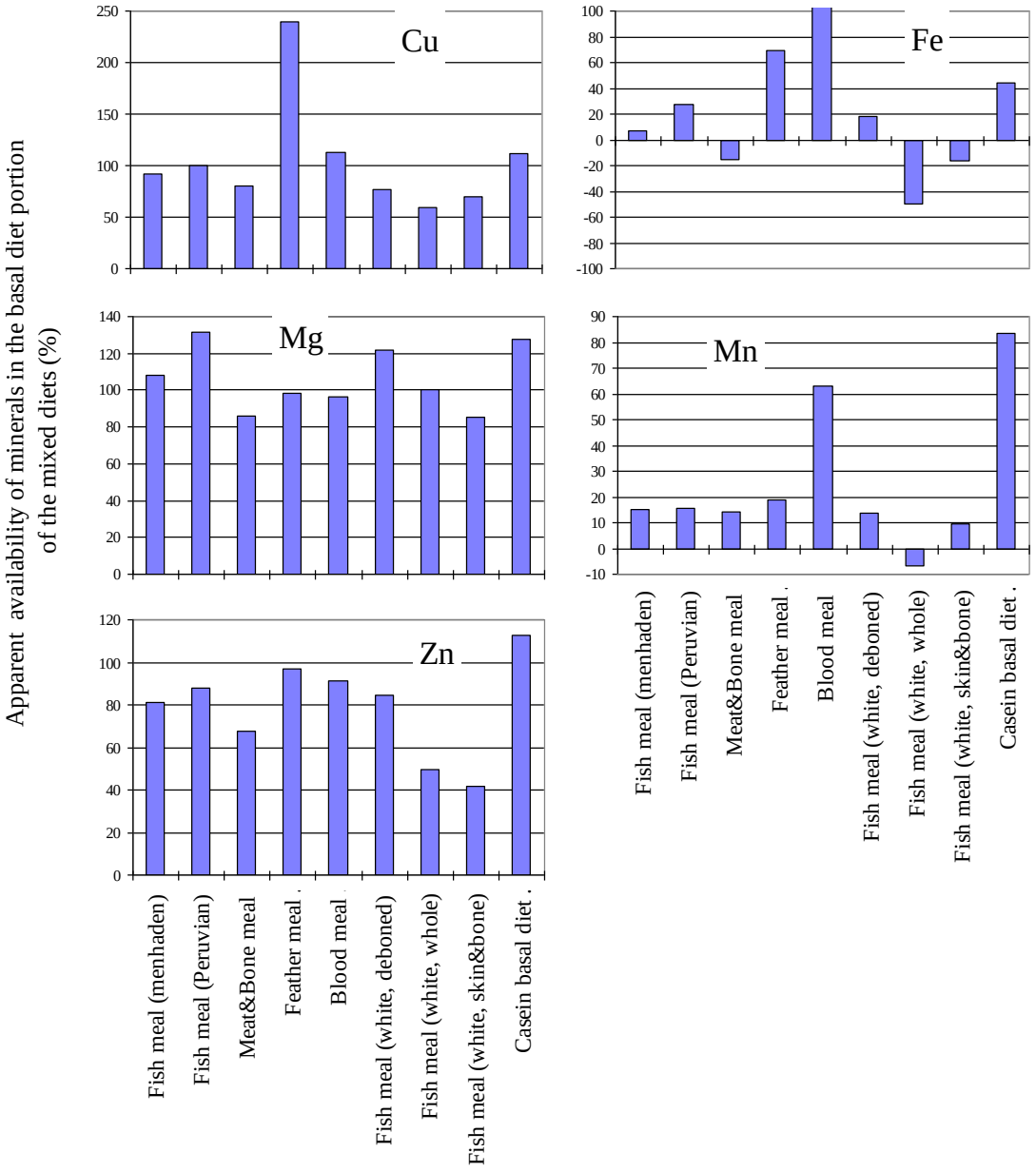


Figure 12. Apparent availability of minerals in the basal diet portion of the test diets (assuming minerals supplied from the test ingredient portion in the diet are totally unavailable). Note: the apparent availability of minerals is often lowered when mixed with test ingredients than when the casein basal diet was fed alone (right end).

3. Availability of Minerals in Fish Bone at Various Dietary Levels

ABSTRACT The apparent availability of macro (Ca, K, P, Mg, Na) and micro (Cu, Fe, Mn, Sr, Zn) elements in fish bone was determined using rainbow trout (initial mean body weight 110 g). Yttrium oxide (Y_2O_3) and chromium oxide (Cr_2O_3) were used as the inert markers, and passive feces collection tanks were used to collect fecal samples. The basal diet was prepared using deboned whitefish meal, and the intrinsic fish bone was replaced to make experimental diets containing incremental concentrations of fishbone. The apparent availability was determined as the fractional net absorption of dietary minerals. The apparent availability of phosphorus, calcium, magnesium, iron, zinc, and strontium in fish bone decreased ($P < 0.05$) as the fish bone content in the diet increased.

BACKGROUND

Fishbone is the major source for macro and micro elements in commercial fish feeds. The apparent availability of minerals in fish meal was, however, found to be quite low. The low availability might be explained by two possible effects; 1) fish are simply unable to efficiently digest those minerals contained in fishmeal or 2) fish have received sufficient minerals from other sources such as inorganic mineral supplements added to the diet and regulated to digest or absorb excess amounts from fish meal to maintain homeostasis of those minerals. The purpose of this research was to examine efficiency of digestion and absorption of minerals in fishbone tissues at different dietary concentrations.

MATERIALS AND METHODS

Whitefish meal was used as the basal ingredient. Fish bone was obtained by sieving the fishmeal, repeatedly washing with tap water, and air-drying for two days. This intrinsic fish bone was returned to the deboned meal at 0, 2, 5, and 10 percent per dry diets to prepare experimental diets differing in the bone contents (Tables 29 and 30). No chemical supplements for macro and micro elements was used to assure that most elements in the test diets were from the fish bone and the fish meal. Five rainbow trout (initial mean body weight: 110.85 g, s=13.35, n=60) were stocked in each of twelve digestibility tanks (150 L, modified Guelph type). Three tanks were randomly assigned for each diet. Fish were fed once daily at 1800 h at the rate of 1.35 % (dry basis) per body weight. Feces were almost totally collected twice daily at 0800 h and 1730 h for five consecutive days following seven days of acclimation to each test diet. Biofiltered and recirculated water with temperature-controlled at 17.0 ± 0.5 °C was supplied to each tank at 3 L/min with continual auxiliary supply of dechlorinated municipal water to the entire system to maintain optimal water quality and to prevent accumulations of any nutrients in the recirculating water. The fecal samples collected over the period of five consecutive days were pooled and analyzed for minerals by inductively coupled plasma emission spectrophotometry. The

apparent availability coefficients were calculated as the fractional net absorption (net absorption/intake) of nutrient based on chromium (Cr) and yttrium (Y) as inert markers.

RESULTS AND DISCUSSION

The apparent availabilities of Ca, P, Mg, Fe, Zn, and Sr decreased ($P < 0.05$) as the bone content increased in the diets (Table 31). When phosphorus concentration in the diet was 0.60% (control diet) that was the level equivalent to the dietary requirement (NRC, 1993), the availability was 72.0% (Cr basis) or 72.9% (Y basis). When dietary phosphorus concentration was 1.84% (10% diet) that was about three times the requirement level, however, the availability decreased to 15.8% (Cr basis) or 18.9% (Y basis). If the dietary concentrations are multiplied by the corresponding availability values to express actual amount absorbed by fish, they will be 0.44% P per diet (control diet) and 0.32% P per diet (10% diet). These two values indicate that the fish fed high fish bone diet will be more vulnerable to the phosphorus deficiency than the fish fed deboned fishmeal. Deboned fish meal which served as the control in this trial still contained substantial amount of the residual bone. Figure 13 shows the prediction of P availability when bone content in diets is approaching zero based on the Ca concentration as proportional to the bone level. The figure indicates that the availability of P in fish bone could be above 90 % when its concentration is very low, whereas the availability decreases linearly ($P < 0.01$) thereafter. In either case, the requirement can not be met by the intrinsic fish bone alone, which indicates the necessity of external P supplements. Wilkinson (1976) states that the gastrointestinal absorption of P is unregulated in that net absorption is directly proportional to the amount ingested, and that the phosphorus homeostasis depends primarily on the mechanisms that govern the renal excretion of phosphorus. This unregulated uptake of P, however, has been disproved in the present study, possibly because bone was used as the source of P rather than soluble inorganic phosphates. Calcium ions contained in hydroxyapatite or bone phosphate might exert an antagonistic effect for the absorption of P (Nakamura, 1982; Hoek et al., 1988; Al-Masri, 1995). Also, hydroxyapatite might not be efficiently solubilized at the normal gastric pH of fish or possibly soluble phosphates might be precipitated as Ca-phosphates in the intestine upon neutralization by pancreatic juice and bile.

The addition of fish bone at dietary levels as low as 2 % induced the dispossession (negative balance) of Ca, Cu, Fe, Mn, and Zn from the fish. Relatively large deviations within treatment indicated that the apparent absorption of minerals, unlike macro nutrients, may not be constant but the fish may discharge some mineral elements periodically The fecal samples in this study were collected over five consecutive days following a seven day acclimation period to the test diet. In the previous study with coho salmon (ref. Study 1 in Ch. 2), large day-to-day variations of mineral availability (absorption) were observed, indicating that a longer monitoring period might be required and also that diet history could be of significant importance in the mineral availability studies as suggested by Schwartz et al. (1986). This

is, in fact, a critical point in relation to the study of the dietary requirement of micronutrients by means of the balance technique, where experimental animals need to be well-acclimatized and adapted to the experimental diet. To determine dietary nutrient availability, however, any balance or acclimation needs to be eliminated (for detail, see Iron section, p 61).

The apparent availabilities of most minerals decreased as the bone level increased as was indicated by the inverse correlations (Table 32). It may not be possible, however, to determine exactly which specific element was responsible for the antagonistic behavior to the absorption of those elements because the dietary concentration of most elements increased in proportion to the bone level in the diet. It is therefore only appropriate to state, in general terms, that the bone per se is responsible to the reduced availability of minerals. Although mineral availabilities (% of intake) were significantly and negatively correlated to the dietary bone content, the actual amount absorbed by fish (g or mg of minerals per kg of feeds) was not largely different among the various treatments (dietary bone content), suggesting that fish were simply not utilizing any excess portion of minerals in diets (bones), which may be related to the limited secretion of gastric acid and the gastric retention time.

The present study, however, provides another important suggestion for the practical feed formulation in that increasing bone concentration in fishmeal not only reduces the absorption of some essential trace elements but it may induce the dispossession of those elements which fish originally retain (endogenous minerals). Long term effects of feeding high levels of fish bone in diets need to be studied since the continual discharge of those elements will eventually result in a clinical deficiency and the impairment of normal growth of fish.

Table 29. Composition of the experimental diets.

Diet	C	2%	5%	10%
Deboned fishmeal[1]	50	50	50	50
Gelatin[2]	10	10	10	10
Dextrin[3]	12	12	12	12
Fish Bone[1]	0	2	5	10
α-cellulose[4]	10	8	5	0
CMC[5]	1	1	1	1
Vitamins[6]	2	2	2	2
Vitamin C[7]	0.1	0.1	0.1	0.1
Choline (70%)	0.5	0.5	0.5	0.5
Fish oil	14	14	14	14
Y_2O_3	0.2	0.2	0.2	0.2
Cr_2O_3	0.2	0.2	0.2	0.2
(Water)	40	40	40	40

[1] From whitefish meal.
[2] Type B, bovine, 225 bm (Sigma Chemical Co., St. Louis, MO).
[3] From corn, 12 % water-soluble (ICN Biomedicals, Inc., Aurora, OH).
[4] Alpha-cellulose powder (Sigma Chemical).
[5] Carboxymethyl cellulose (Sigma Chemical).
[6] Abernathy No.2 pre-mixture (Hoffmann-La Roche, Inc.).
[7] Fat-coated ascorbic acid; 90% active; F-90 (Takeda USA, Inc.).

Table 30. Concentrations[1] of minerals in the experimental diets.

Diet	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn	Y
C	10284	1539	37	145	3040	1050	5.5	7115	6006	57	105	1663
2%	14935	1562	53	123	3236	1121	6.3	7696	8063	77	62	1641
5%	24496	1532	93	127	3944	1272	10.5	7247	12482	124	122	1631
10%	36379	1539	42	124	3635	1469	16.4	8324	18384	183	31	1613

[1] μg/g dry diet.

Table 31. Apparent availability[1] of minerals in the fish bone-gradient diets.

	Fish bone[2] %			
	0	2	5	10
DM	78.1	79.7	81.6	80.0
	(77.3)	(80.1)	(81.7)	(79.2)
Ca	22.0 a	14.6 ab	25.2 a	-6.4 b
	(17.9) a	(15.8) a	(25.5) a	(-10.5) b
Cr	4.36	-1.53	-0.45	3.75
	(0.00)	(0.00)	(0.00)	(0.00)
Cu	39.4	-353.5	34.5	-275.9
	(40.4)	(-348.6)	(34.2)	(-295.3)
Fe	9.69 a	-2.45 a	-1.61 a	-35.44 b
	(4.90) a	(-0.81) a	(-1.13) a	(-40.79) b
K	114.8	92.9	105.4	119.8
	(115.8)	(92.9)	(105.5)	(120.4)
Mg	70.7 a	65.1 a	62.6 a	47.6 b
	(69.0) a	(65.7) a	(62.8) a	(45.6) b
Mn	16.81	-7.94	4.83	-37.75
	(7.45)	(-6.80)	(5.27)	(-42.86)
Na	45.0	48.9	59.7	49.1
	(43.0) a	(50.0) a	(59.8) b	(47.2) a
P	72.9 a	60.5 ab	52.8 b	18.9 c
	(72.0) a	(61.3) ab	(52.9) b	(15.8) c
Sr	34.6 a	24.3 a	30.9 a	1.3 b
	(31.0) a	(25.5) a	(31.2) a	(-2.5) b
Zn	115.6 a	-170.6 bc	49.8 ac	-375.8 b
	(111.4) a	(-169.3) bc	(49.6) ac	(-398.4) b
Y	0.00	0.00	0.00	0.00
	(-7.25)	(0.92)	(0.44)	(-3.95)

[1] The apparent availability values (% of intake) were determined based on yttrium (values in parentheses were those based on chromium). Each value represents the average of three tanks. Values in rows with common letters are not significantly different ($P > 0.05$) by Duncan's new multiple range test.

[2] Purified ground fish bone from whitefish. Expressed as the percentage of fish bone added to the basal diet (air-dry basis).

Table 32. Correlation coefficients[1] (r) between dietary concentration of minerals and the apparent availability[2] of minerals.

	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
Diet					Availability					
Bone	-0.62 *	-0.26	-0.80 **	0.35	-0.91 **	-0.53	0.18	-0.92 **	-0.73 **	-0.67 *
Ca	-0.59 *	-0.22	-0.78 **	0.35	-0.89 **	-0.51	0.21	-0.91 **	-0.70 *	-0.64 *
Cu	0.40	0.26	0.15	-0.29	0.06	0.14	0.56	0.04	0.27	0.28
Fe	0.37	0.53	0.52	0.30	0.58 *	0.43	-0.27	0.59 *	0.49	0.63 *
K	-0.08	0.10	-0.37	0.07	-0.51	-0.20	0.51	-0.53	-0.23	-0.16
Mg	-0.59 *	-0.22	-0.79 **	0.36	-0.90 **	-0.51	0.20	-0.91 **	-0.71 **	-0.64 *
Mn	-0.61 *	-0.19	-0.79 **	0.42	-0.90 **	-0.51	0.16	-0.91 **	-0.71 **	-0.63 *
Na	-0.77 **	-0.60 *	-0.80 **	0.18	-0.84 **	-0.60 *	-0.09	-0.83 **	-0.82 **	-0.88 **
P	-0.59 *	-0.23	-0.79 **	0.36	-0.90 **	-0.51	0.20	-0.91 **	-0.71 **	-0.65 *
Sr	-0.59 *	-0.22	-0.79 **	0.36	-0.90 **	-0.51	0.20	-0.91 **	-0.71 *	-0.64 *
Zn	0.75 **	0.66 *	0.69 *	-0.13	0.68 *	0.55	0.25	0.66 *	0.75 **	0.84 **

[1] Simple linear correlation. Values with asterisks are statistically significant (* P< 0.05, ** P< 0.01).
[2] Fractional net absorption (%/ intake) based on yttrium.

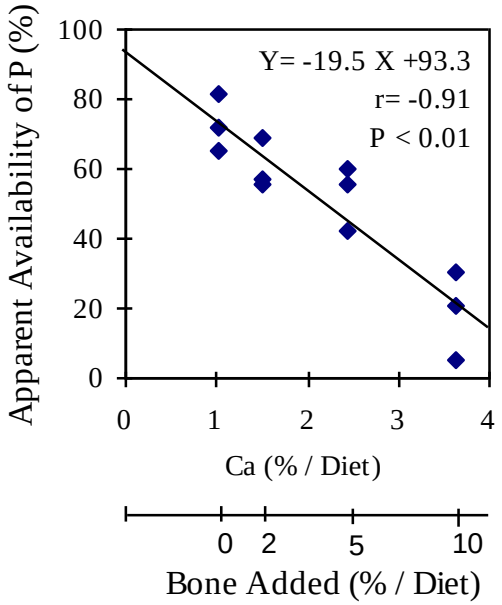


Figure 13. Apparent availability of phosphorus in fish bone.

4. Availability of Minerals in Diets at Different Water Hardness

BACKGROUND

Dietary calcium and other bone minerals exert antagonistic effects on the absorption of many minerals by reducing their absorption from the digestive tract, presumably by direct- or co-precipitation at neutral pH in the intestine. Because fish absorb and utilize not only dietary calcium, but water-borne calcium at least equally well (Steffens, 1989), calcium in ambient water could also reduce absorption of dietary minerals. When calcium concentration in water is low, fish need to absorb dietary calcium, which reduces the concentration of calcium in the intestinal lumen and thus may reduce its antagonistic effect to the other minerals. Also, physiological response to the reduced calcium intake from diets may elevate the concentration of hormones such as calcitriol (1,25-dehydroxycholecalciferol) in the circulatory system of fish, which also has an effect to increase phosphorus and magnesium absorption at the intestinal brush border. Conversely, when calcium concentration in the ambient water is high, fish may reduce absorption of calcium from the gastrointestinal tract. Although the result is highly inconclusive, Scarpa and Gatlin (1992) reported somewhat different dietary zinc requirements and tissue mineralization in channel catfish reared in hard and soft water. This may indicate that the water-borne calcium also affect trace mineral availabilities in diets. There is little information currently available in the literature regarding the effect of water-borne calcium or water hardness on the absorption of dietary minerals in fish.

This study was conducted to determine whether water-borne calcium influences the absorption of dietary minerals, with particular interest in phosphorus absorption, and whether different dietary regimes are required at different water hardnesses.

MATERIALS AND METHODS

A casein-gelatin semi-purified test diet was prepared as cold-extrusion pellets (Table 33) and stored at –20°C until fed. Fifteen rainbow trout (initial mean weight 236.3 g) were stocked into three 150 L-FRP digestibility tanks (modified Guelph type; Hajen et al., 1993), each of which was supplied with soft-water (total hardness ca. 34 ppm as $CaCO_3$) during the first two weeks, then hard water (total hardness ca. 310 ppm as $CaCO_3$) during the following two weeks, and again soft water (total hardness ca. 34 ppm as $CaCO_3$) during the last two weeks at 5 L/min./tank from a common recirculation-filtration system. Water hardness was adjusted by dissolving calcium sulfate ($CaSO_4 \cdot 2H_2O$, min. 99%, obtained from Sigma Chemical) into the recirculating water. During the 6 week-feeding period, water temperature, pH, total alkalinity, total ammonium nitrogen ranged 18 ± 1°C, 7.2-7.5, 25-40 ppm $CaCO_3$, 0.2-0.7 ppm NH_4-N, respectively. Fish were fed once daily a constant amount of feed (18 g per tank; approximately 1.15 % per body weight). The fecal samples were collected twice daily at day-9, -14 (1st

and 2nd week with soft water), day-1, -6, -14 (3rd and 4th week with hard water), and day-1, -6, -14 (5th and 6th week with soft water), and analyzed for mineral concentrations as described previously (ref. Study 1 in Ch. 2).

RESULTS AND DISCUSSION

There was no notable difference in the fecal mineral content during the soft or hard water periods (Fig.14), indicating that the absorption (availability) of dietary minerals is unaffected by the calcium concentration in the ambient water. The diet used in the present study was, however, prepared with purified ingredients with very low calcium content. In practical feeds, the content of minerals and their inherent availability are considerably different, suggesting there could be a different effect of water-borne calcium with practical feeds. Also, gypsum was used (due to its high solubility) in this study to increase calcium hardness, whereas under practical situations, calcium in hard water is more likely to be contributed from less soluble calcium carbonate with proportionately higher alkalinity. Differences in calcium source, alkalinity and, possibly, pH might otherwise affect the absorption of dietary minerals. The present research did not cover these potentially important water quality parameters.

Table 33. Composition of the diet.

Ingredient	%
Casein[1]	35
Gelatin[2]	8
Dextrin[3]	15
Alpha-cellulose	8
Fish oil	25
Vitamin mix.[4]	4
Mineral mix.[5]	4
Amino acid mix.[6]	1
Yttrium oxide (Y_2O_3)	0.05
Trace mineral soln.[7]	30
Total dry matter	100

[1] Low trace element (ICN Biomedicals).
[2] 225 bloom, type IV (Sigma Chemical Company).
[3] 80% soluble, corn (ICN Biomedicals).
[4] Vitamin mix. supplied the following amount (g per 100g diet, dry basis): Abernathy No.2 formula (Hoffmann-La Roche, Inc.), 2.8; coated ascorbic acid (Takeda USA, Inc.), 0.2; choline-HCl, 0.8; inositol, 0.2.
[5] Mineral mix. supplied the following amount (g per 100g diet, dry basis): $MgSO_4.7H_2O$, 0.5; NaH_2PO_4, 1.6; KCl, 1.4; NaCl, 0.5.
[6] Amino acid mix. supplied the following amount (g per 100g diet, dry basis): L-Arg, 0.5; L-Met, 0.5.
[7] Supplied following minerals per kg dry diet: Cu 3.0 mg (as $CuSO_4 \cdot 5H_2O$); I 1.1 mg (as KI); Fe 60.0 mg (as $FeSO_4 \cdot 7H_2O$); Mn 13.0 mg (as $MnSO_4 \cdot H_2O$); Zn 30.0 mg (as $ZnSO_4 \cdot H_2O$); Se 0.3 mg (as Na_2SeO_3); Co 0.86 mg ($CoCl_3 \cdot 6H_2O$).

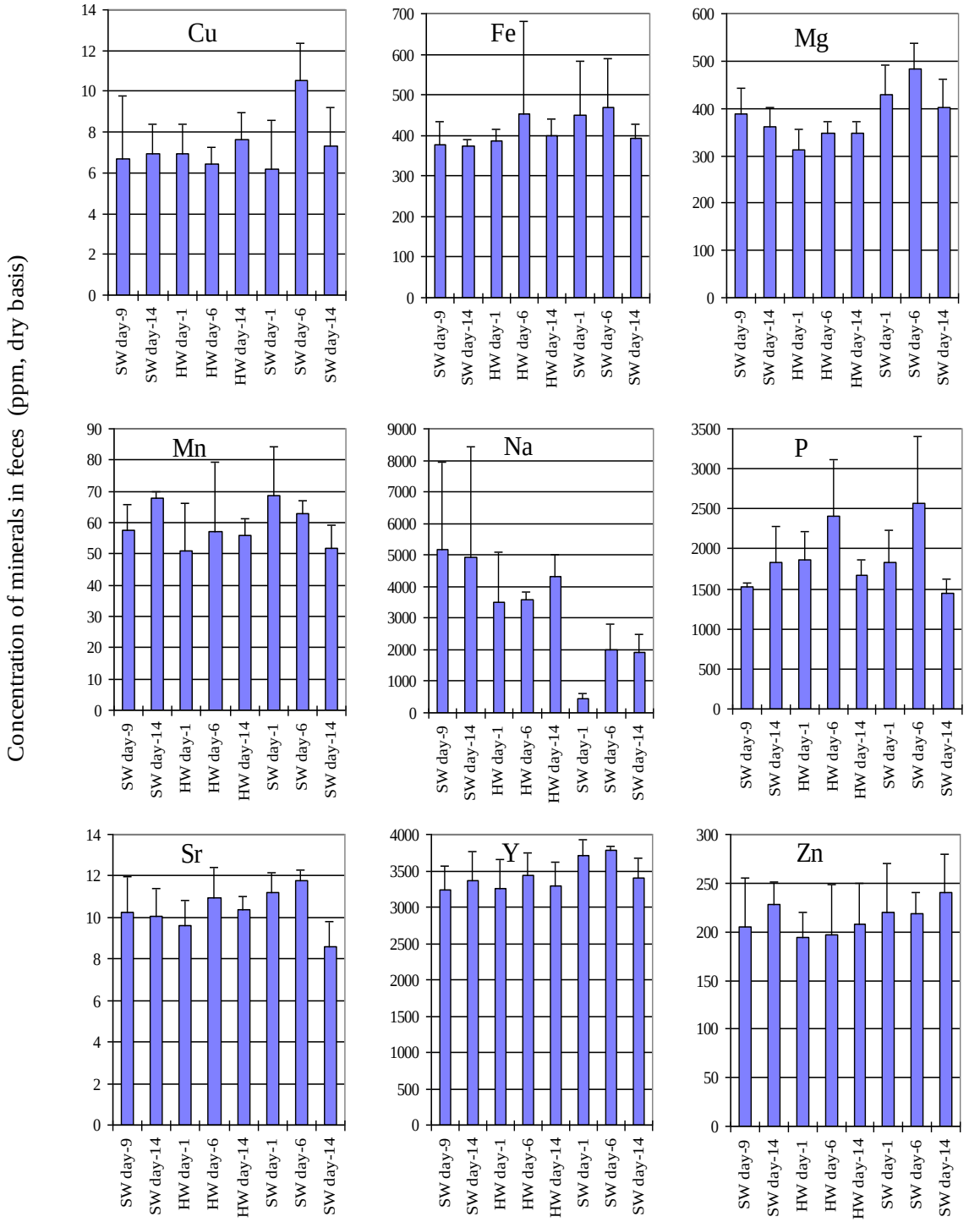


Figure 14. Fecal mineral content (ppm, dry basis) in rainbow trout placed in soft-water (SW) and hard-water (HW). Fish received the same diet (Table 33) throughout the period. Each bar represents the average of 3 tanks (sem. as error bars).

5. Availability of Minerals in Mutant Low-Phytic Acid Grains

ABSTRACT Approximately two-thirds of total phosphorus in various grains is present as phytate, which is not well-utilized by fish and many other monogastric species. Besides its low availability of phosphorus, phytate has been known to interact with various dietary nutrients to reduce their availability to the animals. Certain single-gene non-lethal mutations cause the plant to store most of the phosphorus as inorganic-phosphorus instead of as phytate-phosphorus. Theoretically, using these low-phytate mutant grains in animal feeds should reduce phosphorus excretion by the animals without any additional cost and effort. This study was conducted as the first step in this direction to determine the biological availability of phosphorus in the low-phytate mutants of barley, dent corn and flint corn, and their effect, if any, on the availability of other minerals in complex feeds using rainbow trout as a model species. Feeding trials demonstrated that the apparent availabilities of phosphorus in low-phytate grains were significantly higher than that in the ordinary grains of the same variation when they were mixed with low-ash ingredients. Fecal phosphorus content (in average) decreased 50.2% (in phytate-phosphorus) or 42.9% (in total phosphorus) by replacing ordinary grains with low-phytate grains in the low-ash diets. The apparent availabilities of calcium, iron, zinc and strontium also were significantly higher in the low-ash diet containing low-phytate dent corn than that containing ordinary dent corn; however, no such increase was observed with low-phytate barley or low-phytate flint corn over their counterpart grains. The apparent availability of strontium was higher in all of the three mutant grains than in the ordinary grains. The apparent availabilities of copper, manganese, magnesium, potassium and sodium, and the apparent digestibility of dry matter were not significantly different between ordinary and low-phytate grains. In contrast to the low-ash diets, no difference on the apparent availabilities of phosphorus and other minerals was observed when they were included in either casein- or fish meal-based diets that had higher mineral or ash contents than the dietary requirements for fish (typical in commercial fish feeds). The present study holds an important implication to achieve a substantial reduction of phosphorus discharge from fish, poultry and animal farms without any additional cost or effort but by simply replacing ordinary grains with low-phytate mutant grains in the production feeds of marginal phosphorus content, which we call environmentally friendly feeds.

BACKGROUND

In most bodies of water, especially in freshwater, phosphorus is the most important limiting nutrient for the growth of phytoplankton and algae, which reduce dissolved oxygen levels in aquatic ecosystems (Miller et al. 1974; Beveridge 1984; Boyd 1990). This eutrophication thereby alters local habitats and, in extreme cases, causes a destruction of the environment. The most logical and

efficient way to reduce the environmental impact of intensive aquaculture is, therefore, to reduce phosphorus discharge into the effluent water.

The ultimate source of phosphorus in aquaculture effluent is feeds. The concentration and availability of phosphorus in the feed are the two most important factors that affect the retention of phosphorus in ingested feeds by the body of the fish. Approximately two-thirds of total phosphorus in various grains is present as phytate or inositol hexaphosphate, which is not well-utilized by fish (Ogino et al., 1979; NRC, 1993) and other monogastric species (NRC, 1984; NRC, 1988). Besides its low availability of phosphorus, phytate has been shown to interact directly and indirectly with various dietary components to reduce their availability to the animals. For example, calcium-bound phytate increases chelation with trace minerals such as zinc to form co-precipitates (Anon., 1967). Phytate may decrease endogenous zinc reabsorption as well as affect bioavailability of dietary zinc (Morris, 1986). Increasing the phytate level from 1.1 to 2.2% in channel catfish diets containing 50 mg zinc/kg decreased weight gain, feed efficiency and zinc content in the vertebrae (Satoh et al., 1989). With 1.1% phytate in diets, channel catfish require about 200 mg zinc/kg feed, which is 10 times higher than the dietary requirement of available zinc (Gatlin and Wilson, 1984).

Certain single-gene mutations in corn, barley, rice and soybeans cause the plant to store most of the phosphorus as inorganic phosphate instead of as phytate phosphorus (Raboy et al., 1984; Raboy et al., 1989; Raboy, 1990, 1997; Ertl et al., 1998). Theoretically, using these mutant grains containing lower levels of phytate in fish or animal feeds should reduce phosphorus excretion by the fish or animals without any additional cost and effort. This study was conducted as a first step in this direction to determine the biological availability of phosphorus in the mutant low phytate grains, and their effect, if any, on the availability of other minerals in complex feeds using rainbow trout as a model species.

MATERIALS AND METHODS

Test ingredients (ordinary grains vs. low-phytate mutant grains)

Six ingredients (grains) were evaluated: three ordinary grains --- barley (B), dent corn (DC) and flint corn (FC) --- and three low-phytic acid mutant grains of the same variety --- low-phytic acid barley (BLP), low-phytic acid dent corn (DCLP) and low phytic acid flint corn (FCLP). The analytical compositions of these grains are presented in Tables 34 and 35. Prior to mixing with basal diets or other ingredients, test ingredients (grains) were finely ground using a Wiley mill (97%, < 1 mm), weighed and steam-cooked (with added water, 1:1 ratio, 0.7kg/cm^2 = ca. 115°C) for 10 min. to gelatinize the starch portion of the grains. In previous experiments with soybean meal, this procedure did not affect phytate or inorganic phosphorus content (ref., Study 5, Ch.3).

Availability of minerals in complex diets I (Grain 30% + Casein diet 70%)

A casein-gelatin based semi-purified diet (basal diet; Table 36) was mixed with test ingredients (barley or corn) at 7 to 3 ratio (by weight, dry basis), cold-extruded into pellets, dried at room temperature, and stored at 0-4°C until fed. The analytical compositions of the casein diet and the complex diets are given in Table 37. Forty-two rainbow trout (initial mean body weight, 275.3g; sem, 3.88) were stocked into seven 145L-fiberglass tanks supplied with spring water (14.5°C) at ca.10L/min. Each fish was fed the casein diet once daily at 1% of the body weight (dry basis) for 7 days by the method of Post et al. (1965). On day eight, the casein diet was switched to one of the complex diets in six of the tanks with one tank continuing to receive the casein diet (the control group), and the feeding was continued for another seven days. The feeding level for different dietary treatments was not adjusted although they had different digestible energy levels. After seven days of feeding the complex diets, fish were anesthetized with tricaine methane sulfonate (MS-222), feces were collected by stripping, pooled by treatment (6 fish per treatment), dried (105°C-12h), and analyzed for mineral content.

Availability of minerals in complex diets II (Grain 30% + Fishmeal diet 70%)

A fish meal-based diet (basal diet; Table 36) was mixed with each of the six test ingredients at 7:3 ratio (by weight, dry basis), cold-extruded into pellets containing approximately 30% moisture, and stored at -20°C until fed. The analytical compositions of the fishmeal diet and the complex diets are given in Table 37. Two hundred rainbow trout (initial mean body weight, 167.7g; sem, 3.01) were stocked in 21 145L-fiberglass tanks (10 fish per tank) receiving spring water (14.5°C) at ca. 10L/min.. Three tanks were randomly assigned to each dietary treatment, and fish were fed the fishmeal diet once daily, as much as they would consume, for seven days. On day eight, the fishmeal diet was replaced with one of the complex diets except for the control group that continually received the fishmeal diet, and the feeding was continued for another seven days before fecal samples were collected as described above. Samples from each fish were pooled by tank, and analyzed for mineral content.

Availability of minerals in low-ash diets (Grain 50% + low-ash ingredients 50%)

Test diets were formulated with semi-purified ingredients of low mineral contents except for the test ingredients (barley or corn) that comprised 50% of the diets (Tables 36 and 37). The test diets were prepared as moist pellets, and stored at -20°C until fed. One hundred and eighty rainbow trout (initial mean body weight, 175.4; sem, 6.4) were stocked in 18 145L-fiberglass tanks (10 fish per tank) supplied with 10L/min of spring water (14.5°C), and three tanks were randomly assigned for each of six dietary treatments. Fish were fed the test diets once daily to apparent satiation for seven days before fecal samples were collected by stripping. Fecal samples of each fish were pooled by tank. A portion of the fecal samples from each tank was analyzed for phytate, and the remaining portion was dried, ashed and analyzed for minerals.

Analysis and calculation

Samples of test ingredients and fresh feces were acid-extracted (0.65N-HCl, 3 h at room temperature with continuous shaking), centrifuged (1000 × g, 10 min), eluted through anion exchange column chromatograph (AG1-X4 resin, 100-200 mesh, Bio-Rad Laboratories, Hercules, CA) by the procedure of Harland and Oberleas (1986), and analyzed for phytate content according to Latta and Eskin (1980). The concentration of phytate was determined using sodium phytate (dodecasodium salt, from rice, purity 99%, water 12%, Sigma Chemical, P3168) as the standard. A remaining portion of the acid extract of test ingredients was neutralized and analyzed for the inorganic phosphorus content (Taussky and Shorr, 1953).

Fecal and diet samples were dried (105°C-6h), ashed (550°C-12h), and dissolved in a hydrochloric-nitric acid mixture (1:1, v/v) and left at room temperature for 12 h (or when samples contained added chromium, dissolved in a sulfuric-perchloric acid mixture (1:1, v/v) and heated to boiling to oxidize chromium in the samples). The acidified samples were diluted to an appropriate volume and analyzed for minerals as described previously (ref. Study 1 in Ch. 2).

Apparent availability (digestibility) of minerals in diets was determined as a fractional net absorption of each mineral from diets based on yttrium as the non-absorbable indicator. Apparent availability of minerals in ordinary and low-phytic acid grains was compared using a two tailed t-test. The difference was considered significant at $P< 0.05$. Fish were handled and treated in accordance to the guidelines approved by the Animal Care & Use Committee of the University of Idaho.

RESULTS

In the complex diets (with casein diet or fishmeal diet), there was little or no effect of low-phytate grains on the apparent availabilities of minerals (Table 38). The casein diet also had similar values to the complex diets in mineral availabilities. The fishmeal diet had lower availabilities of Fe and P than did the complex diets. The apparent digestibility of dry matter was similar in all dietary treatments.

In the test diets (low-ash), however, there was a marked positive effect of low-phytate grains on the apparent availabilities of phosphorus and strontium (Table 38; Fig.1). Fecal phytate-P and total-P were significantly lower in fish fed low-phytate grains than those fed ordinary grains in the low-ash test diet (Table 39; Fig.1). Fecal phosphorus content decreased ca. 50.2% in average (in phytate-phosphorus) or 42.9% in average (in total phosphorus) by replacing ordinary grains with low-phytate grains in the low-ash diet. The apparent availabilities of Ca, Fe and Zn also were significantly higher in the test diet containing low-phytate dent corn than that containing ordinary dent corn; however, no such increase was observed with low-phytate barley or low-phytate flint corn over their counterpart grains in either Ca, Fe or Zn. The apparent availabilities of Cu, Mn, Mg, K and Na were not significantly different between ordinary and low-phytate grains. The apparent availabilities

of Ca, Na and Sr were negative in all treatments with low-ash test diets, and those of Cu and Fe were either negative or close to zero. The apparent digestibility of dry matter also was not significantly different between ordinary and low-phytate grains; however, the values of barley were apparently higher than those of corn. No significant difference in the calculated availability (digestibility) value was found when yttrium or acid-insoluble ash was used as the inert nonabsorbable markers in any minerals (n=18) except for Mn which showed higher ($P< 0.05$) availability values when acid insoluble ash was used as the indicator than when yttrium was used. Apparent availability values calculated based on chromium frequently gave lower values ($P< 0.05$) than those determined based on yttrium or acid-insoluble ash (data not given).

DISCUSSION

Because of its strong chelating property, phytate has been known to interact with other dietary components, especially with divalent cations, reducing their bioavailability to the animals and humans. This trial was designed to measure (1) its undesirable property to bind trace metals as well as (2) the low availability of inherent phosphorus (phytate phosphorus) in the grains. The methodological approach to answer these two purposes was to use complex diets; i.e., mixing test ingredients with a basal diet (casein-based diet or fish meal-based diet). No difference, however, was observed in the apparent mineral availabilities between ordinary and low-phytate grains. This result may be best explained by the content of phosphorus (and other minerals) in the grains, which was considerably lower than that in the basal diets.

A completely different approach therefore was taken to determine nutrient availability. The test diets were formulated with semi-purified ingredients of low mineral content, and the test ingredients (grains) were incorporated at levels as high as possible in the test diet, so that essentially all minerals in the diets were inherent in the grains and not from other dietary components, except for the trace mineral supplements that were added to meet the minimum dietary requirements for the fish. An extreme example of this approach is the feeding of a single ingredient, often by force feeding. This approach is highly sensitive to differences in availability of nutrients in the test ingredients; however, its most serious drawback is that this approach totally ignores any nutrient interactions by totally eliminating the possibility of such interactions. Also, a single-ingredient “diet” is generally nutritionally defective, and the use of such a diet in nutrition study is questionable since it cannot support normal physiology and the growth of the fish for any extended period. Using a single ingredient in practical feed formulations is very unlikely, suggesting that the availability of minerals in feed ingredients should always be considered as the total of their inherent availability plus any antagonistic or synergistic interactions in compound feeds. The analytical composition of ordinary and low-phytate grains clearly indicates their differences, and the feeding trial with low-ash test diets

demonstrated the significant difference in their availabilities; however, the feeding trials with complex diets, that was similar to commercial trout feeds, the difference was minimal. This indicates that the use of low-phytate grains does not offer any benefit if they are used as a simple replacement for the ordinary grain in the commercial high-ash feeds.

Fish meal is the primary protein source in practical salmonid feeds. Even low-ash fish meals (herring meal, deboned fish meal, etc.) supply more than enough quantities of phosphorus to the fish. The availability of minerals in fish meal (mainly associated with the bone fraction) is normally low, ranging from 15 to 60%; however, when the level of fish meal or fish bone in the feed is reduced, availability (%) of phosphorus (and some other minerals) increases (ref. fish-bone study, p.71). This indicates that the bone phosphorus is "conditionally available" depending on the concentration in the feed. This pattern was observed with the fishmeal-based feeds, where the availability of phosphorus in the basal fishmeal diet was lower than that in the complex diets. This is primarily due to the dilution effect (of phosphorus) in the complex diets, which was apparently more important than the inherent availability of phosphates in the grains. By reducing the phosphorus concentration in the diet to the minimum required levels of fish, the difference of the inherent availability of phosphorus in the grains should become more significant. At present, because most salmonid feeds still contain abundant phosphorus from fish meal, the advantage of low-phytate grains is not fully appreciated. Conversely, for catfish, tilapia and various species of carps, fish meal is a minor feed ingredient and grains such as corn often comprise more than 50% of the feeds, which certainly is an ideal formulation to take full advantage of the low-phytate grains.

The use of low-phytate grains in fish and animal feeds will be increasingly important in the future because reducing phosphorus discharge into the environment is rapidly increasing its priority as a part of the global environmental awareness for which many researchers in many sectors of industries are grouping for the way to reduce phosphorus discharge into the environment in the most effective and economically feasible means. The present study in which rainbow trout was used as a model species to evaluate the effect of low-phytate grains holds an important implication to achieve a substantial reduction of phosphorus discharge from fish, poultry and animal farms without any additional cost or effort but simply by replacing ordinary grains with low-phytate mutant grains in the production feeds of marginal phosphorus content, namely, the environmentally friendly feeds.

Table 34. Concentration of phytate-P and inorganic-P in the test ingredients (g/100g dry matter).[1]

	Phytate-P	Inorganic-P (Pi)	Phytate-P + Pi
Barley (B)	0.29	0.06	0.35
Barley, low-phytate (BLP)	0.12	0.16	0.28
Dent corn (DC)	0.24	0.06	0.30
Dent corn, low-phytate (DCLP)	0.08	0.21	0.29
Flint corn (FC)	0.24	0.08	0.32
Flint corn, low-phytate (FCLP)	0.11	0.25	0.36

[1] Determined on the acid extracts (0.65N-HCl, 3 h). Each value represents the average of duplicate determinations extracted separately.

Table 35. Mineral composition of the test ingredients.[1]

	Ash	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
B	1.97	601.6	1.56	23.9	71.9	3860	1700	17.96	532	3290	1.79	35.3
BLP	1.85	582.5	1.51	25.1	40.6	3010	1670	13.89	1060	2800	1.79	49.8
DC	0.99	tr.[2]	0.25	18.6	16.7	1880	1600	2.33	1170	2570	0.30	14.6
DCLP	0.95	tr.	0.44	21.8	14.7	1020	1560	1.49	1180	2820	0.22	23.7
FC	1.19	tr.	0.19	29.2	33.5	1550	2130	7.62	1760	3390	0.62	34.7
FCLP	1.20	tr.	0.35	28.3	48.7	1530	2100	7.26	1430	3370	0.50	35.0

[1] Determined on the ashed samples. Values are expressed as mg/kg dry ingredient (g/100g dry ingredient for ash).

[2] Trace amount (less than 50 mg/kg dry ingredient).

Table 36. Composition of the basal diets (Casein diet and Fishmeal diet)[1] and the test diets.

Ingredients	Casein diet	Fish mealdiet	Test (low-ash)diet
	---------- g/100g diet ----------		
Barley or Corn	---	---	50
Casein[2]	45	---	---
Herring meal	---	60	---
Egg white[3]	---	---	10
Gelatin[4]	10	10	10
Dextrin	12	10	---
Carboxymethyl cellulose[5]	1	1	---
Cellulose	5[6]	5[6]	4[7]
Mineral mixture	4.5[8]	---	2[9]
Vitamin mixture	2[10]	2[10]	3[11]
Amino acid mixture	4.5[12]	---	1[13]
Herring oil	16	12	20
Water	(30)	(30)	(30)
Trace mineral solution	(10)[14]	(10)[15]	(10)[15]

[1] The basal diets were fed either as-is or as complex diets (70% basal diet + 30% barley or corn).
[2] Vitamin-free casein (USB; United States Biochemical Corporation).
[3] Spray-dried egg white (ICN); contained 0.106% P.
[4] From porcine skin, 300 bloom (USB); contained 0.011% P
[5] Sodium salt (Nutritional Biochemicals Corp.).
[6] Celfil (USB).
[7] Alpha-cellulose (Sigma) contained 0.0012% P. Also supplied the following (g/100g dry diet): Cr_2O_3, 0.5; SiO_2, 0.5; Y_2O_3, 0.05.
[8] Provided the following amount (g/100g dry diet): KCl, 1.5; $CaHPO_4$, 1.2; MgO, 0.2; NaCl, 1.5; Y_2O_3, 0.1.
[9] Provided the following amount (g/100 g dry diet): KCl, 0.9; MgO, 0.1; NaCl, 1.0.
[10] Provided the following amount (g/100 g dry diet): #30 pre-mixture (Hoffmann-La Roche) 1.5; choline chloride, 0.3; inositol, 0.1g; L-ascorbyl polyphosphate, 0.1.
[11] Provided the following amount (g/100 g dry diet): #30 vitamin pre-mixture, 2.0; choline chloride, 0.5; inositol, 0.2; L-ascorbyl polyphosphate, 0.3. #30 pre-mixture contained 0.071% P.
[12] Provided the following amount (g/100 g dry diet): DL-methionine, 1.0; L-arginine HCl, 1.0; L-histidine, 0.3; L-lysine HCl, 1.0; Glycine, 1.0; L-threonine, 0.2.
[13] Provided the following amount (g/100 g dry diet): DL-methionine, 0.5; L-lysine HCl, 0.5.
[14] Provided the following amount (mg/kg dry diet): KI, 1.9; $MnSO_4 \cdot H_2O$, 40; $ZnSO_4 \cdot H_2O$, 44; Na_2SeO_3, 0.9; $CoCl_3 \cdot 6H_2O$, 4; $CuSO_4 \cdot 5H_2O$, 5; $FeSO_4 \cdot 7H_2O$, 300.
[15] Provided the following amount (mg/kg dry diet): KI, 1.9; $MnSO_4 \cdot H_2O$, 40; $ZnSO_4 \cdot H_2O$, 85; Na_2SeO_3, 0.9; $CoCl_3 \cdot 6H_2O$, 4; $CuSO_4 \cdot 5H_2O$, 12; $FeSO_4 \cdot 7H_2O$, 300.

Table 37. Analytical composition[1] of the basal diets, complex diets and the test diets.

	Ash	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	P	Sr	Y	Zn
Basal diets													
Casein diet	5.00	4190	0.312	29.3	75.5	8150	1110	15.9	8190	6310	2.18	804	64.1
Fishmeal diet	11.11	22800	nd	nd	120.0	2300	924	nd	20900	14400	40.2	801	nd
Complex diets[2] (Grain 30% + Casein diet 70%)													
B	4.08	3110	0.689	27.7	74.4	6860	1290	16.5	5880	5400	2.06	561	55.4
BLP	4.05	3110	0.673	28.0	65.0	6610	1280	15.3	6040	5260	2.06	562	59.8
DC	3.85	2990	0.293	26.3	58.7	6370	1250	12.1	6190	5250	1.64	575	50.0
DCLP	3.79	2960	0.349	27.1	57.4	6030	1240	11.6	6100	5270	1.59	564	52.1
FC	3.90	2990	0.277	29.3	63.3	6240	1400	13.5	6330	5470	1.73	572	55.6
FCLP	3.88	2970	0.323	29.0	67.6	6210	1400	13.4	6200	5450	1.68	567	55.6
Complex diets[2] (Grain 30% + Fish meal diet 70%)													
B	8.52	16700	nd	nd	103.6	2940	1180	nd	15200	11500	29.4	563	nd
BLP	8.49	16800	nd	nd	99.0	2520	1150	nd	15300	11300	30.2	566	nd
DC	8.10	16300	nd	nd	88.3	2260	1120	nd	15600	11200	29.0	582	nd
DCLP	8.16	16400	nd	nd	84.4	2220	1100	nd	15300	11200	29.5	571	nd
FC	7.88	15100	nd	nd	88.7	2560	1260	nd	14800	10700	26.9	547	nd
FCLP	8.28	16400	nd	nd	91.9	2520	1290	nd	15000	11400	29.3	559	nd
Test diets[3] (Grain 50% + low-ash ingredients 50%)													
B	5.40	841	3320	18.0	86.5	7910	1600	21.6	7050	2550	1.80	375	59.8
BLP	5.30	800	3330	15.5	76.8	7150	1530	20.7	6840	2280	1.77	384	39.3
DC	4.81	514	3390	14.1	56.5	6500	1490	14.9	7230	2060	1.06	384	17.2
DCLP	4.72	509	2530	16.6	69.9	6210	1560	16.5	7090	2220	1.00	380	45.9
FC	4.97	549	2450	16.8	77.8	6830	1820	18.5	7370	2510	1.29	395	54.7
FCLP	4.80	528	2210	17.6	81.2	6510	1840	19.1	6980	2600	1.22	386	61.6

[1] Values are expressed as mg per kg dry diet (g/100 g dry diet for ash). nd: not determined.

[2] Complex diets were prepared by mixing one of the test ingredients (barley or corn) with the basal diet (casein diet or fishmeal diet; Table 3) at a ratio of 7:3 (weight, dry basis).

[3] Test diets were prepared by mixing one of the test ingredients (barley or corn) with low-ash (semi-purified) ingredients (Table 3) at a ratio of 1:1 (weight, dry basis).

Table 38. Apparent availability[1] of minerals in the basal diets, complex diets and the test diets.

	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
					Basal diets					
Casein diet[2]	32.9	60.8	19.0	97.6	85.6	28.2	10.2	87.1	1.5	67.9
Fishmeal diet[3]	12.4	*nd*	6.99	*nd*	61.9	*nd*	*nd*	48.1	*nd*	*nd*
					Complex diets[2] (Grain 30% + Casein diet 70%)					
B	18.5	47.1	16.7	99.5	81.3	12.3	-40.2	77.9	-4.8	45.1
BLP	-2.6	40.9	12.5	97.3	80.2	-5.2	-57.4	77.9	-38.9	49.0
DC	32.0	44.7	-20.5	98.4	82.3	36.3	-25.1	83.6	-6.3	66.9
DCLP	29.7	55.8	-11.2	98.3	81.1	21.5	-11.1	89.3	-10.6	73.3
FC	11.9	54.3	6.2	98.9	73.1	15.4	-17.2	78.3	-15.4	46.2
FCLP	34.0	55.2	24.6	99.4	79.3	12.2	-1.8	85.3	9.9	55.2
					Complex diets[3] (Grain 30% + Fish meal diet 70%)					
B	14.6	nd	17.6	nd	62.9	nd	nd	54.0	nd	nd
BLP	11.4*	nd	14.1*	nd	61.3	nd	nd	53.2	nd	nd
DC	13.0	nd	16.7	nd	63.7	nd	nd	52.3	nd	nd
DCLP	12.8	nd	15.5	nd	63.0	nd	nd	52.1	nd	nd
FC	10.6	nd	24.2	nd	59.7	nd	nd	53.1	nd	nd
FCLP	13.4	nd	20.2	nd	59.5	nd	nd	54.8	nd	nd
					Test diets[4] (Grain 50% + low-ash ingredients 50%)					
B	-162	8.9	-6.7	100	65.6	13.4	-23.3	47.1	-69	56.0
BLP	-137	-8.7	-15.4	100	75.5	7.3	-25.4	64.9**	-20**	35.6*
DC	-269	-80.6	-32.7	101	64.1	12.9	-55.1	36.7	-143	-70.1
DCLP	-178**	-33.4	2.6*	100	68.3	18.4	-29.4	69.0***	-70***	48.8**
FC	-340	-33.7	3.8	100	62.3	19.8	-34.6	36.3	-136	42.3
FCLP	-219	-26.2	8.7	100	71.1	20.0	-26.5	65.6*	-70***	51.1

[1] Expressed as the fractional net absorption per intake (diet). nd: not determined.

[2] Each value represents pooled sample of six fish fed 1%BW for each fish daily. Dry matter digestibility (%) of casein diet and the complex diets was 82.4, 76.9, 75.2, 77.7, 82.1, 79.7, 80.8% for Casein diet, B, BLP, DC, DCLP, FC, FCLP diets, respectively.

[3] Each value represents the average of three tanks. Values with asterisk are significantly different from those of the ordinary grain (* $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$). Dry matter digestibility (%) of fishmeal diet and the complex diets was 79.2, 75.7, 73.3, 74.5, 73.8, 74.7, 74.7% for Fishmeal diet, B, BLP, DC, DCLP, FC, FCLP diets, respectively.

[4] Each value represents the average of three tanks. Values with asterisk are significantly different from those of the ordinary grain (* $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$). Dry matter digestibility (%) was not significantly different between ordinary and low-phytate grains; 61.8, 60.6, 40.7, 46.8, 45.0, 48.2 % for B, BLP, DC, DCLP, FC, FCLP, respectively.

Table 39. Fecal contents of phytate-P and total-P in fish fed test diets of low-ash content.[1]

	Phytate-P	Total P
B	0.34	0.36
BLP	0.16 **	0.20 **
DC	0.21	0.22
DCLP	0.11 **	0.13 **
FC	0.30	0.30
FCLP	0.15 *	0.17 *

[1] Determined on the acid extracts of fresh feces, duplicate determinations on a single extract. Expressed as the percentage in dried feces. Each value represents the average of 3 tanks. Values with asterisk are significantly different from those of the ordinary grains (* P< 0.05, ** P< 0.01).

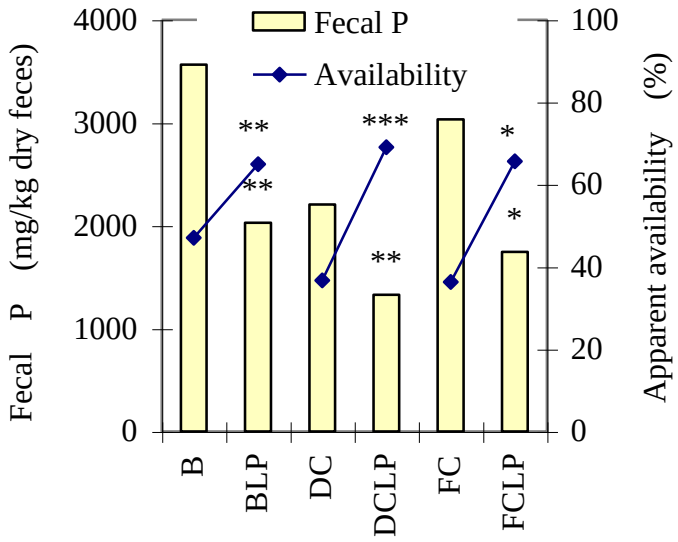


Figure 15. Fecal content and apparent availability of phosphorus in the low-ash test diets containing either ordinary grain or low-phytic-acid-mutant-grain. The diets contained one of the following test ingredients (grains) at 50% in diets (dry basis); B (ordinary barley); BLP (low-phytic acid barley); DC (ordinary dent corn); DCLP (low-phytic acid dent corn); FC (ordinary flint corn); FCLP (low-phytic acid flint corn). Columns or plots of low-phytic-acid-mutant grains with asterisks are significantly different from those of the ordinary grains by the two-tailed t-test (* P< 0.05; ** P< 0.01; *** P< 0.001). n=3 (tanks) per treatment.

Chapter III: Effect of Dietary Supplements and Processing on the Availability of Minerals

1. Effect of Various Dietary Supplements on the Availability of Minerals in Fish Meal

ABSTRACT Several feed supplements with the potential to improve dietary mineral availabilities in fish meal were tested using rainbow trout (initial mean body weight 232 g). Eleven supplements were tested: citric acid; sodium citrate; potassium chloride; sodium chloride; histamine dihydrochloride; EDTA disodium salt; sodium bicarbonate; a mixture of amino acids; ascorbic acid; a mixture of inositol and choline; and cholecalciferol. Apparent availability of calcium, phosphorus, magnesium, sodium, iron, manganese and strontium in fish meal-based diets was determined using both yttrium oxide (Y_2O_3) and chromium oxide (Cr_2O_3) as inert dietary markers. Apparent availability was expressed as the fractional net absorption (%) of mineral elements from diets. After a 7-day acclimation period with test diets, fecal samples were collected for five consecutive days using passive collection systems. Apparent availability of calcium, phosphorus, magnesium, iron, manganese and strontium was increased by citric acid supplementation. Apparent availability of manganese also was increased by EDTA and sodium citrate. The other supplements had no measurable effect on the apparent availability of minerals in fish meal.

BACKGROUND

Reducing the amount of phosphorus in salmon and trout hatchery discharge water is a priority both in Europe and in North America (Kossmann, 1990; Cowey and Cho, 1991). Phosphorus in fish feeds is the source of phosphorus in hatchery effluents, and strategies to lower phosphorus levels in hatchery effluents include capturing uneaten feed and fecal solids, lowering dietary phosphorus levels, avoiding feed ingredients that contain phosphates of low availability, and increasing availability of those phosphate compounds by chemical, physical or enzymatic treatments. Capturing uneaten feed and fecal solids involves settling, screening and other treatment processes (Piper et al., 1982). Lowering dietary phosphorus levels involves modifying feed ingredients such as fish meal to reduce bone content (Babbitt et al., 1994) and using low-phosphorus feed ingredients to formulate aquatic feeds (Ketola, 1985). Increasing availability of phosphates in feed ingredients involves the use of supplements such as phytase to hydrolyze phytate phosphorus in plant feed ingredients, making it available to fish (Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995; Schaefer et al., 1995; Jackson et al., 1996; Riche and Brown, 1996). In salmonid feeds, however, fish meal is the main ingredient, and the intrinsic phosphate in fish meal is the major source of dietary phosphorus. In a series of digestibility trials in the previous chapter, it has been shown that phosphates in various fish meals are not efficiently utilized by fish (range 11.8 - 50.2% as apparent phosphorus availability). Improving availability of phosphates in fish meal was, therefore, considered essential to reduce phosphorus levels in effluents from salmonid aquaculture.

Numerous factors have been shown to affect gastrointestinal absorption of mineral elements in animals and fish. After an extensive review of the literature, 11 supplements were selected which appeared to offer the possibility of increasing digestion and/or absorption efficiency of minerals. The modes of action of these supplements are diverse and are summarized in Table 40. Before this study, the effects of some of these supplements on the availability or absorption of mineral elements in practical rations had not been reported for any animal species. The purpose of this study was to determine if common feed supplements listed in Table 40 had positive (or negative) effects on the availability of phosphorus and other minerals in fish meal to rainbow trout.

MATERIALS AND METHODS

Test diets were formulated with semi-purified ingredients, except fish meal, and without mineral supplements. All diets were made as cold-extruded moist pellets, air-dried overnight, and stored at -20°C until fed. Composition of the basal diet and the amount of supplements added to the basal diet are given in Tables 41 and 42, respectively. Yttrium oxide and chromium oxide were used as inert unabsorbable dietary markers at identical concentrations (0.5%) to determine net absorption (apparent availability) of other mineral elements in the diets.

Sixty rainbow trout (initial mean body weight 232.0 g ± 37.08 SD) were stocked into twelve feces-collection tanks (150 L, modified Guelph type; Hajen et al., 1993) and acclimated to each experimental diet for seven days. For five consecutive days, feces were collected twice daily at 0800 h and 1730 h from the collection cylinder that was separated from the fish tank. Biofiltered, recirculating, temperature-controlled (16.5 ± 0.5°C) water was supplied to each tank (5 L/min/tank) with auxiliary input of dechlorinated municipal water to the entire system at 5 L/min to maintain optimal water quality and to minimize accumulation of elements in the recirculating water. Fish in each tank were fed once daily at 1800 h with equal amounts of diets for each treatment (0.7 - 0.8% of body weight on a dry basis). Photoperiod was held constant at a 14-h day length. No mortality or anorexia were observed in any treatment group at any time. Diet and fecal samples were dried for 12 h at 105°C to a constant weight by a forced air convection oven. Portions of the dried samples were wet-ashed and digested with perchloric acid (Furukawa and Tsukahara, 1966), diluted with deionized distilled water, and analyzed for minerals by an inductively-coupled plasma emission spectrophotometer (Jarrell-Ash Plasma Atom Comp., Waltham, MA). The dried diet and fecal samples were also analyzed for crude protein contents using a LECO FP-428 Nitrogen determinator (Leco Instruments, St. Joseph, Michigan, USA). Apparent availability (%) of minerals was expressed as the fractional net absorption of minerals from diets based on either yttrium or chromium as the unabsorbable inert markers. Fish were handled in accordance with the guidelines approved by the Animal Care Committee of the University of Washington.

Apparent digestibility and availability data were subjected to Wilcoxon signed rank test using GraphPad Prism, version 2.0 (GraphPad Software, Inc., San Diego, CA). Treatment effects were considered significant at $P<0.05$.

RESULTS

Apparent availability (%) of phosphorus increased from 68.3% (control diet; no supplements) to 84.4% by supplementing the diet with 5% citric acid (Table 43). The concentration of phosphorus in feces decreased from 32,000 μg /g dry feces (control diet) to 14,300 μg /g dry feces by supplementing the diet with citric acid (Fig.16). Citric acid also increased the apparent availability (net absorption) of calcium (from 22.0% to 52.2%), iron (from 1.7% to 10.9%), magnesium (from 69.4% to 86.6%), manganese (from -83.8% to 7.2%) and strontium (from 22.0% to 44.3%) in the fish meal diet (Table 43; Fig.17). Apparent availability of manganese was negative in many dietary treatments including the control diet, but was increased in diets supplemented with citric acid, sodium citrate or EDTA. Apparent availability of iron was decreased by many supplements. Apparent availability values calculated using chromium showed consistently lower values than those determined by yttrium. Apparent absorption of chromium calculated based on yttrium was 4.8% ± 1.7 (average ± SD of 12 diets in Table 43). Apparent digestibility of protein was similar among treatments; ranged between 94.4 and 96.0 (%, yttrium basis).

DISCUSSION

Although no tank replication was employed in the supplement study, the effects of citric acid supplementation on apparent availabilities of Ca, P, and Sr were large enough to be easily detectable and thus satisfy the purpose of this preliminary effort to screen candidate supplements. Further, with the exception of $NaHCO_3$ supplementation, which had a negative effect, none of the supplements had much influence on apparent availability of minerals. The similar apparent availability values observed among treatments indicates that the system and approach employed in this study yielded consistent and reproducible results, supporting the conclusion that citric acid supplementation did indeed affect apparent availabilities of key minerals, despite the absence of true replication.

The effect of citric acid on availability of mineral elements in fish meal appeared to be due mainly to an acidifying effect which solubilized bone minerals rather than to a chelating effect. Sodium citrate added at an identical concentration to the basal diet had a minor effect on increasing mineral availability except for manganese. Calcium availability was also increased significantly by supplementing the diet with 5% citric acid, and the corresponding increase of strontium availability provided additional evidence to support the hypothesis that availability was increased by bone mineral solubilization. Sodium bicarbonate (the only base or alkali used in this study) decreased availability of

minerals. This suggests that the availability of minerals increases when diets are acidified and decreases when base is added to the diets.

Although concentrations of each supplement added to the basal diet were chosen to be high enough to be able to detect possible effects but not so high as to cause toxicity, some supplements might have affected mineral availability had they been supplemented at different dietary levels. For example, the level of histamine added to the diet was chosen based on the level present in low-quality fish meal. To stimulate gastric acid secretion in trout, a higher dose might be needed (Fairgrieve et al., 1994).

Apparent availability less than zero was induced by some supplements, indicating a possibility of negative balance of those minerals unless they were compensatorily supplied from the ambient water. The adverse effects of those supplements fed over long periods of time could be of potential significance since the loss of those elements over an extended period could result in growth depression and clinical deficiency signs. Long-term effects were beyond the scope of the present study; however, studying the balance of elements over an extended period of time is obviously of enormous importance. For the determination (comparison) of dietary nutrient availabilities, however, the acclimatization period should be minimized to reduce the effect of biological adaptive (compensatory) responses to a reduced nutrient intake or to a nutrient of lower bioavailability. Feeding fish for an extended period with test diets could permit fish to change the enzymatic or hormonal concentrations in their digestive or circulatory systems (compensatory response), which could alter absorption efficiency of dietary nutrients and lead to an over-estimation of inherent availability of dietary nutrients.

The use of chromium oxide as an inert non-absorbable dietary marker in nutrient digestibility studies was questioned by some investigators (NRC, 1980; Shiau and Chen, 1993; De Silva et al., 1997). Apparent availability coefficients calculated using chromium and yttrium consistently indicated that chromium was absorbed in small amounts by the fish, but no such effect was observed when yttrium was added to the diets at the same concentration. Thus, the use of yttrium oxide as an inert indicator may yield more accurate and reliable values than the usual method using chromium oxide as the inert indicator.

The results of this study, while preliminary, suggest that citric acid warrants further investigation as a dietary supplement to fish meal-based diets to enhance availability of phosphorus and other mineral elements. Sodium citrate and EDTA demonstrated potential to enhance manganese availability. Further studies are needed to determine optimum levels and long-term effects of dietary acidification, to study effects of dietary acidifiers on the digestion and absorption of minerals in other species, particularly agastric fishes, and to examine the effect of citric acid supplementation in practical salmonid feeds.

Table 40. Mode of action and potential effects of supplements on bioavailability of minerals.

Supplement	Mode of action	Ref.
Citric acid	Acidifier in weaning pig diets, Aid gastric digestion	1
	Chelate mineral ions	2
	Increase $H_2PO_4^-/HPO_4^{2-}$ ratio ($H_2PO_4^-$ is absorbed preferentially)	3
Sodium citrate	Chelate mineral ions	4
KCl	Potassium ions necessary for gastric H^+/K^+ ATPase (gastric acid pump)	5
NaCl	Increase Na-dependent active transport of P in the intestinal brush border.	6
Histamine	Induce gastric acid secretion	7
EDTA	Chelate mineral ions	8
Sodium bicarbonate ($NaHCO_3$)	Intestinal pH affects P absorption	9
Amino acid mixture	Chelate mineral ions	10
	Induce gastric acid secretion	11
Ascorbic acid	Reducing agent, increase absorption of iron	12
Inositol + Choline	If deficient, cause slow gastric emptying, inefficient digestion and feed utilization, gastrointestinal abnormalities	13
Cholecalciferol (vitamin D_3)	Increase Ca, P, and Mg absorption as calcitriol or $1,25(OH)_2D$	14

[1] Ravindran and Kornegay (1993).
[2] Pak et al. (1987); Crawford (1995); Misra (1996).
[3] Berner et al. (1976).
[4] Furia (1972); Cuche et al. (1976).
[5] Helander and Keeling (1993).
[6] Hildmann et al. (1982); Lee et al. (1986); Cross et al. (1990).
[7] Holstein (1975); Holstein (1976); Hiramatsu and Okabe (1994).
[8] Vohra and Kratzer (1964).
[9] Danisi et al. (1984); Quamme (1985); Danisi and Murer (1991); Dennis (1992).
[10] Ashmead et al. (1985).
[11] Feldman et al. (1978); Strunz et al. (1978); Taylor et al. (1982).
[12] Johnson (1986); Monsen (1988); Lonnerdal (1989).
[13] Halver (1972).
[14] Harrison and Harrison (1961); Peterlik and Wasserman (1978); Breves and Schröder (1991).

Table 41. Composition of the basal diet.[1]

Ingredients	%
Herring meal[2]	63
Gelatin[3]	11
Dextrin[4]	12
CMC[5]	1
Vitamin mixture[6]	1
Ascorbic acid[7]	0.01
Canola oil	11
Y_2O_3 [8]	0.5
Cr_2O_3 [9]	0.5
(Water)	(31)

[1] Mineral content (μg/g dry matter; analytical values): Ca, 15400; Cr, 3800; Fe, 173; Mg, 1360; Mn, 4.23; Na, 7950; P, 11400; Sr, 35.2; Y, 3950. Protein content (g/100g dry matter; analytical value): 58.91.
[2] Nelson & Sons, Inc., Murray, UT.
[3] Type B, from bovine skin, 225 bloom (G-9382: Sigma Chemical Co., St. Louis, MO).
[4] Type-3, from corn white powder, 12% water-soluble (160057: ICN Biomedicals, Inc., Cleveland, OH).
[5] Carboxymethyl cellulose, sodium salt (C-5013: Sigma Chemical Co.).
[6] Oregon Moist Pellet formula No.2, vitamin D and C free (Hoffmann-La Roche Inc., Nutley, NJ).
[7] Ascorbic acid crystal (Takeda U.S.A., Inc., Orangeburg, NY).
[8] Yttrium oxide (Y-3500: Sigma Chemical Co.).
[9] Chromium oxide (1616-01: J.T.Baker Inc., Phillipsburg, NJ).

Table 42. Levels of supplements added to the basal diet.

Supplements[1]	g/100 g dry diet
Citric acid[2]	5.0
Na-citrate[3]	5.0
KCl[4]	3.0
NaCl[5]	5.0
Histamine[6]	0.3
EDTA[7]	1.0
$NaHCO_3$[8]	5.0
Amino acid mixture[9]	5.0
Ascorbic acid crystal[10]	1.0
Inositol[11] + Choline[12]	0.5+1.0
Vitamin D_3 [13]	0.05

[1] Added with 25 ml of water.
[2] (0627: Mallinckrodt Chemical Works, St. Louis, MO).
[3] Dihydrate (3646-05: J.T.Baker Inc.).
[4] (3040-05: J.T.Baker Inc.).
[5] (S-9625: Sigma Chemical Co.).
[6] Dihydrochloride (H-7250: Sigma Chemical Co.).
[7] Disodium salt (BP120-500: Fisher Scientific, Fair Lawn, NJ).
[8] (S-8875: Sigma Chemical Co.).
[9] L-Met, 1.0; L-Arg, 1.0; L-Lys, 1.0; L-Thr, 1.0; L-Gly, 1.0 (M-9625, A-5006, L-5626, T-8625, G-7126: Sigma Chemical Co.).
[10] (Takeda U.S.A., INC.).
[11] Myo-inositol (I-5125: Sigma Chemical Co.).
[12] Chloride salt (C-1879: Sigma Chemical Co.).
[13] Cholecalciferol, 0.05g = 50,000 IU (13393: United States Biochemical Corporation, Cleveland, OH).

Table 43. Apparent digestibility of dry matter and availability of minerals in fish meal diets.[1]

Supplement	Dm[2]	Ca	Cr	Fe	Mg	Mn	Na	P	Sr	Y
Control (none)	88.7***	22.0	6.4**	1.7**	69.4	-83.8	71.2***	68.3	22.0	0
	(88.0)	(16.6)	(0)	(-5.0)	(67.3)	(-96.4)	(69.2)	(66.2)	(16.7)	(-6.9)
Citric acid	87.6**	52.2***	4.7	10.9***	86.6***	7.2**	63.9*	84.4***	44.3***	0
	(87.0)	(49.9)	(0)	(6.6)	(86.0)	(2.7)	(62.1)	(83.6)	(41.6)	(-4.9)
Na citrate	87.2*	36.1**	4.3	-30.6	82.6***	32.1***	50.5	69.3*	32.2**	0
	(86.6)	(33.2)	(0)	(-36.4)	(81.8)	(29.0)	(48.3)	(68.0)	(29.1)	(-4.5)
KCl	86.9	16.3	3.5	-5.6*	65.9	-51.5	63.0*	64.2	14.5	0
	(86.4)	(13.2)	(0)	(-9.4)	(64.7)	(-57.1)	(61.6)	(62.9)	(11.3)	(-3.7)
NaCl	87.3*	22.8	4.1	-14.5	70.3	-58.6	64.9**	66.7	21.5	0
	(86.8)	(19.5)	(0)	(-19.4)	(69.0)	(-65.4)	(63.4)	(65.3)	(18.1)	(-4.3)
Histamine	86.3	15.6	7.7***	-39.3	69.6	-135	58.3	65.1	15.7	0
	(85.2)	(8.5)	(0)	(-50.9)	(67.0)	(-155)	(54.8)	(62.2)	(8.7)	(-8.4)
EDTA	87.0	24.6	6.8**	-7.7	63.7	38.7***	57.6	70.8*	22.3	0
	(86.0)	(19.1)	(0)	(-15.5)	(61.1)	(34.2)	(54.5)	(68.7)	(16.6)	(-7.3)
$NaHCO_3$	85.0	6.0	6.7**	-30.6	66.6	-59.2	52.6	56.4	7.3	0
	(83.9)	(-0.7)	(0)	(-39.9)	(64.2)	(-70.6)	(49.3)	(53.3)	(0.6)	(-7.1)
Amino acids	85.4	9.2	3.1	-39.8	66.9	-69.9	58.6	61.5	8.7	0
	(84.9)	(6.3)	(0)	(-44.3)	(65.9)	(-75.3)	(57.3)	(60.3)	(5.8)	(-3.2)
Vitamin C	85.9	26.9	3.5	-37.9	75.1	-49.7	60.1	67.0	24.6	0
	(85.4)	(24.2)	(0)	(-42.9)	(74.2)	(-55.1)	(58.6)	(65.8)	(21.8)	(-3.6)
Inositol, choline	86.0	20.7	4.3	-1.3**	69.6	-76.2	58.9	65.3	19.4	0
	(85.3)	(17.2)	(0)	(-5.8)	(68.2)	(-84.1)	(57.1)	(63.7)	(15.7)	(-4.5)
Vitamin D_3	83.7	11.8	2.2	-6.5	68.0	-58.6	54.4	61.9	12.8	0
	(83.3)	(9.9)	(0)	(-8.9)	(67.3)	(-62.1)	(53.4)	(61.0)	(10.8)	(-2.2)

[1] Determined on a pooled fecal sample per treatment collected for 5 consecutive days following 7 days of acclimatization to each test diet. Fecal samples were collected by settling from one tank per treatment containing five fish each (initial mean body weight 232.0g). Values are expressed as fractional net absorption (%) of nutrients from diets based on yttrium as a non-absorbable dietary marker (values in parentheses are based on chromium). Values in each column with asterisks are significantly higher than the median value of the same column (*, P<0.05; **, P<0.01; ***, P<0.001).

[2] Dm: Dry matter.

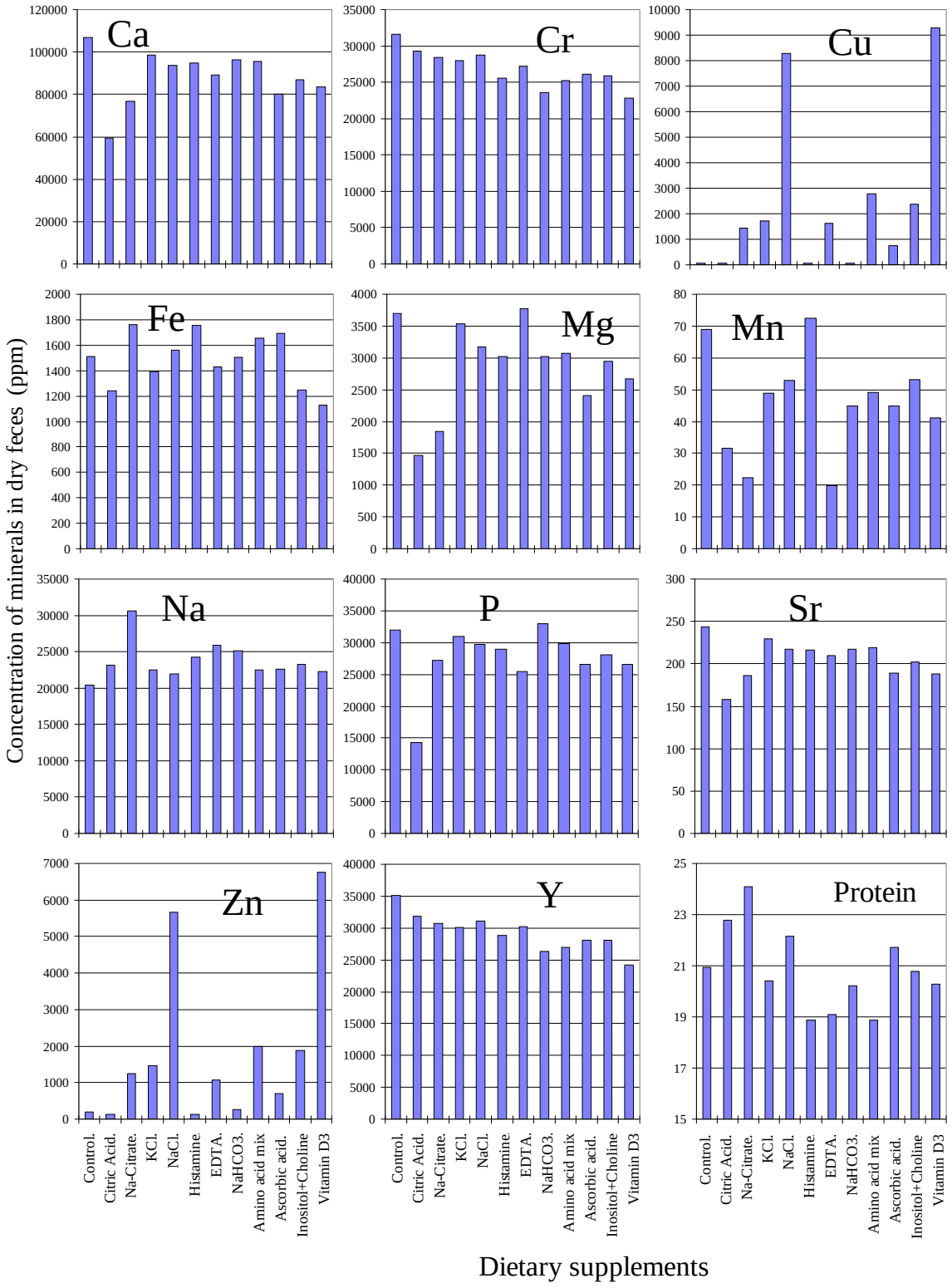


Figure 16. Effects of supplements on the fecal concentration of minerals and protein in fish fed fish meal-based diets. Values for protein (in the Y-axis) are percentage in dry feces.

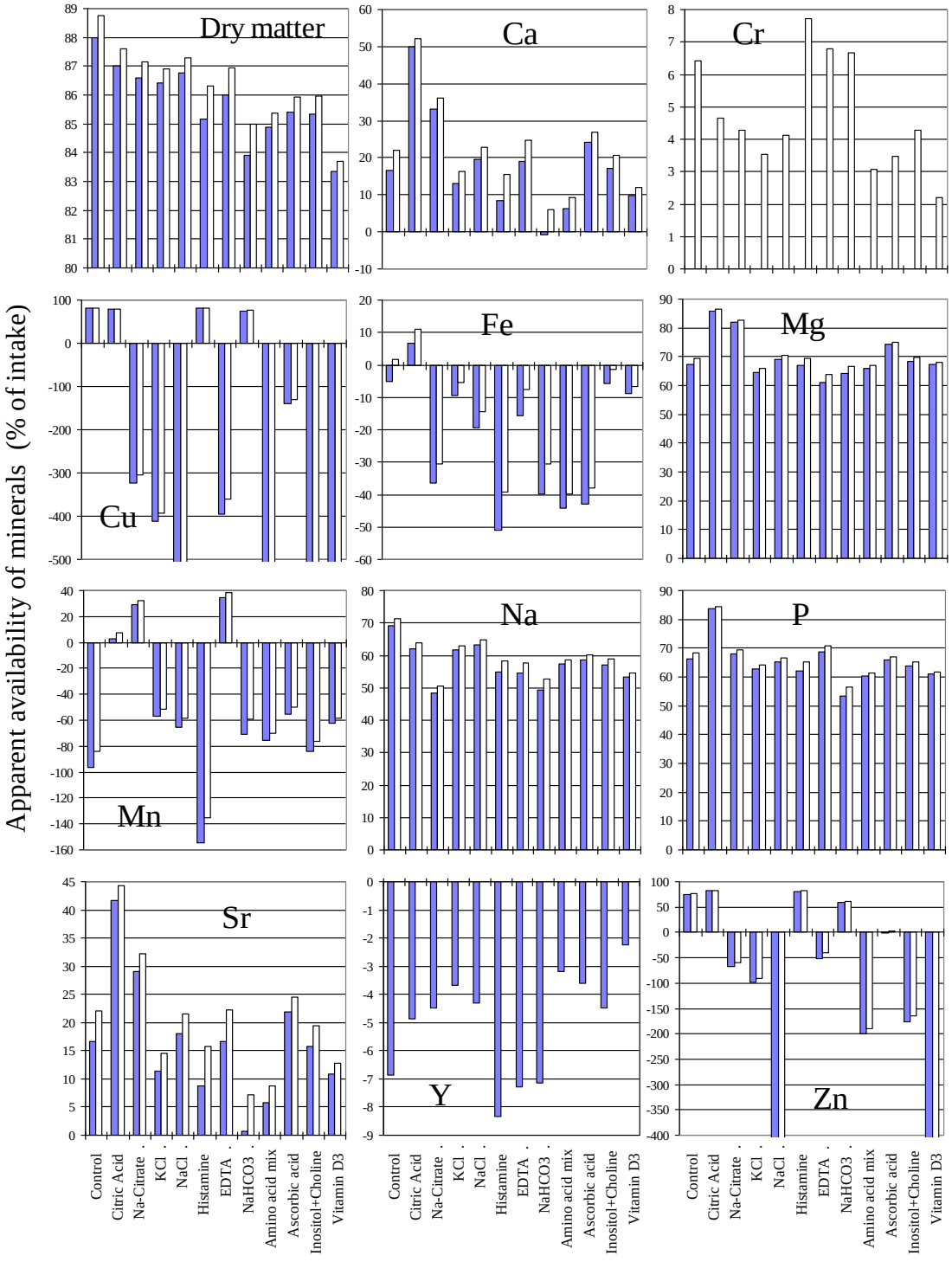


Figure 17. Effect of dietary supplements on the apparent availability of minerals in fish meal. The solid bars show the apparent availability of minerals determined based on chromium oxide (Cr_2O_3); the hollow bars show those determined based on yttrium oxide (Y_2O_3) as inert non-absorbable dietary markers. The apparent availability was expressed as a fractional net absorption of minerals from diets (net absorption %/ Intake).

2. Effect of Citric Acid on the Availability of Dietary Minerals

ABSTRACT The present study was conducted to confirm a previously observed effect of supplemental citric acid on digestion and absorption of minerals in fish meal using monogastric (rainbow trout) and agastric fish (goldfish). Fish were fed for 5 weeks (rainbow trout) or 3 weeks (goldfish) with fish meal-based diets containing either 0% (control), 2% or 5% citric acid on a dry basis. Feces were collected by settling and by stripping. Apparent availability (net absorption) of minerals (Ca, Mg, P, Na, K, Fe, Cu, Mn, Sr, Zn) was determined by using yttrium oxide as an inert unabsorbable dietary marker. Apparent availability (%) of mineral elements was expressed as a fractional net absorption from diets. Apparent availabilities of calcium and phosphorus were most affected by citric acid supplementation in rainbow trout but not in goldfish. Phosphorus levels in feces of fish fed a diet with 5% citric acid were approximately half of that of fish fed the control diet (0% citric acid) in the rainbow trout trial. This pattern was consistent during the 5-week feeding trial. A dietary supplement of citric acid as high as 5% did not reduce feed intake or appetite of rainbow trout. Conversely, this level of dietary acidification led to a reduction of feed intake in goldfish. Dietary supplementation of citric acid at a 2% level did not reduce feed intake of goldfish; however, this level of dietary acidification had little effect on the apparent availability of major minerals in fish meal-based diet. Levels of non-fecal excretion of calcium and phosphorus, inorganic phosphorus in urine, and citric acid in feces were increased in rainbow trout fed 5% citric acid. The pH values of the feces and urine were decreased in rainbow trout treated with citric acid. Plasma bicarbonate, plasma calcium and phosphorus, and blood pH of rainbow trout tended to increase by a 5% dietary supplementation of citric acid. The soluble inorganic phosphorus content increased in the diets and decreased in the feces of rainbow trout by supplementing the diet with 5% citric acid. Feces samples of rainbow trout collected by stripping provided similar availability values to data collected by settling for most elements except sodium, which had negative values in all dietary treatments.

BACKGROUND

Citric acid and some other dietary acidifiers have been shown to improve performance of weaning pigs (Ravindran and Kornegay, 1993), although the mode of action for this effect has not been elucidated. Previously, citric acid was shown to improve availability of some minerals in fish meal (ref. Study 1 in Ch. 3). The experiment, however, was continued no longer than 2 weeks, determination of minerals was made for a pooled (5 days) fecal sample per treatment, and no other measurement except availability of minerals was made. To demonstrate its practical feasibility, more information on the use of citric acid was needed. Feeding acid for a prolonged period of time could be stressful to the fish. In animals, mobilization of bone calcium and phosphates to neutralize H^+ may take place before the body

becomes unable to manage H^+ influx, causing metabolic acidosis (Lemann et al., 1965, 1966, 1967; Barzel and Jowsey, 1969; Petito and Evans, 1984; Ching et al., 1989). For fish, however, calcium or bicarbonate in the ambient water may have some buffering effects to counteract the excess influx of H^+ from acidified feeds.

Carp and other agastric species of fish secrete little or no gastric acid, which has been considered the primary reason for the inefficient utilization of phosphates in fish meal in those fish species (Ogino et al., 1979). Inclusion of acid in diets or pre-digestion of bone phosphate by acid could improve availability of phosphates in fish meal for those fishes.

The primary purposes of the present study were to confirm previously observed effects of citric acid on the digestion and absorption of minerals, to investigate long-term effects of feeding citric acid, to test different dietary concentrations of citric acid, and to examine the effect of citric acid supplementation using a stomachless species of fish.

MATERIALS AND METHODS

Diet preparation

Fish meal composed 50% of the test diets and was the main source of dietary minerals. Three test diets were prepared differing only in citric acid content (0, 2 and 5%). Yttrium oxide (Y_2O_3) was added to the test diets as an inert marker to determine apparent availability (net absorption) of minerals. A previous study had demonstrated the advantage of using yttrium over chromium as an inert unabsorbable dietary marker (ref. Study 1, Ch.3). Test diets were formulated with semi-purified ingredients, except fish meal, and no mineral supplements were added. Composition of the basal diet and the content of mineral elements are given in Table 44. All diets were made as cold-extruded moist pellets and stored at -20°C until fed. Both rainbow trout and goldfish were fed the same diets.

Fish and feeding

Rainbow trout Oncorhynchus mykiss (used as a monogastric fish; initial mean body weight 256.4 g) and goldfish Carassius auratus (used as an agastric fish; initial mean body weight 149.7 g) were obtained from local sources. Five fish were stocked in each aquarium (150 L), which had a specifically designed feces collection system and air-lifting self-recirculating system (modified Guelph type, Fig. 33, p.198). The system collected feces in a long thin collection tube as soon as the feces were discharged by the fish (within one minute). Although soluble components in feces might leach into the water, they would remain in the tube separated from the water in the tank. Fish were acclimatized to each test diet and the rearing environment for one week (week-1), and the collection of feces and water samples began the second week (week-2). Fish were fed the same amount of test diet each day during 1800 h and 1900 h (10 g/tank for goldfish; 20 g/tank for rainbow trout, on an as-is basis). After feeding, each tank and the connected feces collection system were cleaned and flushed thoroughly with excess de-chlorinated

municipal water (23 ± 1°C for goldfish and 19 ± 1°C for rainbow trout), and the initial water sample was collected at 1900 h from each tank. From this time on, the tank was operated with a recirculation system and no external water was supplied until 1800 h of the following day, at which time water was sampled again from each tank, and feeding, cleaning and flushing were completed within an hour. Water temperatures during the self-recirculating hours remained constant to the initial temperature indicated above. Fecal and water samples collected daily (7 days/week for goldfish and 5 days/week for rainbow trout) were pooled by week and stored refrigerated (0-2°C) until the determination of mineral contents. Fecal and water samples were collected for 2 weeks (week 2 and 3) with goldfish and 4 weeks (week 2, 3, 4 and 5) with rainbow trout. At the end of the feeding trial with rainbow trout, fish were anesthetized with tricaine methane sulfonate (MS-222) (150 mg/L), fecal and urine samples were collected by stripping, and blood was drawn into heparinized syringes from the caudal blood vessels of each fish. Non-fecal excretion of minerals was estimated by determining the net excretion of mineral elements into the water, which was calculated as the difference between concentrations of elements in the recirculating water sampled initially and finally (23 h after the initial sampling). During the final week (week 5) of feeding the rainbow trout, calcium sulfate ($CaSO_4$, Sigma Chemical Co., C3771, min. 99%) was dissolved into the water at 250 mg/L (as $CaCO_3$) to see the effect of increased water hardness. Alkalinity and pH of the water were not altered by applying calcium sulfate into the water. Total hardness, alkalinity, pH, nitrite and ammonia levels were monitored during the feeding trial using commercial test kits (HACH Company, Loveland, CO). Elemental composition of the water (mg /L water; mean values; n=15) in the goldfish and rainbow trout trials (week 1-4) was Ca, 10.0; Cu, 0.00736; Fe, 0.0271; K, 0.259; Mg, 1.425; Mn, 0.00140; Na, 2.39; P, 0.0287; Sr, 0.0298; Zn, 0.00961. Elemental composition of the water (mg/L water; mean values, n=3) in the rainbow trout trial during the final week (week-5) was Ca, ca.100; Cu, 0.00738; Fe, 0.0439; K, 0.354; Mg, 1.63; Mn, 0.00631; Na, 2.72; P, 0.0491; Sr, 0.334; Zn, 0.00835. Fish were handled in accordance with the guidelines approved by the Animal Care Committee of the University of Washington.

Analyses and calculation

The fecal suspension (feces + tube water; approx. 1% feces) was weighed and dried in a forced air convection oven at 105°C for 12 h, weighed again (to determine feces concentration) and ground using a mortal and pestle to prepare for mineral analysis. The amount of minerals contributed from the tube (tank) water was determined separately and subtracted from the mineral content of the dried feces to obtain true content of minerals in the feces. Dried feces, diet and water samples were ashed in a muffle furnace at 550°C for 12 h, dissolved in a concentrated acid (hydrochloric acid and nitric acid mixture, 1:1), diluted with deionized-distilled water to an appropriate concentration, and analyzed for mineral content using an inductively-coupled plasma emission spectrophotometer (ICP; Jarrell-Ash Plasma Atom Comp., Waltham, MA). Concentrations of inorganic phosphorus in the water were determined

according to the standard method (Stannous chloride method; #424 E, American Public Health Association et al., 1981). When the concentration was low, phosphorus was condensed using a magnesium co-precipitation method (Karl and Tien, 1992) prior to analysis. The inorganic phosphorus concentrations in urine, dietary extracts and fecal solutions were determined according to Taussky and Shorr (1953). Dietary pH was determined according to AOAC #943.02 (AOAC, 1995). This diet solution (dietary extract) was assayed for inorganic phosphorus as mentioned above. The pH of the fecal solution and blood was determined using a pH electrode. Urinary pH was determined with pH strip paper for clinical diagnostics. Concentrations of citric acid in dietary extracts, fecal solutions, urine and plasma were determined by the method of Beutler and Yeh (1959). Apparent availability (%) of mineral elements was expressed as a fractional net absorption from diets based upon yttrium as an inert unabsorbable marker.

Apparent availability and non-fecal excretion data were subjected to linear regression and correlation using GraphPad Prism, version 2.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Citric acid increased the apparent availability of many minerals in fish meal as previously observed (ref. Study 1, Ch.3). Regression coefficients (slopes) between dietary citric acid levels (X) and the apparent availabilities of dietary minerals (Y), which were positive for most minerals studied, indicated the favorable effects of citric acid supplementation on the availability of dietary minerals except for the wk-5 during which ambient calcium concentrations were increased ca. 10 times over the previous weeks. Of the minerals measured, the apparent availabilities of Ca, P and Mg were most affected by citric acid supplementation (Table 45; Fig. 18). Fecal concentration of P from fish fed the diet with 5% citric acid was approximately half of that in feces from fish fed the control diet (0% citric acid) (Fig. 19). The apparent availability of K was high and unaltered by dietary citric acid, while the availability of Na was lower and more variable than that of K and not associated with dietary citric acid level (Table 46). Among trace elements, the apparent availability of Mn and Sr tended to increase but that of Cu, Fe and Zn was variable and less affected by dietary citric acid (Table 47). The apparent digestibility of the diet (dry matter) was not affected but the digestibility of ash was increased by citric acid (Table 46). Although fish were not fed to satiation in the present study, a dietary supplement of citric acid as high as 5% did not reduce feed intake or appetite of rainbow trout during 5 weeks of feeding. Conversely, this level of dietary acidification led to a reduction of feed intake in goldfish. Dietary concentrations of citric acid at the 2% level did not affect the feed intake in goldfish; however, this level of dietary citric acid did not noticeably affect the apparent availabilities of major minerals (Table 48). In the group of rainbow trout that received 5% citric acid, non-fecal excretion of Ca and P, inorganic P in urine, and the citric acid content in feces were increased (Tables 49 and 50). Concentrations of bicarbonate, Ca, P and citric acid

in plasma of rainbow trout tended to increase and the blood pH also increased by the supplemental citric acid. The pH of feces and urine was decreased in the group of rainbow trout receiving 5% citric acid (Table 50). Dietary pH decreased and the soluble inorganic P content in diets increased with citric acid (Table 50). Non-fecal excretion of Cu and Fe in the rainbow trout often showed negative values, indicating that a portion of the element initially present in the tank water was absorbed by the fish. During week-5 in the rainbow trout study, when the concentration of the water-borne Ca was increased 10 fold over the previous weeks, non-fecal excretion of Fe, Mg and Mn became highly negative, whereas non-fecal excretion of K, Sr and Zn noticeably increased (Table 49). Feces samples of rainbow trout collected by stripping provided similar availability values to those obtained by settling for most elements except Na, which showed negative values in all dietary treatments. Digestibility of dry matter, ash, and absorption of K also showed somewhat lower values by stripping than those obtained by settling during the preceding weeks.

DISCUSSION

Citric acid has been demonstrated to increase availability of P and several other minerals in fish meal as previously reported (ref. Study 1, Ch.3). This result could be attributed to two related factors; (1) effect of dietary acidification and solubilization, (2) effect of subsequent chelation of released metal ions. Because of this, other acidifiers such as inorganic acids may be less effective than citric acid at increasing the availability of minerals (this assumption was later disproved, Tables 74-76, p 245). The mode of chelation for increasing phosphorus and trace element absorption is likely to be indirect; i.e., chelation of Ca ions and thereby reducing antagonistic interactions (including precipitation and co-precipitation) between Ca and phosphates or trace elements at the intestinal brush border.

Dietary acidification of feeds for agastric species was hypothesized to be effective at increasing availability of minerals in fish bone and fish meal. However, because of the concomitant reduction of feed intake associated with acidification, the study with goldfish was terminated in 3 weeks. Negative values of apparent availability (net absorption) of P recorded with goldfish were presumably due to the insufficient acclimation period to the test diets rather than to the dietary acidification since those negative numbers were not associated with dietary treatment. Omnivorous goldfish, having a long intestine, might require an appreciably longer period for dietary acclimation than carnivorous rainbow trout.

The dietary requirement of P for rainbow trout is approximately 0.6% (NRC, 1993). The P content of whole body of rainbow trout was reported to be 0.47-0.48% when P-sufficient feeds were fed (Shearer, 1984; Shearer and Hardy, 1987). If feed efficiency is 100%, this is the level of available P needed in the feed (assuming no absorption of P from water, renal regulation for P is normal and endogenous fecal loss is negligible). If feed efficiency is lower than 100%, which is common in practical situations, the fish may require even less than this amount of available P in diets. The basal

diet used in the present study contained approximately 1.1% P on a dry basis (Table 44). Apparently, rainbow trout do not require this much P in the diet. By adding citric acid in feeds and increasing the bioavailability of P in fish meal which is otherwise less available to fish, the fish absorbed more P than they required. This "excess" amount of P must be discharged from the body to maintain P homeostasis. Thus, the increase of P concentrations in urine and in the tank water (Tables 49 and 50) can be considered a natural consequence of increased bioavailability and absorption of P by citric acid. Increased absorption of Ca from diets in the 5% citric acid group, and the concomitant increase of non-fecal excretion of Ca (excretion into the tank water) is in agreement with the observed behavior of P.

Apparent availabilities of Zn and Fe were not markedly increased by the dietary supplementation of citric acid as indicated by the relatively small values of the regression coefficients, which might be due to the fact that the levels of those minerals in the diet were above the requirements of fish (NRC, 1993), and the fish might regulate the absorption and excretion of those elements at the gastrointestinal level as reported with experimental animals and humans (Weigand and Kirchgessner, 1980; Kirchgessner and Weigand, 1983; Morris, 1987; Sandstrom, 1988). Schwarz et al. (1983) reported that the availability of inorganic Zn in diets was not increased by the supplementation of citric acid in rats. Also, dietary supplementation of EDTA did not increase availability of inorganic Zn in diets for rainbow trout (Hardy and Shearer, 1985). Conversely, chelated Zn (Zn proteinate) has been demonstrated to be more available than inorganic sources to rainbow trout (Hardy and Shearer, 1985) and to channel catfish (Paripatananont and Lovell, 1997), suggesting that the lack of effects of dietary citric acid or EDTA on the availability of Zn could be attributed to its chelating effect; i.e., either low affinity of those ligands to dietary Zn or low absorption of the chelates.

Non-fecal excretion of elements as estimated by sampling tank water often showed negative values, which means that a portion of the tank amount of element initially present was absorbed by the fish (e.g., Cu and Fe). During week-5, non-fecal excretion of Fe, Mg and Mn became highly negative. Increasing the Ca concentration in the water during week-5 by dissolving calcium sulfate, which was not ultra pure grade, resulted in increased concentrations of some other elements (Fe, K, Mn and P). Apparently, fish "filtered" these water-borne elements quite efficiently. In addition, dietary concentrations of some elements were low, which resulted in highly negative non-fecal excretion values since they were expressed per dietary intake basis of that element. Conversely, non-fecal excretion of some elements was found to be more than 100% of dietary intake. This pattern did not persist in any element or treatment during week 2-4 since it is evidence of negative balance of the element. During week-5 when water-borne Ca concentration was elevated about 10 fold over the previous weeks, however, non-fecal excretion of some elements (K, Sr and Zn) noticeably increased. Certainly, this was an acute response of fish to the elevated Ca concentrations in water, and did not indicate a long-term effect.

The possibility of metabolic acidosis caused by feeding acid for prolonged periods of time was hypothesized initially, and to counteract the potential acidosis problem, calcium hardness of the water was elevated experimentally (during week-5). Apparently, fish fed acidified diets remained normal and no physiological disorders were observed. Increasing the Ca concentration in the water, however, affected apparent availability or net absorption of some minerals. The positive effect of dietary citric acid on the digestion of ash, and the availabilities of Ca, Mg, Mn and Sr observed during the previous weeks was decreased by elevating water-borne Ca during week-5. The balance of Sr and Mn became negative or quite low during this week. Since fecal samples collected by stripping after week-5 showed positive values that were similar to those obtained during weeks-2, 3 and 4, the negative values for Sr and Mn during week-5 may be an artifact caused by increased concentrations of those elements in the water, precipitation from the water or their contamination in the settled feces.

Negative apparent availability (net absorption) of Na resulted from its high content in feces collected by stripping. Since data from previous weeks all yielded positive values for apparent availability, the odd values should be attributed to the different fecal collection method. Leaching of Na in feces collected by the settling method should be suspected; however, the stripping method involves its inherent problems of squeezing incompletely digested materials, disturbing the unstirred water layer of the intestinal mucosa and collecting intestinal fluid by applying external pressure.

The present study confirmed previously observed effects that citric acid increases the availability of phosphorus and several other minerals in fish meal. The effect was consistent over the 5-week feeding trial with rainbow trout. However, a concomitant increase in renal excretion of P was observed as a result of the increased absorption of phosphorus by citric acid supplementation. To lower phosphorus levels in trout hatchery effluents, it will be necessary to reduce both fecal and urinary excretion of phosphorus. Further research is, therefore, needed to determine the extent to which dietary P levels must be lowered in feeds in which dietary P availability is increased by citric acid supplementation.

Table 44. Composition of the basal diet.[1]

Ingredient	%
Fish meal[2]	50.0
Fish oil	5.0
Soybean oil	10.0
Alpha-cellulose[3]	10.0
Dextrin[4]	10.0
Corn starch[5]	10.0
Carboxymethyl cellulose[6]	2.0
Choline chloride[7]	1.0
Vitamin mix.[8]	1.8
Vitamin C[9]	0.1
Yttrium oxide (Y_2O_3)[10]	0.1
Total	100.0
(Water)	(40.0)

[1] The basal diet contained the following: Ca, 14400; P, 10900; Mg, 1040; K, 3270; Na, 4970; Cu, 2.19; Fe, 133; Mn, 5.90; Sr, 24.9; Zn, 78.6; Y, 796 (µg /g dry matter); and ash, 5.78; crude protein, 37.6 (%/dry matter).
[2] Herring meal, Silver Cup brand (Nelson & Sons, Inc., Murray, UT).
[3] Alpha cellulose fiber (C 8002: Sigma Chemical Co., St. Louis, MO).
[4] Type-3, from corn white powder, 12% water-soluble (160057: ICN Biomedicals, Inc., Cleveland, OH).
[5] Gelatinized, from corn (S 4126: Sigma Chemical Co.).
[6] Carboxymethyl cellulose, sodium salt (C 5013: Sigma Chemical Co.).
[7] 70% solution (C 1879: Sigma Chemical Co.).
[8] Abernathy No. 2 formula (Hoffmann-La Roche Inc., Nutley, NJ).
[9] Coated ascorbic acid (Takeda U.S.A., Inc., Orangeburg, NY).
[10] Yttrium oxide (Y 3500: Sigma Chemical Co.).

Table 45. Effect of dietary citric acid supplementation on the apparent availability of major elements in fish meal determined with rainbow trout.[1]

	wk	Citric acid (%)			r^2	slope[3]
		0	2	5		
Ca	2	18.5	46.2	63.0	0.97	8.63
	3	35.9	45.7	59.3	1.00	4.67
	4	34.5	33.5	59.7	0.90	5.32
	5	28.8	37.0	39.3	0.91	2.00
	S	30.3	39.4	65.1	0.99	7.08
P	2	70.4	79.6	87.8	0.99	3.41
	3	75.8	79.0	87.6	0.99	2.39
	4	75.0	73.4	89.5	0.88	3.09
	5	75.8	78.0	85.6	0.98	2.00
	S	73.9	78.5	92.7	0.99	3.83
Mg	2	59.1	73.1	81.3	0.97	4.31
	3	69.2	70.6	79.2	0.96	2.08
	4	68.8	67.4	76.3	0.85	1.62
	5	50.9	58.3	50.1	-0.20	-0.36
	S	55.3	57.7	83.0	0.95	5.76

[1] Values indicate apparent availability of nutrients determined on a pooled fecal sample of five consecutive days for each week (fish were acclimatized to each diet during week 1; no fecal samples collected). Fecal samples were collected by settling (week 2, 3, 4 and 5) or by stripping (at the end of the feeding experiment) from one tank per treatment containing five fish each (initial mean body weight 256.4g). During week 5, water hardness was increased ca. 10 times of the previous weeks by using calcium sulfate.

[2] Correlation coefficient between citric acid % and apparent availability (digestibility) % of dietary nutrients (n=3).

[3] Regression coefficient (X, citric acid %; Y, apparent availability %; n=3).

Table 46. Effect of dietary citric acid supplementation on the apparent digestibility and availability of dry matter (Dm), ash and electrolytes in fish meal determined with rainbow trout.[1]

	wk	Citric acid (%)			r^2	slope[3]
		0	2	5		
DM	2	76.7	79.1	78.5	0.63	0.31
	3	77.8	83.3	78.9	0.06	0.07
	4	77.1	76.4	80.3	0.84	0.69
	5	79.2	77.9	76.7	-0.99	-0.49
	S	73.3	72.9	75.2	0.85	0.42
Ash	2	46.5	65.0	72.9	0.94	5.08
	3	55.4	70.2	71.4	0.84	2.98
	4	52.5	52.1	68.7	0.91	3.42
	5	54.2	58.5	55.8	0.25	0.22
	S	24.3	30.0	51.1	0.98	5.50
K	2	89.0	90.5	90.9	0.92	0.37
	3	90.1	92.8	90.4	-0.01	-0.01
	4	89.9	90.3	92.1	0.98	0.44
	5	89.3	88.5	87.2	-1.00	-0.42
	S	82.7	81.9	75.4	-0.95	-1.52
Na	2	37.6	74.3	81.5	0.89	8.28
	3	51.0	58.4	79.8	0.99	5.87
	4	57.8	49.4	50.2	-0.75	-1.38
	5	50.4	52.7	36.5	-0.86	-2.99
	S	-87.3	-82.2	-49.7	0.96	7.78

[1] See footnote 1 of Table 45.
[2] See footnote 2 of Table 45.
[3] See footnote 3 of Table 45.

Table 47. Effect of dietary citric acid supplementation on the apparent availability of trace elements in fish meal determined with rainbow trout.[1]

	wk	Citric acid (%)			r^2	slope[3]
		0	2	5		
Cu	2	35.6	36.1	52.3	0.93	3.51
	3	44.8	27.0	43.2	0.03	0.12
	4	37.2	32.9	44.9	0.72	1.75
	5	25.5	28.0	31.4	1.00	1.17
	S	9.3	39.8	43.9	0.86	6.47
Fe	2	-2.5	17.0	13.1	0.68	2.77
	3	15.8	-6.6	32.9	0.53	4.19
	4	8.8	-12.4	7.6	0.06	0.30
	5	-14.9	7.3	-15.7	-0.15	-0.75
	S	11.5	9.9	12.2	0.40	0.19
Mn	2	17.5	45.8	44.6	0.78	4.96
	3	35.3	43.3	50.8	0.99	3.07
	4	28.0	23.8	47.7	0.84	4.25
	5	-24.7	-9.1	-50.0	-0.70	-5.73
	S	14.8	45.5	67.1	0.98	10.22
Sr	2	15.8	40.6	57.9	0.98	8.21
	3	34.4	40.9	54.8	1.00	4.12
	4	33.0	28.2	55.8	0.84	4.94
	5	-10.3	3.7	-8.0	0.04	0.12
	S	22.6	31.1	60.1	0.98	7.67
Zn	2	44.0	50.5	54.5	0.97	2.04
	3	56.8	55.2	49.4	-0.98	-1.51
	4	53.9	35.2	44.7	-0.39	-1.44
	5	42.6	43.4	44.7	1.00	0.43
	S	39.7	47.8	59.9	1.00	4.03

[1] See footnote 1 of Table 45.
[2] See footnote 2 of Table 45.
[3] See footnote 3 of Table 45.

Table 48. Effect of dietary citric acid supplementation on the apparent availability of major elements in fish meal determined with goldfish.[1]

	wk	Citric acid (%)			r^2	slope[3]
		0	2	5		
Ca	2	-137.87	-85.26	-51.88	0.97	16.72
	3	-46.65	-44.57	-48.18	-0.52	-0.38
P	2	-33.16	-3.47	19.38	0.98	10.28
	3	16.53	20.08	23.82	0.99	1.44
Mg	2	33.15	45.28	52.50	0.97	3.75
	3	51.64	60.04	48.12	-0.40	-0.96

[1] Values indicate apparent availability (%) of dietary minerals determined on a pooled fecal sample of seven days for each week (fish were acclimatized to each diet during week 1; no fecal samples collected). Fecal samples were collected by settling from one tank per treatment containing five fish each (initial mean body weight 149.7g).

[2] See footnote 2 of Table 45.

[3] See footnote 3 of Table 45.

Table 49. Effect of dietary citric acid supplementation on the non-fecal excretion[1] of minerals (% of dietary intake; mean ± s.d.) from rainbow trout fed fish meal-based diets.

	wk	Citric acid (%)			r^2	slope[3]
		0	2	5		
Ca	2	-3.7	6.0	79.4	0.96	17.25
	3	15.7	21.2	61.9	0.96	9.58
	4	6.3	14.2	53.6	0.97	9.75
	5[4]	ND[5]	ND[5]	ND[5]		
P	2	44.6	45.9	60.2	0.94	3.26
	3	38.4	45.4	55.9	1.00	3.49
	4	36.3	45.9	57.7	1.00	4.24
	5[4]	43.4	51.9	87.7	0.97	9.09
Mg	2	46	43.2	117.0	0.90	15.04
	3	55	57.3	83.5	0.94	5.86
	4	43	23.9	94.4	0.78	11.33
	5[4]	-371	-356	-2321	-0.91	-411
Fe	2	-77	-30	8	0.99	16.63
	3	-103	-88	-72	1.00	6.06
	4	-110	-100	-80	1.00	6.09
	5[4]	-452	-464	-1605	-0.92	-242
Mn	2	-105	-67	-36	0.99	13.56
	3	-82	-189	-172	-0.70	-15.96
	4	-267	-294	-232	0.66	8.20
	5[4]	-1940	-1825	-6948	-0.91	-1057
Sr	2	5.3	1.7	113.1	0.91	22.79
	3	28.4	35.0	80.4	0.96	10.78
	4	31.1	26.0	88.1	0.89	12.14
	5[4]	5686	3871	3741	-0.84	-362
Cu	2	-340	414	-714	-0.43	-98.5
	3	-741	-1051	-2649	-0.97	-393.6
	4	-424	-153	-587	-0.48	-41.6
	5[4]	-632	-72	-10424	-0.90	-2076
Zn	2	-157	68	144	0.92	57.52
	3	-2	0	-39	-0.90	-7.76
	4	-6	-50	23	0.50	7.29
	5[4]	69	263	528	1.00	91.54

[1] Values indicate non-fecal excretion (presumed to be a total of gill and urinary excretion) of nutrients determined on a pooled water sample of five consecutive days for each week (fish were acclimatized to each diet during week 1; no samples collected). Water samples were collected twice a day (0h and 23h post feeding) from one tank per treatment containing five fish each (initial mean body weight 256.4g). Non-fecal excretion of nutrients were estimated by the difference of nutrient concentrations in tank water sampled at 0 h and 23 h post feeding.

[2] Coefficient of determination between citric acid % and the non-fecal excretion of minerals (n=3).

[3] Regression coefficient (X, citric acid %; Y, non-fecal excretion of minerals %; n=3).

[4] During week 5, water hardness was increased by using calcium sulfate.

[5] Not determined.

Table 50. Effect of dietary citric acid supplementation on the pH, bicarbonate, calcium, phosphorus, potassium, soluble inorganic phosphorus and citric acid levels in diets, feces, scales, urine and blood of rainbow trout.

		0% (control)	2% citric acid	5% citric acid
pH	diet	6.46	4.71	4.03
	feces solution[1] (wk 4)	7.00	6.47	6.11
		6.92	6.71	6.04
	(wk 5)			
	urine[2]	5.9	ND[6]	5.5
	blood[3]	7.12	7.17	7.21
Bicarbonate	plasma (mEq. HCO_3^-/L)[4]	12.5 ± 2.8	11.6 ± 1.4	14.8 ± 1.6
Ca	plasma (μg /g)[5]	118	118	129
	scales (% air dry basis)[5]	15.2	15.3	16.4
P	plasma (μg /g)[5]	756	736	803
	scales (% air dry basis)[5]	7.47	7.48	8.02
K	plasma (μg /g)[5]	80.6	80.2	78.9
	scales (% air dry basis)[5]	1.19	1.31	1.52
Soluble inorganic phosphorus (μg /g)				
	diet (dry basis)	1800	2630	3210
	feces (dry basis)[5] (wk 4)	6370	6200	2580
		5220	6270	3540
	(wk 5)			
	urine[2]	112	ND[6]	652
Citric acid (μg /g)				
	diet	387	10900	17900
	feces (dry basis)[5] (wk 4)	tr.[7]	218	6970
		tr.[7]	tr.[7]	10600
	(wk 5)			
	plasma[4]	17.5 ± 5.0	17.7 ± 4.0	18.4 ± 11.6

[1] On a pooled sample of 5 fish per treatment, contained approximately 0.8-1.6% feces (dry basis).
[2] On a pooled sample of 3 fish per treatment.
[3] Average of 2 fish per treatment.
[4] Average (± s.d.) of 5 fish per treatment.
[5] On a pooled sample of 5 fish per treatment.
[6] Not determined (urine collection unsuccessful).
[7] Below detection limit (< 100 μg/g).

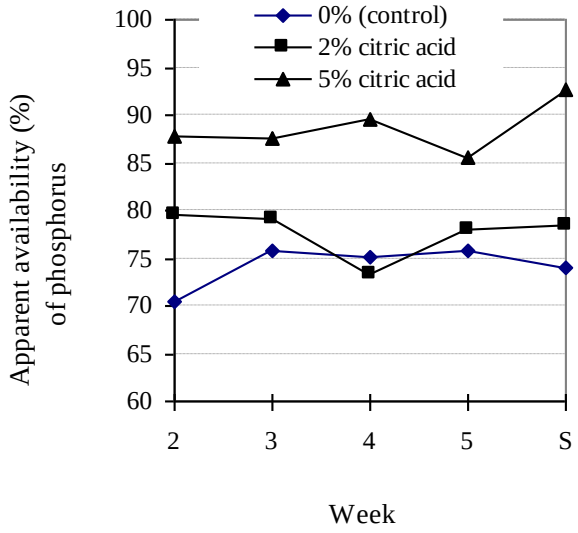


Figure 18. Effect of dietary citric acid supplementation on the apparent availability (%) of phosphorus in fish meal determined with rainbow trout.

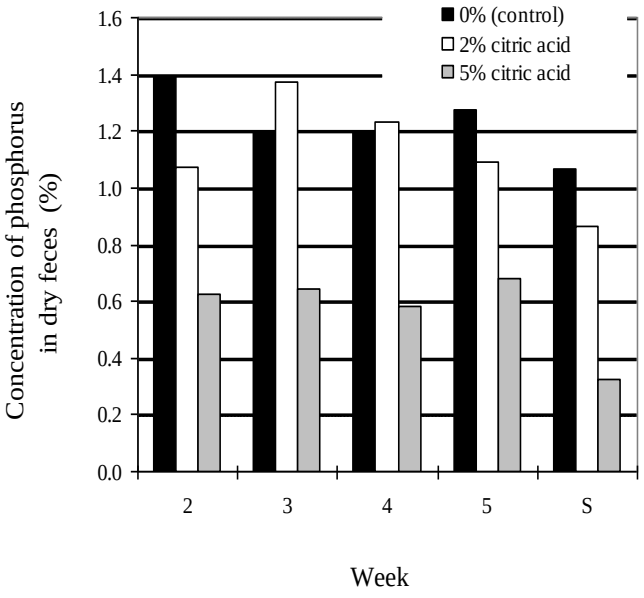


Figure 19. Concentration of phosphorus in dried feces (%) of rainbow trout fed fish meal-based diets with varied levels of supplemental citric acid.

3. Effect of Citric Acid on the Performance of Fish

ABSTRACT Effects of dietary citric acid on P and Ca digestibilities and fish performance were studied on low- and high-ash diets using rainbow trout. In high-ash diets, supplementation with citric acid significantly increased net absorption of P (i.e., reduced fecal excretion), which, however, resulted in a marked increase of urinary excretion of P. The net P retention was, therefore, similar regardless of citric acid supplementation. In low-ash (LA) diets, dietary P was almost completely absorbed and retained regardless of the citric acid supplementation, and both fecal and urinary excretion were negligible. Dietary supplementation of citric acid up to 10% (dry basis) apparently did not affect feed intake, feed utilization and weight gain during 35 days of satiation feeding. The pH values of chyme and feces were significantly lower in fish fed citric acid-supplemented diets than in fish fed the control diet (no supplemental citric acid). Fecal Ca and P levels were much lower and citric acid content was higher in fish fed diets supplemented with citric acid than in fish fed the control diet. Plasma Ca, citric acid and alkaline phosphatase levels were significantly higher in fish fed diets supplemented with citric acid. Urinary P level was higher in the citric acid groups than in the control group. Total cholesterol in plasma was slightly higher in fish fed citric acid-supplemented diets than in fish fed the control diet. No notable difference was observed in a 35 day feeding trial with up to 10% supplemental citric acid in the pH values of blood, urine and fillets, in the Ca content in bone, urine and chyme, in the P content in bone, chyme and plasma, in the citric acid content in bone, urine and fillets, in the plasma bicarbonate, chloride, acetoacetate and total cholesterol levels, in the chloride content in chyme, in the total lipid content in fillets, in the peroxide value of the lipids in fillets, or in the liver glycogen content. This study demonstrated that a significant reduction of both fecal and urinary excretion of P was possible without any adverse effects in feed intake, feed utilization, growth and health of rainbow trout by reducing total amount of P in diets and by increasing availability of P with citric acid supplementation.

BACKGROUND

The dietary supplementation of citric acid increased availability of minerals in fish meal (ref. Study 1 and 2 in Ch. 3). The possible effects were solubilization of fish bone (hydroxyapatite) in fish meal by dietary acidification and the chelation of calcium (as Ca-citrate) and of other minerals. The high bone content in practical fish meal-based diets that supplied higher amounts of phosphorus than fish required, however, resulted in a concomitant elevation of urinary P excretion in the previous trial (ref. Study 2 in Ch. 3). In order to minimize both fecal and urinary excretion of P, reducing bone content in the basal diet is essential. Dietary or water-borne calcium presumably buffers or neutralizes dietary acid. Consequently, if dietary Ca level is low and dietary acid level is high, the acid could be more toxic to fish.

Citric acid is a key intermediary compound in energy metabolism; it inhibits phosphofructokinase in glycolysis and activates acetyl-CoA carboxylase in lipogenesis. Thus, the cellular concentration of citric acid is important in the allosteric control of key enzymes and de novo lipid synthesis (Goodridge, 1973; Donaldson, 1979). The effect of dietary citric acid on lipid synthesis or proximate body composition has not been reported in fish. The objective of this study was to reduce both fecal and urinary excretion of P by supplementing low ash (low P) diets with citric acid, and to investigate the potential side effects of high levels of dietary citric acid intake in low-ash diets.

MATERIALS AND METHODS

Experiment 1 (Partition of dietary P in High-ash vs Low-ash diets)

Two basal diets containing either low- or high-levels of ash (Ca and P) were formulated (Table 51). Low-ash diet (LA) contained a marginal level of P, while the high-ash diet (HA) had a P level similar to commercial salmonid feeds. The two basal diets were supplemented with either 0, 5 or 10% citric acid (dry basis), cold extruded into pellets containing ca. 30% water, and stored at –20°C until fed.

One hundred eighty rainbow trout (initial mean body weight, 167.7g; sem, 3.01) were randomly stocked in 18 145 L-fiberglass tanks (ten fish per tank) receiving a continuous flow of spring water (temperature, 15 ± 0.5°C; Ca, ca. 35 mg/L; pH, 8.1; dissolved oxygen, 8.5-9.5 mg/L) at ca. 5 L per min. Three tanks were randomly assigned to each dietary treatment; i.e., LA0, LA5, LA10 (low-ash diet with 0, 5 or 10% citric acid, respectively) and HA0, HA5, HA10 (high-ash diet with 0, 5 or 10% citric acid, respectively). Fish were fed the test diets once daily for 10 days at 1.13% (dry basis) of body weight per day. At the end of the feeding trial (10th day), fish were gently transferred to a metabolic tank (Fig. 33, p.198) 5 min after feeding, and the fecal and nonfecal (urine) wastes were collected for 24 h. There was no mortality during the feeding trial.

The amount of P excreted into feces was calculated based on the apparent digestibility of P using acid-insoluble ash (as the indigestible indicator) and the amount of diet given. The amount of P excreted via urine was estimated based on the concentration of P in the tank water. Both fecal and dietary samples were ashed (550°C-12h), dissolved in a concentrated acid solution (hydrochloric / nitric acid, 1/1), diluted to an appropriate concentration, and determined for P and Ca by the molybdovanadate method (APHA, 1989) and the o-cresolphthalein complexone method (Sigma Diagnostics #587), respectively. The concentration of P in water samples were determined by the stannous chloride method (APHA, 1989).

Experiment 2 (Fish performance with Low-ash fish meal diets)

A low-ash diet (FM) was formulated using herring meal and wheat gluten meal as the major protein sources (Table 51). The diet contained a slightly higher level of P and much higher levels of Ca

than the LA diets used in the Experiment 1. The test diets with/without supplemental citric acid (0, 5, 10%, dry basis) were prepared and stored as described above.

Ninety rainbow trout (initial mean body weight, 164.6g; sem, 2.52) were randomly stocked in 9 fiberglass tanks (10 fish/tank) and 3 tanks were randomly assigned to each dietary treatment; i.e., 0, 5, 10% citric acid in the FM diet. The rearing system and methods were the same as described above except fish were fed to apparent satiation for 35 days. There was no mortality during the feeding period. Eight hours after the last feeding, all fish were anesthetized and urine, feces, blood and chyme were sampled (10 fish per tank). The urine and feces were collected by stripping, blood was withdrawn from caudal vessels into heparinized syringes, chilled on ice, and the plasma was separated by centrifugation (1,000 × g, 3 min) within 2 hours of collection. The chyme was obtained by inserting a glass tube from mouth into the stomach. Fish were weighed after sampling of urine, feces, blood and chyme, and stored at –20°C for one week before fillets, livers and vertebrae were separated for further analyses. The fillet (with skin and ribs attached) and liver samples were obtained from the 7 largest fish per tank, pooled by tank, minced twice without thawing completely. The vertebral samples were collected from the 7 largest fish per tank (from the fillet scraps), pooled by tank, dipped in a boiling water, muscles were removed, washed with tap water several times, air-dried, ground, washed twice with chloroform-methanol (1/1), and dried (105°C, 1 h) to obtain bone samples for Ca, P and citrate analyses.

The pH of blood, urine, chyme, and feces were measured immediately after sampling using a pH electrode (Accumet®, model-50, Fisher Scientific, Pittsburgh, PA). The pH of diets and fillets were determined on frozen stored samples. The plasma samples were stored at 0-4°C for no longer than 2 days before analysis for acetoacetate (Schilke and Johnson, 1965), alkaline phosphatase (Sigma Diagnostics #104, incubated at 15°C), bicarbonate (Segal, 1955), and glucose (Hyvarinen and Nikkila, 1962). The aliquot of plasma samples for the determination of citric acid, Ca, P, chloride and total cholesterol concentrations were stored at –20°C up to 2 weeks. The concentration of citric acid in dried bone, feces (wet samples), fillets (wet samples), plasma and urine were determined on TCA extracts by the procedure of Beutler and Yeh (1959). Ca and P contents in bone, feces, chyme, plasma and urine were determined on ashed (550°C-12h) samples by o-cresolphthalein complexone method (Sigma Diagnostics #587) and phosphomolybdate-ferrous sulfate method (Taussky and Shorr, 1953), respectively. Chloride content in plasma and chyme were determined according to Van Slyke and Hiller (1947). Total cholesterol in plasma was determined by the procedure of Assous and Girard (1962). Liver glycogen content was determined on frozen-stored samples by the method of Oser (1965). Total lipids in the minced fillets were determined according to Bligh and Dyer (1959). The extracted lipids were titrated with standard sodium thiosulfate solution for the determination of peroxide value (AOAC, 1984). The crude protein in diets and feces was determined (on dried samples) using a LECO FP-428 Nitrogen determinator (Leco Instruments, St. Joseph, Michigan, USA). Apparent digestibility of protein and

availabilities of calcium and phosphorus were determined as the fractional net absorption from diets based on acid-insoluble ash as the non-absorbable, inert dietary marker.

The analytical data were subjected to a single factor ANOVA and a linear regression. The treatment effect was considered significant at $P< 0.05$. Fish were handled in accordance with the guidelines approved by the Animal Care Committee of the University of Washington.

RESULTS

Experiment 1 (Partition of dietary P in High-ash vs Low-ash diets)

In high-ash (HA) diets, the supplementation of citric acid significantly increased net absorption of P (i.e., reduced fecal excretion), which, however, resulted in a marked increase of urinary excretion of P. Because of the renal regulation, net P retention was similar regardless citric acid supplementation (Fig. 20). In low-ash (LA) diets, dietary P was almost completely absorbed and retained regardless of citric acid supplementation, and excretion of P in feces and urine were negligible.

Experiment 2 (Fish performance with Low-ash fish meal diets)

The dietary supplementation of citric acid up to 10% (dry basis) apparently did not affect feed intake, feed utilization and weight gain during 35 days of satiation feeding (Table 52). The pH values of chyme and feces were significantly lower in fish fed citric acid-supplemented diets than in fish fed the control diet (no supplemental citric acid). Fecal Ca and P levels were much lower and citric acid content was higher in fish fed diets supplemented with citric acid than in fish fed the control diet (Fig. 21). Plasma Ca, citric acid and alkaline phosphatase levels were significantly higher in fish fed diets supplemented with citric acid. Urinary P levels were higher in the citric acid groups than in the control group. Total cholesterol in plasma was slightly higher ($P< 0.05$ by regression; $P >0.05$ by ANOVA) in fish fed citric acid-supplemented diets than in fish fed the control diet. No notable difference was observed among treatments in the pH values of blood, urine and fillets, in the Ca content in bone, urine and chyme, in the P content in bone, chyme and plasma, in the citric acid content in bone, urine and fillets, in the plasma bicarbonate, chloride, acetoacetate and total cholesterol, in the chloride content in chyme, in the total lipid content in fillets and the peroxide value of the lipids in fillets, and in the liver glycogen content (Table 52).

DISCUSSION

Experiment 1 (Partition of dietary P in High-ash vs Low-ash diets)

In the high-ash diet treatment, the supplementation of citric acid markedly increased availability (digestibility) of dietary P by increasing net absorption of P, which agrees with the previous observation (ref. Study 1 and 2, Ch.3). In the high-ash diets; however, marked increase of urinary excretion of P was noted as the result of citric acid supplementation presumably due to the excess amount of P absorbed

from acidified diets. Studies in humans indicated that the intake of citric acid increased absorption of $CaHPO_4$ and increased urinary excretion of Ca, P and citrate (de Leacy et al., 1989). They assumed that dietary citric acid acted by solubilizing insoluble salts in the gut lumen rather than by mobilizing calcium from bone or promoting its renal excretion.

In the low-ash diet treatment; however, fish absorbed dietary P quite efficiently without assistance of citric acid. The low Ca content in the low-ash diet indicated that the P source was mostly organic P of high availability rather than inorganic bone phosphate (hydroxyapatite). The result, therefore, may not be predictive for the effect of citric acid in "low-fish bone" diets. However, fish apparently did not discharge P even when the diet was highly acidic and low in Ca content, indicating bone dissolution (reported by other workers, p 101) did not occur by the dietary supplementation of citric acid up to at least 10% per diet (dry basis).

Experiment 2 (Fish performance with Low-ash fish meal diets)

Fish accepted the highly acidic feed from the first feeding without any sign of hesitation. In addition, no differences in feed intake, feed efficiency and weight gain were observed between the control and citric acid groups during 35 days of satiation feeding. Physiological parameters (Table 52) also indicated no apparent shift from the normal state (control fish). In rats and guinea-pigs, the ingested citric acid is rapidly metabolized by the tissues and there appears to be little evidence that elevated (up to 5% in diet) or prolonged (up to 200d) dietary intakes of citric acid in either low- or high-Ca diet are likely to exert any general toxic effect (Bonting, 1952; Wright and Hughes, 1976). In humans, citric acid does not alter acid-base balance (Sherman et al., 1936; Sakhaee et al., 1992). In broilers, however, significant reduction of feed intake, weight gain and abdominal or carcass fat percentage were reported in birds fed 3-5 % citric acid in a corn-soybean diet for 42 days after birth (Lessard et al., 1993; Gentesse et al., 1993; 1994). In the present study with rainbow trout, neither feed intake nor weight gain was affected after 35 days of satiation feeding with up to 10% citric acid in diets. Although citric acid is known to stimulate lipogenesis by activating the rate-limiting enzyme acetyl-CoA carboxylase, there was no measurable increase of fat deposition in fish fillets. Also, although citric acid is known to inhibit glycolysis by inhibiting phosphofructokinase, there was no significant increase of plasma glucose or liver glycogen. There was a slight increase of plasma cholesterol by citric acid feeding, which might be due to increased availability of cytoplasmic acetyl-CoA (via ATP-citrate lyase) for cholesterol de novo synthesis. Since acetoacetate levels were not altered, dietary citric acid was apparently not utilized for ketogenesis. Plasma citric acid increased with dietary citric acid administration, which was followed by the increase of both fecal and urinary excretion. Little amount was retained in bones or fillets of fish. Sherman et al. (1936) reported a large amount of dietary citric acid was efficiently oxidized by dogs following the oral administration of 0.5-2.0g of citric acid per kg of body weight.

The pH of the stomach content (chyme) sampled 8h after feeding was lower in acidified groups, but the chloride content in the chyme was apparently unaffected by the acidification, which differs from the observation of Hunt and Knox (1973) in humans who reported less output of acid and chloride with slower gastric emptying by increasing citric acid levels in diets. Higher levels of P (not significant) and Ca in plasma could be due to the increased absorption of dietary P and Ca. Alkaline phosphatase activity in plasma was increased with increasing percentages of dietary citric acid, but the exact mechanism for this effect is not clear. Pinchasov and Elmaliah (1995) also noted increased plasma alkaline phosphatase activities in broiler chicks by dietary acetic and propionic acids. The activity of alkaline phosphatase generally increased in P deficiency in various tissues as a compensatory response to lower intake of dietary P (Pileggi et al., 1955; Davies et al., 1970; Kempson et al., 1979; Birge and Avioli, 1981). In rainbow trout and catfish, the plasma alkaline phosphatase activity increased as the dietary P level increased (ref. Table 4, p 26; Eya and Lovell, 1997a), which disagrees with findings reported earlier by other workers with other species but might agree with the present observation since fish absorbed more P when diets were supplemented with citric acid. Fecal P contents were significantly and linearly decreased by the supplemental citric acid. This indicates that the use of citric acid in diets containing marginal levels of P (as bone P or hydroxyapatite) is an effective approach to increase its availability. Fecal content of Ca was less affected than P by the supplemental citric acid, which might be due to the formation of Ca-citrate precipitate in the intestine, while P might be liberated from hydroxyapatite as either HPO_4^{2-} or $H_2PO_4^-$ (Misra, 1996). In spite of the distinctive difference of P content in feces, P content in the chyme was little changed by the dietary citric acid. This indicates that little or no absorption of P occurs in the stomach under these conditions. Phillips et al. (1964) and Sugiura (ref. Fig.4, p.31; Fig.43, p.207; Fig.45-48, p.209-216) observed that P excretion (via urine) peaked at 6-8 h after feeding. Because total passage time of feed through the gastrointestinal tract in actively-feeding fish is often less than 24 h (personal observation), the early excretion of P could be due to the rapid gastric evacuation followed by the absorption of P via intestine rather than via stomach.

CONCLUSION

Dietary supplementation of citric acid markedly reduced fecal excretion of P without any adverse effects in feed intake, feed utilization, growth and health of rainbow trout after 35 days of satiation feeding with fish meal-based diets containing marginal levels of P. By reducing the total amount of P in diets and by increasing the availability of P with citric acid supplementation, reducing both fecal and urinary excretion of P is possible without reducing fish growth or feed efficiency.

Table 51. Composition of the basal diets (Experiment 1 and 2).[1]

	LA[2]	HA[2]	FM[2]
Flesh meal[3]	50	30	-----
Bone meal[3]	-----	20	-----
Herring meal[4]	-----	-----	20
Wheat gluten meal[4]	-----	-----	20
Gelatin[5]	10	10	10
Fish oil[6]	15	15	25
Alpha-cellulose[6]	10	10	10
Dextrin[6]	10	10	10
Carboxymethyl-cellulose[6]	1	1	1
Vitamin mix.[7]	3	3	2
Mineral mix.[8]	-----	-----	1
SiO_2	1	1	1
Total	100	100	100
Citric acid[9]		0 / 5 / 10	
TM soln.[10]	(40)	(40)	(40)
Analytical Composition			
Crude Protein	47.7	41.4	43.1 (40.2, 38.2)[13]
Crude Ash	4.62	11.9	6.40
Total Ca	0.031	3.16	0.614
Total P	0.573	2.04	0.640
Bone P[11]	3.2	92.8	57.4
Dietary pH	ND[12]	ND[12]	6.14 (4.05, 3.47)[13]

[1] Values are g / 100g dry diet.
[2] Diet abbreviations; LA (low-ash diet); HA (high-ash diet); FM (fish meal diet).
[3] Prepared from large rainbow trout (Flesh meal; whole fish were boiled for ca. 5 min, flesh, liver and gonads were separated from skin, bones, fins and head, partially-dried at 130°C-12h (to the moisture level approx. 50%) and then air-dried at room temperature for 24h; Bone meal: skin, bones, fins and head were boiled for ca. 5 min, washed several times with hot water to remove fats and dried as above (P content, %/dry basis; Flesh meal, 1.11; Bone meal, 7.29).
[4] Commercial grade; contained 2.41% P (herring meal) and 0.284% P (wheat gluten meal), (dry basis).
[5] 300 bloom, from porcine skin (United States Biochemicals); contained 0.011% P.
[6] Fish oil, alpha-cellulose, dextrin, CMC contained the following amounts of P, respectively; 0.0018; 0.0012; 0.0196; 0.0013 (%, dry basis).
[7] Provided the following amount per 100g dry diet; (for LA and HA diets) #30 pre mixture (Hoffmann-La Roche), 2g; Choline (60%), 0.6g; Inositol, 0.2g; Ascorbic acid, 0.2g; (for FM diets) #30 pre mixture, 1g; Choline (60%), 0.6g; Inositol, 0.2g; Ascorbic acid, 0.2g. #30 pre-mixture and choline contained 0.0713 and 0.0650% P, respectively.
[8] Provided the following amount per 100g dry diet; NaCl, 0.3; KCl, 0.6; MgO, 0.1.
[9] 0, 5 or 10g of citric acid (Sigma Chemical Company) added to 100g of the basal diet.
[10] Trace mineral solution supplied the following amount (mg/kg dry diet); KI, 1.9; $MnSO_4 \cdot H_2O$, 40; $ZnSO_4 \cdot H_2O$, 85; Na_2SeO_3, 0.9; $CoCl_3 \cdot 6H_2O$, 4; $CuSO_4 \cdot 5H_2O$, 12; $FeSO_4 \cdot 7H_2O$, 300.
[11] Values are the percentage in total P. Calculated based on the Ca/P ratio of hydroxyapatite: 1.67 (Irving, 1973).
[12] Not determined.
[13] Values for the diet supplemented with citric acid at the level of 5% and 10%, respectively. The lower protein content in the citric acid-supplemented diet is due to the citric acid which replaced the whole diet.

Table 52. Physiological parameters[1] of fish fed for 35 days to satiation with fish meal diets (FM) containing citric acid at levels 0, 5 or 10% per diet (dry basis).

		Citric acid level (%, dry feed)						ANOVA	Regression
		0		*5*		*10*		P[3]	P[3]
%-gain (wt gain %/initial wt.)		77.6		70.2		72.2			
Feed efficiency (%, gain/feed)		110		107		108			
Feed intake (%/BW/d)[2]		1.43		1.38		1.40			
Crude protein (g/100g)	feces, dry	10.8		10.6		10.1			
	digestibilit	93.7		93.2		94.6		*	
Dry matter (g/100g)	fillets	28.9		28.5		28.3			
	chyme	26.8		25.7		23.1			*
pH	blood	7.59		7.57		7.55			
	chyme	3.88		3.29		2.88		**	***
	feces	8.38		7.92		7.80		***	***
	fillet	6.45		6.46		6.47			
	urine	7.66		6.37		6.82			
Ca (mg/g)	bone, dry	225	(408)	239	(421)	230	(412)		
	chyme, dry	25.0	(147)	25.5	(136)	22.7	(129)		
	feces, dry	24.8	(168)	18.9	(130)	16.6	(113)	***	***
	plasma	0.136		0.147		0.147		**	**
	urine	0.139		0.195		0.197			
P (mg/g)	bone, dry	122	(221)	125	(220)	126	(225)		
	chyme, dry	22.8	(134)	23.2	(124)	21.5	(122)		
	feces, dry	8.88	(59.9)	2.53	(17.3)	1.11	(7.5)	***	***
	plasma	0.123		0.150		0.150			
	urine	0.011		0.342		0.173		**	
Citric acid (μg/g)	bone, dry	10500		11200		10600			
	feces, dry	1340		35000		57700		***	***
	plasma	15.0		17.2		24.7		**	**
	urine	23.0		17.9		469.3			
	fillet, wet	75.7		97.2		90.8			
Alkaline phosphatase (U/L)	plasma	46.4		49.0		62.3		**	**
Bicarbonate	plasma	12.6		12.6		12.9			
Chloride (mM)	chyme, wet	108.7		114.8		98.2			
	plasma	69.7		70.1		68.6			
Glucose (μg/g)	plasma	1088		864		916			
Glycogen (g/100g)	liver	0.94		0.59		1.34			
Acetoacetate (mM)	plasma	0.60		0.39		0.62			
Total cholesterol (μg/g)	plasma	4450		4640		5030			*
Total lipids (g/100g)	fillets, dry	36.1		34.1		34.3			
	fillets, wet	10.4		9.7		9.7			
Peroxide (mEq./kg lipid)	fillet, lipid	11.2		13.7		11.1			

[1] Samples were taken 8h after feeding. Each value is the average of 3 tanks. Values in parentheses are expressed on the ash basis.

[2] Amount of feed intake at satiation feeding (dry feed consumed %/body weight/day).

[3] Single factor ANOVA and linear regression; * P< 0.05; ** P< 0.01; *** P< 0.001. n=3 (tanks) per treatment.

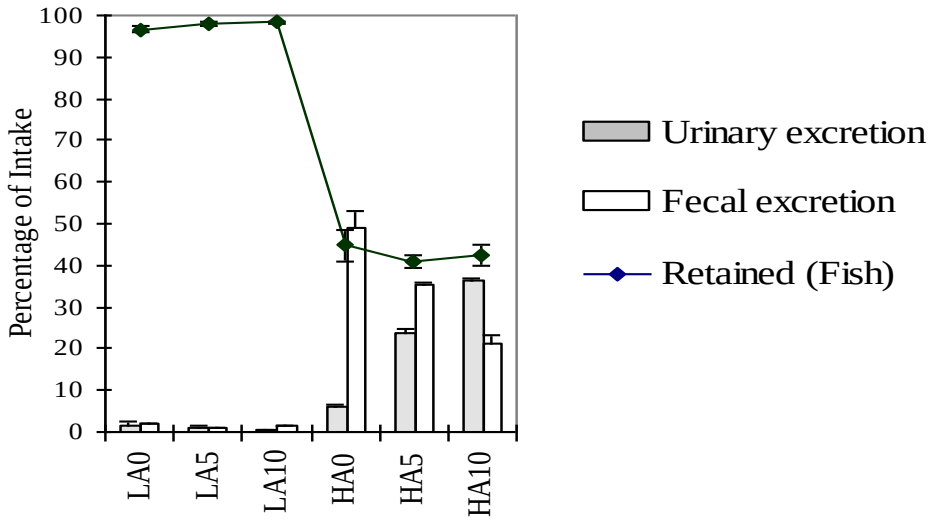


Figure 20. Partition of dietary P in low/high ash diets with/without supplemental citric acid (Experiment 1). Diet abbreviations: LA0 (low-ash diet + 0% citric acid); LA5 (low-ash diet + 5% citric acid); LA10 (low-ash diet + 10% citric acid); HA0 (high-ash diet + 0% citric acid); HA5 (high-ash diet + 5% citric acid); HA10 (high-ash diet + 10% citric acid).

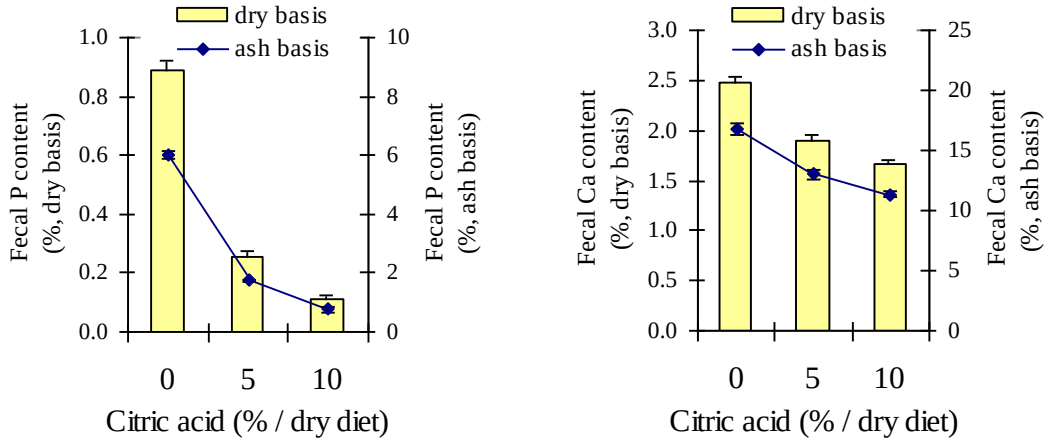


Figure 21. Fecal P and Ca content in fish fed fish meal diet (FM) supplemented with 0, 5 or 10% citric acid. Each value is the average of 3 tanks (sem as error bars). Fish were fed test diets to apparent satiation for 35 days. Feces were collected by stripping from all fish (10 fish) and pooled by tank.

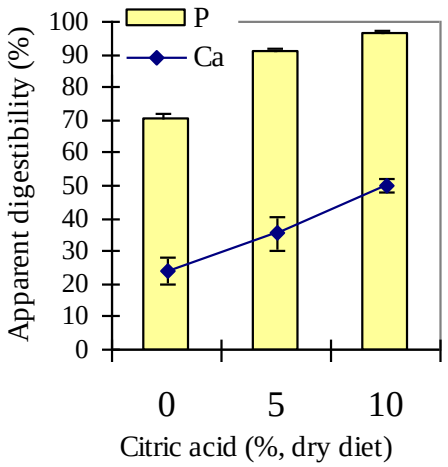


Figure 22. Apparent digestibility of P and Ca of fish meal diets (FM); Effect of supplemental citric acid. Each value is the average of 3 tanks (sem as error bars). Fish were fed test diets to apparent satiation for 35 days. Feces were collected by stripping from all fish (10 fish) and pooled by tank. Apparent digestibility was determined as the fractional net absorption of minerals from diets based on acid-insoluble ash as the indigestible dietary marker.

4. Use of Citric Acid in Practical Feeds

BACKGROUND

In a series of previous experiments, citric acid has been shown to increase the availability of phosphorus in fish meal. The increased absorption of phosphorus by fish resulted in a reduced excretion of phosphorus into feces, which was, however, accompanied by a concomitant increase in urinary excretion of phosphorus. The elevated urinary excretion of phosphorus was postulated to be a result of the regulatory response of fish to the excess phosphorus absorbed from feeds supplemented with citric acid. This suggests that for supplemental citric acid to be effective in reducing P excretion in fish meal-based feeds, the total phosphorus level in the feeds needs to be low. The present study was conducted to confirm the effect of citric acid to increase phosphorus availability using a practical low-phosphorus feed, and to reduce both fecal and urinary excretion of phosphorus to the lowest possible level.

MATERIALS AND METHODS

Experimental Feeds

Basal diet containing a marginal level of phosphorus (0.66 % by calculation) was formulated with practical ingredients (Table 53). Two experimental diets, one of which was not supplemented with citric acid (C0) and the other was supplemented with citric acid at 5 % (C5), were prepared as moist pellets. The ingredient mixtures (containing water) were placed in plastic bags and briefly heated in a microwave oven to gelatinize the starch portion of wheat flour in the diet. Feed pellets were prepared using a cold pellet extruder, air-dried for 24 h, and stored at 0-5°C until fed.

Feeding and Sampling

Forty-two rainbow trout (initial mean body weight ± sem, 59.6 g ± 2.0) were randomly stocked into six 140L-fiberglass digestibility tanks (seven fish per tank; three tanks per treatment). Each tank was equipped with a feces-collection device (modified Guelph type; Fig.33) and with an air-lifting self-recirculating system. The system collected feces into a long thin collection tube within one minute after being defacated by fish; thus, soluble components in feces might leach out into the water (in the tube) but were still separated from the tank water. Fish were acclimatized to the test diet and the environment for 10 days before sampling of feces and water started (week-1). The fecal and urine samples were collected by stripping during week-2. During week-3, the fecal and water samples were collected by the same procedure as week-1 except that fish from each treatment tank were pooled into one tank to increase response by increasing the number of fish. Between 1800 h and 1900 h each day, fish were fed a constant amount of test diet (6 g on an as-is basis per tank during week-1 and -2 and 18 g during week-3, which was ca. 1.5 % per body weight). After feeding, each tank and the attached

feces-collection system were cleaned and flushed thoroughly with plenty of water (15 ± 1°C) supplied from a common recirculation system, and the initial water sample was collected at 1900 h. From this time on, each tank was separated by an individual-recirculation and aeration system. No external water was supplied until 1800 h of the following day at which time the tank water was sampled again, and feeding, cleaning and flushing were completed within an hour as described above. Amounts of phosphorus and ammonia excreted into the tank water over a 23h period were determined by difference between the concentration in the tank water sampled initially (1900 h) and finally (1800 h). Fish were treated and handled in accordance with the guidelines approved by the Animal Care Committee of the University of Washington.

Analysis

Inorganic phosphorus concentrations in water were determined by the standard method (Stannous chloride method; 4500-P D., American Public Health Association et al., 1989). When the concentration was low, phosphorus was condensed by the magnesium co-precipitation method (Karl and Tien, 1992) prior to the analysis. Inorganic phosphorus concentrations in urine and total phosphorus concentrations in ashed feces and feeds were determined according to Taussky and Shorr (1953). Calcium contents in ashed feeds were determined by the o-cresolphthalein complexone method (Sigma Diagnostics, Procedure No. 587). Ammonium concentrations in the tank water were measured at 410nm using Nessler's reagent (HACH Company, Loveland, CO), EDTA, and NH_4Cl (in tank water) as the internal standard (Direct Nesslerization method, 4500-NH3 C., American Public Health Association et al., 1989). Total lipids in dry feces were extracted by an EtOH-Ether-7% HCl solvent system (Sobel, 1964). Total cholesterol was determined on the extracted lipids according to Kates (1972). Mean values were subjected to two-tailed t-test between control and citric acid groups. Differences were declared to be statistically significant at $P < 0.05$.

RESULTS & DISCUSSION

Fecal phosphorus concentrations in the citric acid-supplemented group were not largely different from those in the control (unsupplemented) group (Table 54), which was not expected since, in the previous three trials with citric acid, a difference was clearly and repeatedly demonstrated. The crude ash content in dry feces was similar in the two groups. Since dietary citric acid indeed lowered the pH value of the diet (Table 53), the marginal effect of dietary citric acid on the availability of phosphorus observed in the present experiment was unlikely due to insufficient dietary acidification. Another important effect of citric acid could, therefore, be responsible, i.e., the chelating effect. This chelating effect of citric acid may have been disabled or highly blocked by some unidentified compound in the feed.

Two practical ingredients, blood meal and wheat flour, were introduced in the present trial to replace semi-purified ingredients used in the previous trials (gelatin, dextrin, α-cellulose and wheat gluten). Blood meal supplied a large amount of iron in the diet (ca. 449.0mg Fe/kg dry diet). Ferrous sulfate supplied additional iron (60.1mg Fe/kg dry diet) to meet the dietary requirement by itself. Thus, an excess amount of dietary iron, whether absorbed or excreted, might bind with most of the citric acid, leaving little citric acid to bind with calcium (to sequester it) in the intestinal lumen. Phosphates in the gastric chyme might, therefore, be precipitated upon neutralization in the intestine as calcium phosphates since calcium was not sequestered away by the supplemental citric acid. In other words, the excessive amount of dietary iron may have blocked the expected effect of citric acid (as Ca-sequestrant) by forming ferrous citrate. Blood meal has been shown to have relatively high mineral availability and low antagonistic properties in affecting the absorption of dietary minerals (ref. p 58-70, p 163-167, p 225-231); however, the result of the present study suggests that blood meal might disable the effect of citric acid supplemented to the diet. Further study is required to confirm this hypothesis.

Phosphorus concentrations in the tank water markedly decreased in 23h, while ammonia concentrations increased (Fig. 23). This indicates that fish might absorb (filter) water-borne P very efficiently. This result, however, contradicts the earlier studies conducted at the Cortland Hatchery, NY, by Arthur M. Phillips, Jr. and his associates (Phillips et al., 1958; 1959), who reported that the absorption of dissolved ^{32}P was much lower than that of ^{45}Ca (ca. 1/400), and that the dietary P was the primary source of P in trout. They used, however, previously fasted (16-96 h) fingerling fish having an apparently adequate diet history in terms of P intake (not stated). In the present trial, on the other hand, fish were fed low-P feeds, and they were obviously growing. Since P is used for the growth of the skeletal system and scales, it is very important to use fish which are growing and feeding normally to determine P absorption and P requirement. When dietary P is not adequate to meet the needs for the growth of fish (limiting factor), then the fish may increase the uptake of P from water via the gills. This hypothesis, however, needs to be confirmed by additional experiments.

Table 53. Composition of the experimental diets.

		Diet C0	C5	P[4]
Ingredient		(%)	(%)	(%)
Herring meal		25	25	0.513
Blood meal		15	15	
Fish oil		25	25	
Wheat flour		30	30	0.096
Vitamin. mix.[1]		3	3	
Mineral mix.[2]		2	2	
TM solution (ml)[3]		(20)	(20)	
Total		100	100	0.609
Citric acid		0	5	
Analytical				
Total P (ppm/dry diet)		5867	5853	
Total Ca (ppm/dry diet)		5916	5993	
Crude protein (%/dry diet)		36.7	35.4	
Crude ash (%/dry diet)		5.07	4.95	
Soluble Pi (ppm/dry diet)	(10 min)[5]	892	1373	
	(24 h)[5]	1778	3060	
Soluble Pi (%/total P)	(10 min)	15.2	23.5	
	(24 h)	30.3	52.3	
pH	(10 min)	6.25	3.73	
	(24 h)	5.75	3.99	

[1] Vitamin mix. supplied the following amount (g) per 100g dry diet: Abernathy #2 pre-mixture (prepared by Hoffmann-La Roche, Inc.), 2.3; ascorbic acid, 0.2; choline-HCl, 0.25; inositol, 0.25.

[2] Mineral mix. supplied the following amount (g) per 100g dry diet: KCl, 1.0; NaCl, 0.5; MgO, 0.5.

[3] Trace mineral solution supplied the following amount (mg) per kg dry diet: KI, 1.9; $MnSO_4 \cdot H_2O$, 75.8; $ZnSO_4 \cdot 7H_2O$, 132; Na_2SeO_3, 0.88; $CoCl_3 \cdot 6H_2O$, 4.0; $CuSO_4 \cdot 5H_2O$, 11.8; $FeSO_4 \cdot 7H_2O$, 298.5.

[4] Percentage of phosphorus/diet contributed from each ingredient (values by calculation).

[5] Extraction time in distilled water with continuous shaking at room temperature.

Table 54. Concentrations of inorganic phosphorus (Pi) and total ammonium nitrogen (TAN) in urine and feces solution.[1]

		Citric acid (%)		Significance
		0	*5*	
Urine[2]				
Pi (wk-2)	(ppm)	9.82 ± 3.21	22.41 ± 7.06	ns
TAN (wk-2)	(ppm)	49.7 ± 5.2	70.4 ± 19.5	ns
Feces solution[3]				
Pi (wk-1)	(ppm)	17.91 ± 4.23	9.27 ± 1.32	ns
TAN (wk-1)	(ppm)	6.29 ± 1.98	15.51 ± 3.34	ns
pH (wk-1)		6.43 ± 0.12	5.95 ± 0.11	P < 0.05
Dry feces[4]				
Ash (wk-1)	(%)	7.07 ± 0.09	7.08 ± 0.12	ns
(wk-2)	(%)	9.79 ± 0.90	12.65 ± 0.52	ns
(wk-3)	(%)	7.10 ± 0.19	6.51 ± 0.18	ns
Pi (wk-1)	(%)	0.839 ± 0.015	0.695 ± 0.025	P < 0.01
(wk-2)	(%)	0.705 ± 0.109	0.614 ± 0.016	ns
(wk-3)	(%)	0.851 ± 0.040	0.733 ± 0.027	P < 0.05
Total lipids (wk-1)	(%)	3.61 ± 0.19	6.64 ± 0.97	P < 0.05
Total cholesterol (wk-1)	(%)	0.34 ± 0.03	0.50 ± 0.06	ns
Total cholesterol (wk-1)	(%/lipids)	9.30 ± 0.60	7.59 ± 0.27	ns

[1] Each value represents the mean ± sem of triplicate tanks containing 7 fish per tank (wk-1 and wk-2) or the mean ± sem of 4 consecutive days of a pooled tank containing 21 fish.

[2] The urine samples were collected during week-2 by stripping over 5 consecutive days from all fish, which were pooled by tank.

[3] The feces solution were collected daily during week-1 from the collection tube of the digestibility tank, which were also pooled by tank.

[4] Dry feces were obtained by drying (105°C-12h) the feces solution collected by settling (wk-1 and wk-3) or feces collected by stripping (wk-2). Total lipids in the dry feces were extracted by an EtOH-Ether-7% HCl solvent system. Total cholesterol was determined on the extracted lipids.

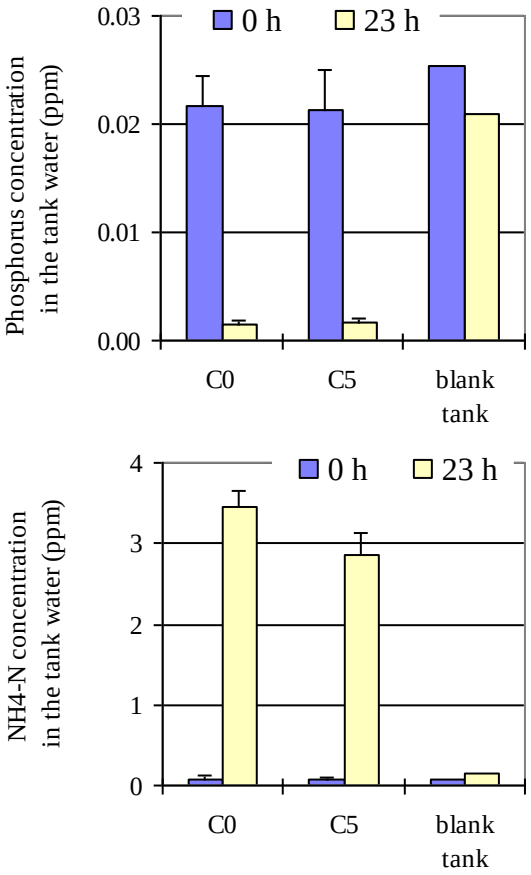


Figure 23. Concentrations of phosphorus and ammonia in the tank water sampled at 0 h (initial) and 23 h (final). Error bars show SEM of three consecutive days during week-3 from a single tank containing 21 fish. The blank tank contained no fish but had the same rearing systems (water, aeration, recirculation, temperature, photoperiod). The value for the blank tank was based on a single determination.

5. Effect of Various Processing Methods on the Availability of Minerals in Soybean Meal

ABSTRACT The effect of various treatments on the hydrolysis of phytate-P in soybean meal was studied. Treatment effects were evaluated based on the fecal content or net absorption of total phosphorus, inorganic phosphorus, phytate phosphorus, protein, calcium, copper, iron, magnesium, manganese, potassium, sodium, strontium, and zinc. Yttrium was used as the inert nonabsorbable indicator to determine net absorption of each nutrient. Fecal phytate content was significantly lower in groups of fish fed soybean meal diets supplemented with phytase or those containing phytase-pretreated soybean meal. The thermal treatments (microwaving, dry roasting, steam heating, cooking) had no measurable effect on the fecal phytate levels. In the fish meal-soybean meal based diet, supplemental phytase had no effect on the net absorption (availability) of total phosphorus or any other minerals. Conversely, in low-ash soybean meal diets, dietary phytase supplementation increased the net absorption (availability) of all minerals studied (except for potassium). In addition, phytase increased the digestibility of protein (N), ash and dry matter. Apparent availability of phosphorus was increased proportionately with the supplemental phytase from 26.6% (no phytase added) up to 90.1% (4000U per kg dry diet) or 92.5% (pre-treated 24h at 50°C with phytase, 200U per kg soybean meal; equivalent to 100U per kg dry diet). Urinary P concentrations in phytase treated and untreated groups were 24.7 and 6.7 (mg P/L urine), respectively (P=0.067), corresponding to the increased availability of dietary P by the supplemental phytase. Citric acid strongly inhibited phytase hydrolysis in the fish meal-soybean meal combined diet; conversely, citric acid increased the phytase hydrolysis (ca. 8 times) in the low-ash soybean meal diet. Also, phytase hydrolysis was increased (ca. 2 times) by performing a single feeding per day compared with multiple feedings.

BACKGROUND

In the period 1915-1919, Edward Mellanby pursued landmark research that ultimately resulted in the discovery of vitamin D. He reported that certain diets caused rachitic changes in the bones of puppies, and that the greater the proportion of cereals in the diet, the greater tendency to produce rickets. The inclusion in the diet of certain fats containing “fat-soluble A” led to normal bone development (Mellanby, 1919). In 1926, he introduced the term “toxamin” to designate an unidentified harmful substance in cereals which prevented utilization of calcium and phosphorus. Later, he concluded that this substance was phytic acid or inositol hexaphosphate (Mellanby, 1950; McCollum, 1957). Since Mellanby’s discovery of the rachitogenic property of phytic acid, numerous studies have been conducted, most notably a series of research experiments entitled “Cereals and Rickets” by Harry Steenbock and his associates at the University of Wisconsin. Most of these early studies focused on the elucidation of interactive properties of phytic acid or its Ca-Mg salt phytin with other nutrients in diets, the development

of methods to reduce phytic acid content in bread and cereal products using (endogenous) phytase, soaking, malting or fermentation, and the use of vitamin D or UV-irradiation to increase bioavailability of phytate by animal species.

In commercial aquaculture feeds, fish meal is a major ingredient. The continuous use of fish meal in fish feeds, however, will be subjected to various constraints in the foreseeable future, such as its increasing price, limited supply, high phosphorus content and the public contention for the use of fish meal in fish feeds. Replacing fish meal with plant or grain by-product materials, therefore, has been increasing in priority over the last years in terms of the sustainability of commercial aquaculture. Hardy (1995) suggests that since the production of soybean meal continues to increase, it is likely to be the most promising alternate protein source for fish feeds.

Unfortunately, about 50-58% of total phosphorus in soybeans is present as phytate which is not efficiently utilized by many monogastric animals, including fish (Ogino et al., 1979; NRC, 1993) and poultry (Nelson, 1967; Anon., 1984; NRC, 1984). Besides its low bioavailability, phytate has been shown to interact directly and indirectly with various dietary components to reduce their bioavailability to the animals. Increasing the level of phytate from 1.1 to 2.2% in channel catfish diets containing 50mg zinc/ kg decreased weight gain, feed efficiency and zinc content in the vertebrae (Satoh et al., 1989). With 1.1% phytate in diets, channel catfish require about 200mg zinc/ kg feed, which is 10 times higher than the dietary requirement of available zinc (Gatlin and Wilson, 1984). High phytate content in semi-purified diets (25.8g/ kg) depressed the growth and feed efficiency in chinook salmon (Richardson et al., 1985). These studies suggest that reducing phytate content in diets is essential to increase availability of phosphorus, trace elements and protein.

Phytase is an enzyme specific to phytate hydrolysis. This enzyme is present in the digestive tract of many animals, however, the amount is normally too small to digest dietary phytate to a significant extent (Bitar and Reinhold, 1972). Also, the enzyme is not adaptive or inducible by its substrate (Roberts and Yudkin, 1961). Development of technology to produce phytase at a low cost offered an opportunity to use this enzyme in commercial animal feeds. Recent studies have shown that supplemental phytase increases the bioavailability of phytate-phosphorus in the feed for rainbow trout (Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995; Riche and Brown, 1996), channel catfish (Jackson et al., 1996; Eya and Lovell, 1997b; Li and Robinson, 1997), carp (Schaefer et al., 1995), pigs (Simons et al., 1990; Jongbloed et al., 1992; Cromwell et al., 1993; Ketaren et al., 1993; Lei et al., 1993; Young et al., 1993; Lei et al., 1994; Mroz et al., 1994; Cromwell et al., 1995a,b; Kornegay and Qian, 1996; Yi et al., 1996b; Han et al., 1997; Harper et al., 1997; Liu et al., 1997; Murry et al., 1997; O'Quinn et al., 1997) and chickens (Simons et al., 1990; Edwards, 1993; Perney et al., 1993; Broz et al., 1994; Biehl et al., 1995; Denbow et al., 1995; Kornegay et al., 1996; Mitchell and Edwards, 1996a,b; Qian et al., 1996; Sebastian et al., 1996a,b; Yi et al., 1996a; Qian et al., 1997; Van-Der-Klis et al., 1997).

The purpose of the present study was to determine economically feasible methods for increasing bioavailability of phosphorus, trace elements and protein in soybean meal in order to replace fish meal with soybean meal in aquaculture feeds and to minimize nutrient excretion into the environment.

MATERIALS AND METHODS

Preliminary experiment. (Property and potency of phytase enzyme).

It is essential to understand the properties of phytase enzyme in order to gain maximum activity from minimum supplementation and to reduce unnecessary expense in practical feed manufacturing. The phytase enzyme was obtained from a commercial source (5000U /g, liquid). The properties of phytase enzyme (pH, temperature, moisture optima and potency) were investigated in in vitro settings by Engelen et al. (1994). Sodium phytate (Sigma, Phytic acid dodeca-sodium salt, purity 99%, water 12%) was used as the substrate. For moisture test and processing test, actual soybean meal was used as the substrate. Untreated soybean meal (dehulled, solvent extracted, moisture 11.6%, particle size; 95.1% < #18 mesh, 1 mm) contained 0.58% phytate-P, 0.22% inorganic-P, 0.85% total P, 0.54% Ca per dry matter. Phytate-P and inorganic-P in the enzyme- or heat-treated soybean meal were extracted with 0.65N-HCl for 3 h at room temperature with continuous shaking, separated by anion exchange column chromatography using AG1-X4, 100-200 mesh resin (Bio-Rad Laboratories, Hercules, CA) by the procedure of Harland and Oberleas (1986), and analyzed for phytate-phosphorus according to Latta and Eskin (1980) and for inorganic phosphorus by a molybdovanadate method (APHA et al., 1985).

Experiment 1. (Effects of various processing methods).

Apparent digestibility of protein and availabilities of phosphorus and other minerals were determined using fish meal-soybean meal compound diets (70% fish meal basal diet, Table 55, 30% soybean meal). The soybean meal was heat- or enzymatically-treated under various conditions (Table 56). Phytase-treated soybean meal was prepared according to Nelson et al. (1968) modified by using commercial fungal phytase and using a 24h incubation time. Briefly, per kg soybean meal, 250ml of distilled water containing phytase was added and mixed quickly and thoroughly, then 250ml of 1.2N-HCl was added, mixed thoroughly, incubated at 50-55°C for 24 h in a hermetically sealed flattened plastic bag. Incubation was terminated by increasing the temperature to 100°C and inactivating the enzyme. The treatment effect was evaluated in vitro by measuring phytate and inorganic phosphorus content in the treated soybean meal and in vivo by measuring nutrient content in fish feces and determining their apparent digestibility or availability for fish. Feeds were prepared as cold extrusion moist pellets and stored at -20°C until fed.

Thirteen rainbow trout (initial mean weight 152.7g, sem 8.1) were stocked in each of 33 40L-fiberglass tanks. Three tanks were randomly assigned to each treatment. Each tank was supplied

with spring water (15 ± 0.5°C) at 5 L/min. Fish were fed once daily to apparent satiation for one week with the basal diet, and fed another one week with experimental diets (fish meal-soybean meal combined diets) before collecting fecal samples from all fish by stripping. The fecal samples were collected once and pooled by tank.

Fecal and feed samples were dried in a convection oven (105 ± 5°C, 6 h), finely ground by mortar and pestle, ashed (550°C, 12 h), heated to boil in a sulfuric-perchloric acid (1/1, v/v) mixture, diluted to an appropriate concentration and analyzed for phosphorus, calcium, magnesium, potassium, sodium, copper, zinc, manganese, iron, strontium, chromium and yttrium using an inductively coupled plasma emission spectrophotometer (Jarrell-Ash Plasma Atom Comp., Waltham, MA). Dried fecal and feed samples were also analyzed for total nitrogen content using a LECO FP-428 Nitrogen Determinator (LECO Instruments, St. Joseph, MI).

The treatment effects were evaluated by measuring a fractional net absorption of dietary nutrients (apparent availability) based on yttrium (Y) as the inert nonabsorbable marker, or based on the concentration of minerals in dried feces per se.

Experiment 2. (Optimum level of phytase).

Availability of nutrients studied in the experiment 1 was re-evaluated using low-ash soybean meal diet (Table 57). The diets contained less phosphorus than the level required by rainbow trout (0.6%, NRC, 1993) to assure that fish did not regulate the absorption of available phosphorus from the diet. The low-ash diet did not contain fish meal, and had lower ash, Ca, Na, P, Sr and Zn levels than the fish meal-soybean meal combined diet used in the experiment 1 (Table 58). The test diets were prepared by supplementing the low-ash diet with incremental concentrations of phytase at 0, 500, 1000, 2000, 4000U per kg diet (dry basis). The sixth dietary treatment was pre-treated soybean meal with phytase (200U per kg soybean meal) substituted for untreated soybean meal.

Thirteen rainbow trout (initial mean body weight 150.6g, sem 8.7) were stocked in each aquarium and three tanks were randomly assigned to each treatment. Test diets were fed to fish once daily to apparent satiation for one week and both fecal and urine samples were collected once by stripping. Fecal samples were analyzed for minerals and protein as described above. Urine samples were analyzed for inorganic phosphorus content according to Taussky and Shorr (1953).

Experiment 3. (Effects of dietary acidification).

Interaction of phytase and citric acid were studied in the fish meal-soybean meal combined diet (Tables 55) and also in the low-ash soybean meal diet (Table 57) using a two-factor ANOVA. The feeding trial was conducted in conjunction with experiments 1 and 2 using the same group of fish, feeding, sampling and analytical procedures as already described.

Experiment 4. (Effects of feeding frequency).

A soybean meal based low-ash diet (Table 57) was supplemented with phytase (1000U per kg diet dry basis) and acid-insoluble ash (SiO_2, Mallinckrodt, reagent grade; 0.5% per diet dry basis). Three tanks containing 13 rainbow trout (initial mean BW 207.3, sem 1.3) in each tank were randomly assigned to each treatment. Fish were fed the same feed and the same total amount per day either once (6am), twice (6am, 5pm), or five times (6am, 9am, 12pm, 3pm, 5pm) daily. In a previous experiment with rainbow trout, neither one, two or continual feeding regimes during the diurnal photoperiod made any difference in the feed efficiency and the growth rate of the fish (Mäkinen, 1993). After 7 days of feeding with the test diet, feces were collected once by stripping, 24 h after the last feeding from all fish in each tank. Fecal samples were pooled by tank and analyzed for total P, phytate P and N (protein) contents by the procedures described above. Apparent availability was determined based on the acid insoluble ash as the nonabsorbable marker.

Analytical data were subjected to one-way or two-way ANOVA, Newman-Keuls multiple comparison test, 2-tailed t-test and regression analysis using GraphPad Prism, version 2.01 (GraphPad Software, Inc., San Diego, CA). Treatment effects were considered significant at $P< 0.05$. Fish were treated in accordance with the guidelines approved by the animal care committee of the University of Washington.

RESULTS

Preliminary experiment. (Property and potency of phytase enzyme).

The phytase enzyme used in the present trial had its optimum pH at 5.30, and the optimum temperature at 55°C (Fig. 24, 25). The rate of hydrolysis was highly dependent upon these two factors. The rate of hydrolysis was much faster at the temperature of 37°C (the body temperature of warm-blooded animals) than at 15°C (the "body temperature" of rainbow trout) (Fig. 26). The minimum amount of moisture necessary to maximize phytase activity was 50% (water/ dry soybean meal ratio is 1/2) (Fig. 27). Using fish oil as a carrier for the enzyme solution (applied as water-in-oil emulsion) did not reduce the volume of water required (data not given). There was no significant difference in the rate of hydrolysis between citric acid and hydrochloric acid used to adjust the pH of the reaction mixture (Fig. 27).

Experiment 1. (Effects of various processing methods).

In contrast to the enzymatic treatment with phytase, the various thermal treatments had no significant effect on the phytate and inorganic phosphorus content of soybean meal (Table 56). Fecal phytate content also was significantly lower in phytase supplemented or phytase-pretreated groups, whereas the thermal treatments had no apparent effects on the fecal phytate levels (Fig. 28). Dry matter digestibility (%) of the fish meal-soybean meal combined diets was not significantly different among treatments (mean range, 75.5-77.8, Table 59), which was, however, significantly lower than the value of

the basal fish meal diet (81.3%). Apparent digestibility of protein was not increased by any treatment. Apparent availability (net absorption) of total phosphorus was slightly but significantly increased by the phytase pre-treatment but not by the phytase supplementation or any thermal treatment (Table 59). There were no notable differences on the net absorption (apparent availability) of other minerals in the fish meal-soybean meal combined diets by either thermal or enzymatic treatments (Table 59).

Experiment 2. (Optimum level of phytase).

Except for potassium, dietary phytase supplementation significantly increased the availability of all minerals examined; in addition, phytase increased the digestibility of protein (N), ash and dry matter (Table 60). Apparent availability of phosphorus was increased proportionately with the supplemental phytase from 26.6% (no phytase added) up to 90.1% (4000U per kg dry diet) or 92.5% (pre-treated with phytase, 200U per kg dry soybean meal; equivalent to 100U per kg dry diet). Urinary P concentrations (mg P/L urine) in phytase treated and untreated groups were 24.71 (6.95, sem) and 6.69 (1.88, sem), respectively (P=0.067, by two-tail t-test), corresponding to the increased availability of dietary P due to the supplemental phytase. Fecal phytate-P content decreased proportionally as the dietary phytase level increased (Fig. 29).

Experiment 3. (Effects of dietary acidification).

Citric acid strongly inhibited phytate hydrolysis by the supplemental phytase in the fish meal-soybean meal complex diet (Fig. 28). Although citric acid increased the net absorption (apparent availability) of ash, Ca, Fe, Mn, P, Sr and Zn, phytase did not increase the absorption of any nutrients in the fish meal-soybean meal combined diets (Table 61). There was no significant interaction between supplemental phytase and citric acid except for Fe whose absorption was reduced by phytase or phytase plus citric acid. Conversely, citric acid clearly increased the hydrolysis of phytate by the supplemental phytase in the low-ash soybean meal diet (Fig. 29). The content of phytate-P in the feces of fish fed low-ash soybean meal diet supplemented with 500U of phytase (per kg dry feed) was 0.605 (%/dry feces); however, by supplementing the diet with 500U of phytase and citric acid (5%), the fecal content of phytate-P was decreased to 0.098%, which was close to the value (0.088%) obtained with 4000U of phytase supplementation. Citric acid and phytase increased synergistically the absorption of Fe, Mg, Zn (not significant), Mn and P (significant) (Table 62) in the diet.

Experiment 4. (Effects of feeding frequency).

Feeding frequency had a significant effect on the efficacy of supplemental phytase in the low-ash diet (Fig. 30); fecal phytate-P (and total-P) content was significantly reduced when fish were fed only once instead of twice or five times (per day). Digestibility of protein was unaffected by the frequency of feeding.

DISCUSSION

Preliminary experiment. (Property and potency of phytase enzyme).

The rate of phytate hydrolysis by phytase enzyme was affected not only by the pH, but also by the temperature of the reaction mixture. Nayini and Markakis (1986) reported that the optimum temperature varied among different phytases in the range of 45 to 57°C. For homeotherms, the body temperature appears to be favorable for the enzyme reaction. For poikilotherms, especially for cold-water fishes such as rainbow trout, however, the body temperature could be limiting the enzyme activity, suggesting an economical disadvantage for the use of this enzyme as a supplement in salmonid feeds. Increasing water temperature increases enzyme activity, which, however, simultaneously increases the rate of food passage through the gastrointestinal tract, offsetting the net effect. Since gastric evacuation time is influenced not only by the water temperature but also by the meal size (He and Wurtsbaugh, 1993), increasing meal size or reducing feeding frequency could increase gastric retention time and preserve phytase activity. This subject was studied in experiment 4.

Alternative methods are those which employ preliminary digestion of soybean meal before mixing with other feed ingredients. Use of phytase to digest phytate in plant ingredients has been extensively studied (Nelson et al., 1968; Stone et al., 1984; Cain and Garling, 1995). However, adding water to dry ingredients, which is essential for the enzyme reaction, may be prohibitive for its practical application because of the extra energy and cost required in the succeeding drying process. Thus, knowing the minimum essential amount of water to maximize enzyme activity is practically important when introducing the method involving preliminary digestion.

Experiment 1. (Effects of various processing methods).

Under the processing conditions reported previously (Smith, 1977; Smith et al., 1980) to reduce antinutritional factors in soybean meal, little decomposition of phytate P appears to occur. The microwave heating which was demonstrated effective to reduce phytate in full-fat soybean (Hafez et al., 1989) appears to be ineffective for solvent-extracted soybean meal that was used in the present study. The autoclaving at high temperatures has also been reported to reduce phytate but at the expense of some heat labile amino acids (Rackis, 1974; de Boland et al., 1975). Toma and Tabekhia (1979) also reported a high loss of phytate in milled rice by cooking. None of the thermal treatments described above, however, caused a significant reduction of phytate-P in the present study even if it was at a level of extreme condition (dark browning or smoldering).

The enzymatic method, therefore, appears to be the only way to reduce phytate-P levels in soybean meal. Supplemental phytase certainly reduced fecal phytate content in the fish meal-soybean meal diet; however, the fish concomitantly increased the fecal inorganic (insoluble) P excretion. Consequently, total P levels in feces were not significantly reduced. When diets contain high levels of bone phosphorus, the fractional absorption (availability) is low; however, as its dietary level decreases the

availability increases (see Fish bone study, p 71). This may indicate that the bone P is "conditionally available", and the addition of phytase enzyme in such diets may offer little or no advantage to reduce total P excretion. Thus, as far as the diet contains enough bone P to meet the dietary requirement (typical in most commercial feeds), the effect of supplemental phytase may not be seen.

Experiment 2. (Optimum level of phytase).

Phytic acid has been shown to interact with various nutrients in diets; calcium compounds including bone and the dietary supplements have a strong affinity to phytate, to form acid-insoluble precipitates (Kaufman, 1986) and reduce both calcium and phytate availability (Lowe and Steenbock, 1936; Krieger and Steenbock, 1940; McCance and Widdowson, 1942a,b; Hoff-Jorgensen, 1946; Mellanby, 1949; Taylor, 1965). The calcium-bound phytate increases chelation with trace minerals such as zinc to form co-precipitates (Anon., 1967) or with protein to form phytate-protein or phytate-mineral-protein complexes that are resistant to proteolytic digestion (Cheryan, 1980). Phytate may decrease endogenous zinc reabsorption as well as affect bioavailability of dietary zinc (Morris, 1986). In the present study, the dietary supplementation of phytase increased the availability of all minerals examined (except potassium) and of protein, which clearly indicates that the antagonistic property of phytic acid in diets was effectively reduced by the supplemental phytase.

Since the supplemental phytase in the low-ash soybean meal diet proportionately reduced fecal phytate P (and total P) levels, the definition for "optimum supplemental level" could be highly subjective. The level of 4000U per kg dry feed or more will be necessary to achieve 90% reduction, which is much higher than currently recommended levels for swine and poultry. The difference could presumably be due to the difference of body temperatures. This indicates that the use of phytase as a dietary supplement in salmonid feeds will be economically prohibitive, but the use of phytase to pre-treat soybean meal before mixing with other feed ingredients will be a workable alternative although it depends on the cost of the phytase enzyme and the cost of the phytase treatment process.

Experiment 3. (Effects of dietary acidification).

Fungal phytases have their pH optima in an acidic range (Nayini and Markakis, 1986). Because of this, dietary acidification was predicted to be beneficial for increasing the activity of supplemental phytase. In a previous study, the pH of the feces was reduced in fish fed citric acid at 5% in diets compared with fish fed non-acidified diets (Table 50, p.114), suggesting that the dietary acidification could possibly preserve phytase enzyme activity throughout the digestive tract instead of only in the stomach. In addition, phytate-mineral complexes, which are less soluble at a neutral pH, have increased solubilities at lower pH (Møllgaard, 1946; Tangkongchitr et al, 1982; Grynspan and Cheryan, 1983; Nolan et al., 1987). Also, another benefit of citric acid and other organic ligands, such as EDTA is that they may prevent precipitation of minerals at a neutral pH in the presence of phytate (Vohra and Kratzer, 1964; Nielsen et al., 1966; Lyon, 1984).

In the fish meal-soybean meal combined diets, however, citric acid strongly inhibited the hydrolysis of phytate by the supplemental phytase. This might be due to the increased acidity and the solubilization of hydroxyapatite, resulting in a formation of Ca-phytate precipitate, rendering it more resistant to enzymatic hydrolysis (Lasztity and Lasztity, 1990). In contrast to this observation with fish meal-soybean meal complex formula, citric acid greatly increased the efficiency of phytate hydrolysis by phytase in the low-ash soybean meal diet of low Ca content. The rate of phytate hydrolysis in the low-ash soybean diet approached a level equivalent to 4000U of phytase by supplementing the diet with 500U of phytase plus citric acid, even though citric acid per se had no effect for the hydrolysis of phytate. This remarkable interplay should be a powerful tool in practical feed formulations but the exact mechanism remains to be elucidated.

Experiment 4. (Effects of feeding frequency).

Because of its pH optimum, phytase is active only in the stomach. It should, therefore, be important to increase the stomach retention time as long as possible to keep added phytase active. It has been well recognized before the advent of scientific experimentation that a large single meal stays in the stomach longer than small meals. Also, it is generally agreed that small meals can be more rapidly evacuated from the stomach than large meals (Jobling et al., 1977). As the results of this study clearly indicate, a single feeding per day should be recommended for phytase-supplemented feeds. Significant loss of phytase activity will result by practicing multiple feeding (e.g., by automatic feeder, demand feeder). It should be remembered, however, for plant protein feeds, not only phytase but free amino acids may also be supplemented to correct amino acid imbalance. Because frequent feeding is favorable to counteract different absorption rates between supplemental free amino acids and the amino acids in the intact protein from feed ingredients (Yamada et al., 1981a, b), here is a conflict of introducing single feeding practice. Another way to increase gastric retention time of feeds, and thus potentially increase hydrolysis of phytate, is to increase the energy density of the feeds (Lee and Putnam, 1973; Grove et al., 1978). Also, the gastric evacuation time is affected by the size of fish; i.e., slower in large fish and faster in small fish, thus small fish require more frequent feeding per day than large fish. In summary, the effect of supplemental phytase is predicted to be most efficient in large fish fed high-energy feeds in a single feeding per day.

CONCLUSION

In order to maximize the effects of enzyme supplementation, it is critical that one know the properties of the enzyme; i.e., temperature and pH effects, activators, inhibitors, as well as gastrointestinal physiology for digestion and absorption of dietary nutrients. Because the body temperature of coldwater fishes is much lower than that of pigs and chickens, the use of phytase enzyme as a feed additive may not be economically feasible. Thus, the preliminary treatment of soybean meal with phytase before mixing

with other feed ingredients appears to be a more effective procedure for salmonid feeds than simply adding phytase enzyme to the feed. Nevertheless, the present study demonstrated that phytase activity can be increased ca. two times by simply performing a single feeding per day compared to multiple feeding. Furthermore, the use of citric acid increases the efficiency of supplemental phytase ca. eight times compared to non-acidified feeds containing equivalent amount of phytase. By applying these new approaches, phytase enzyme could be used as an effective supplement for salmonid feeds. Whichever approach is taken, using phytase enzyme will be critical in formulating low-pollution feeds containing soybean meal.

Table 55. Composition of the basal diet (Experiment 1 and 3).[1]

Ingredient	%
Herring meal[2]	60
Gelatin[3]	10
Dextrin[4]	10
Fish oil[5]	10
Cellulose + markers[6]	7
Vitamins[7]	3
Trace mineral solution[8]	(10 ml)
Water[9]	(30 ml)

[1] Test diets were formulated by mixing the basal diet (70%) and treated/untreated soybean meal (30%) on a dry basis.
[2] Commercial grade (West Coast fish meal); contained 2.276% P (as-is basis).
[3] 300 bloom, from porcine skin (United States Biochemical Corporation, Cleveland, OH); contained 0.0106% P.
[4] Technical grade (United States Biochemical Corporation); contained 0.0196% P.
[5] Fish oil contained 0.0018% P.
[6] Chromium oxide 0.5% and yttrium oxide 0.05% (per dry diet).
[7] #30 pre-mixture (Hoffmann-La Roche), 2%; L-ascorbyl-2-polyphosphate (15% active), 0.3%; myo-inositol, 0.2%; choline chloride (60%), 0.5%. #30 pre-mix and choline contained 0.0713 and 0.0650% P, respectively.
[8] Supplied the following per kg dry diet: KI, 2 mg; $MnSO_4 \cdot H_2O$, 40 mg; $ZnSO_4 \cdot H_2O$, 80 mg; Na_2SeO_3, 0.9 mg; $CoCl_3 \cdot 6H_2O$, 4.0 mg; $CuSO_4 \cdot 5H_2O$, 12 mg; $FeSO_4 \cdot 7H_2O$, 300 mg.
[9] With/without phytase.

Table 56. Phytate-P and inorganic-P content in soybean meal treated with various methods and intensity.[1]

treatment	Microwaving	Dry roasting	Steam heating	Cooking	Enzyme
mild	0.56 (0.24)	0.51 (0.26)	0.53 (0.13)	0.53 (0.11)	0.54 (0.33) a
intense	0.58 (0.14)	0.57 (0.25)	0.59 (0.13)	0.57 (0.12)	0.12 (0.65) b
abused	0.59 (0.11)	0.58 (0.20)	0.60 (0.11)	0.57 (0.12)	0.06 (0.67) c
extreme	charcoal	0.55 (0.15)	0.57 (0.12)	0.57 (0.12)	0.04 (0.68) c

[1] Untreated soybean meal (dehulled, solvent extracted, particle size; 95.1% < #18 mesh, 1 mm) contained 0.58% phytate-P, 0.22% inorganic-P and 0.85% total P per dry soybean meal. Values are expressed as phytate-P content (inorganic-P content in parentheses) in treated soybean meals (%, dry basis). Each value represents the average of 3 or 4 samples randomly taken from a single lot of processed soybean meals (extracted separately). Untreated soybean meal was placed (less than 5 mm deep) on an aluminum tray (or in a glass beaker for microwaving) to allow rapid heat transfer. Treatment intensity (respectively: mild, intense, abused, extreme) was 10,15,20,30 min.(microwaving per kg soybean meal; AC1.35kw; 2450MHz); 5,8,12,15 min. (dry roasting, 232°C); 10,15,20,30 min.(steam heating or cooking with 350ml distilled water added per kg soybean meal, 0.70kg/cm^2 , 115°C); 0,250,500,1000U (phytase per kg soybean meal incubated at 50°C for 24h). Values in columns with common letters are not significantly different by the SNK multiple comparison test. Processed soybean meals of the extreme treatment (except for microwaving) was used for the feeding trial. Test diets were prepared by mixing the basal diet (70%, Table 55) and the processed/unprocessed soybean meal (30%) on a dry basis. The original reference methods were for microwaving, 15 min per kg soybean meal by a 650w-microwave oven (Hafez et al., 1989); for dry roasting, 232°C, 8 min. (Smith et al., 1980); for steam-heating 0.70 kg/cm^2 (= ca.115°C), 10 min without water (Smith et al., 1980).

Table 57. Composition of the low-ash soybean meal diet (Experiment 2, 3 and 4).

	%
Soybean meal	50
Gelatin[1]	10
Dextrin[1]	10
Fish oil[1]	20
Cellulose + markers[1]	4
Vitamins[1]	3
Minerals[2]	2
Amino acids[3]	1
Trace mineral solution[1]	(10 ml)
Water[4]	(30 ml)

[1] See footnotes of Table 55.
[2] NaCl, 1.2%; KCl, 0.7%; MgO, 0.1% per diet (dry basis).
[3] DL-methionine, 0.5%; L-lysine HCl, 0.5% per diet (dry basis).
[4] With/without phytase.

Table 58. Analytical composition of the basal diet, test diets, and the low-ash diet used in the experiments 1, 2 and 3.[1]

	Ash	N	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Y	Zn
Basal diet[2]	14.4	8.48	24400	15	133	5480	1440	16	18000	15800	48	318	136
Combined diets[3]	12.3	8.40	19400	19	139	9910	2120	28	14000	14700	37	245	132
SD	0.24	0.34	819	0.85	6.4	257	58	1.4	524	471	2.6	8.0	5.5
Low-ash diet[4]	6.72	6.09	2110	17.8	133	13000	2380	32.8	5440	4210	5.87	370	83.6

[1] Values for ash and N are the percentage in the dry diets; minerals are mg per kg dry diets. The basal diet (fish meal diet, Table 55) had a pH of 6.49; fish meal-soybean meal-combined diets (70% basal diet + 30% soybean meal) had a pH of 6.60 and the addition of phytase enzyme did not alter dietary pH. Fish meal-soybean meal-combined diets supplemented with 5% citric acid had pH values between 4.61-4.65. Low-ash diet (Table 57) had a pH of 6.61-6.67 and the citric acid (5%)-added diets had a pH of 4.22-4.24.
[2] Fish meal diet (Table 55).
[3] Experimental diets (fish meal-soybean meal complex diets). Values are the average (and the standard deviation) of all test diets in Tables 59 and 61.
[4] Low-ash soybean meal diet (Table 57).

Table 59. Effect of various processing methods on the apparent availability of minerals in fish meal-soybean meal diets.[1]

	DM	Ash	N	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
Untreated	76.1	52.3	89.6	25.8	6.1	17.1	93.3	62.2	34.1	52.6	56.3	4.4	42.2
Microwaved[2]	76.7	56.0	89.0	26.6	-1.9	11.8	92.9	61.3	25.5*	54.0	57.1	8.6	45.3
Dry roasted[2]	77.8	56.5*	90.8	24.6	6.1	20.9*	92.1	64.4	33.9	55.1	54.9	8.7	39.0
Steam heated[2]	75.5	51.5	89.7	22.1	0.9	16.3	89.2	62.7	20.7**	53.5	52.2 *	12.6	35.4
Cooked[2]	76.7	53.5	90.3	23.7	-0.5	18.2	88.2	63.2	22.3*	54.2	54.9	10.1	34.9
Phytase-added[3]	75.8	52.8	89.8	20.1	-5.8	14.2	92.8	65.7**	20.3*	56.4	56.2	5.6	36.7
Phytase-treated[2]	75.9	55.5*	89.1	27.9	-6.2*	17.1	88.9	60.7	27.8	55.3	62.2 *	15.2*	39.5

[1] The test diets were the mixture of 70% basal diet (Table 55) and 30% treated/untreated soybean meal on a dry basis. Asterisks indicate a significant difference from the untreated soybean diet by the 2-tailed t-test (* P< 0.05; ** P< 0.01; *** P< 0.001). n=3 (tanks) per treatment.
[2] Soybean meals treated (processed) with the highest intensity (Table 56).
[3] 1000U phytase per kg dry feed.

Table 60. Effect of phytase on the apparent availability of minerals in the low-ash diet.[1]

Phytase unit		DM	Ash	N	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
0U		62.8	20.9	89.0	-75.1	-58.9	-6.8	87.2	10.9	-0.4	-127.0	26.6	-42.8	15.7
500U		69.3	35.4	91.2	-36.6	-26.5	11.9	90.1	37.6	-3.9	-67.2	53.9	-16.7	48.2
1000U		69.0	42.0	91.6	-24.3	-22.5	9.3	87.6	43.2	-15.3	-56.4	68.1	-5.5	45.7
2000U		71.7	50.5	93.3	-7.4	-10.0	16.6	88.9	55.9	0.5	-29.1	82.0	10.0	61.4
4000U		70.7	49.5	92.4	-7.5	-15.3	19.8	87.8	58.0	26.8	-35.8	90.1	8.1	57.7
Pre-treated[2]		68.5	47.0	90.9	-3.0	-35.1	14.6	89.8	48.6	28.3	-69.3	92.5	3.3	47.9
ANOVA-P		*	***	**	***	**			***	**	**	***	**	**
Regression-P[3]	L		**	*	**	*	*		***	*	**	***	**	*
	Q	**	***	***	***	***	**		***		***	***	***	***

[1] The ingredient composition and analytical composition of the diet were given in Tables 57 and 58, respectively. The negative values indicate that a higher amount appeared in the feces (per intake) than in the feed, which, however, does not mean a negative balance since intake from the water (via gills) was not accounted for.
[2] Pretreatment: 200U phytase per kg soybean meal (equivalent to 100U phytase per kg diet), 250ml water and 250ml of 1.0N HCl per kg soybean meal, incubated 24 h at 50°C.
[3] Regression-P does not include pre-treated soybean diet. L; linear effect, Q, quadratic effect. Asterisks indicate the significant difference or effect by one-way ANOVA or regression (* P< 0.05; ** P< 0.01; *** P< 0.001). n=3 (tanks) per treatment.

Table 61. Effect of phytase and citric acid on the apparent availability of minerals in fish meal-soybean meal diets.[1]

	DM	Ash	N	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
Untreated	76.1	52.3	89.6	25.8	6.1	17.1	93.3	62.2	34.1	52.6	56.3	4.4	42.2
Phytase	75.8	52.8	89.8	20.1	-5.8	14.2	92.8	65.7	20.3	56.4	56.2	5.6	36.7
Citric acid	76.8	58.2	88.9	42.1	-5.6	26.7	93.8	65.0	37.1	49.9	75.0	20.1	57.9
Phytase + Citric acid	76.8	56.5	89.6	40.1	-2.5	15.6	93.0	64.0	32.5	47.9	72.7	28.1	53.5
ANOVA-P Phytase						***			**				
		*		***		**			*		***	***	**
Citric acid													
						*							
Interaction													

[1] The diets contained the basal (fish meal) diet (70%, Table 55) and unprocessed soybean meal (30%) on a dry basis with/without phytase (1000U per kg dry diet) or citric acid (5% per dry diet). Asterisks indicate the significant difference or effect by the two-way ANOVA (* P< 0.05; ** P< 0.01; *** P< 0.001). n=3 (tanks) per treatment. Note: significant effects of phytase on the availabilities of Fe and Mn are negative.

Table 62. Effect of phytase and citric acid on the apparent availability of minerals in the low-ash soybean meal diet.[1]

	DM	Ash	N	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
Untreated	62.8	20.9	89.0	-75.1	-58.9	-6.8	87.2	10.9	-0.4	-127	26.6	-42.8	15.7
Phytase	69.3	35.4	91.2	-36.6	-26.5	11.9	90.1	37.6	-3.9	-67	53.9	-16.7	48.2
Citric acid	63.6	13.3	90.2	-97.3	-37.1	7.2	83.3	28.1	-18.8	-160	27.1	-55.5	45.7
Phytase + Citric acid	67.7	30.6	91.7	-36.8	-27.7	23.4	87.9	51.7	33.0	-123	86.9	-16.0	59.3
ANOVA-P	*	**	*	***	*	*		**	*	*	***	**	**
Phytase													
								*		*	**		*
Citric acid													
									*		**		
Interaction													

[1] The diet was supplemented with/without phytase (500U per kg dry diet) or citric acid (5% per dry diet). Asterisks indicate the significant difference or effect by 2-way ANOVA (* P< 0.05; ** P< 0.01; *** P< 0.001). n=3 (tanks) per treatment.

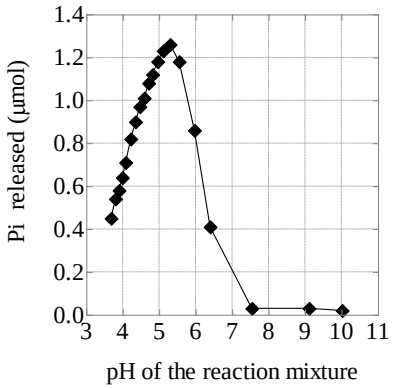


Figure 24. Phytase activity at various pH. Each point represents the average of duplicate determinations. The pH values were measured in the reaction mixture. The Y axis indicates Pi released in 65 min at 37±0.5°C by 5µg liquid phytase using the reaction mixture specified in Engelen et al. (1994).

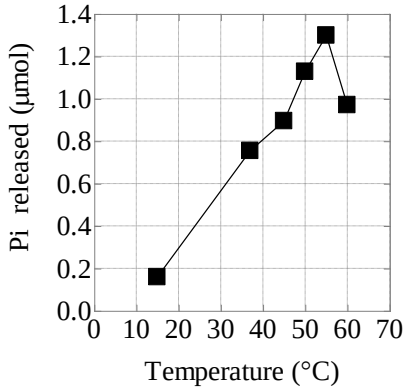


Figure 25. Phytase activity at various temperatures. The pH of the reaction mixture was 5.30. Each point represents the average of duplicate determinations.

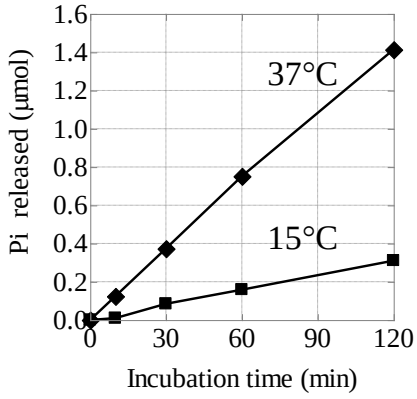


Figure 26. The rate of hydrolysis at two temperatures. The pH of the reaction mixture was 5.30.

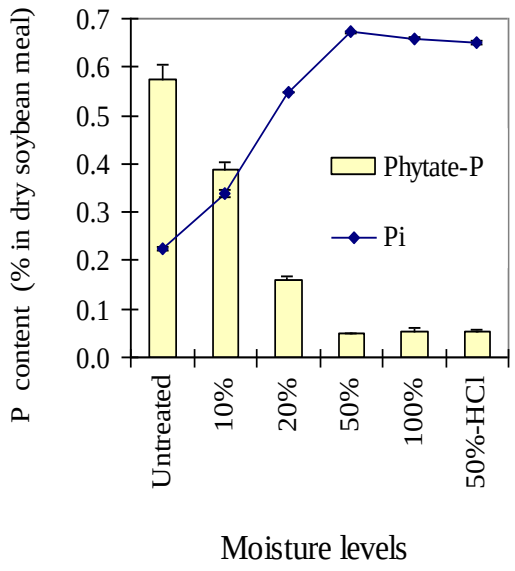


Figure 27. Minimum amount of moisture required for optimum phytase activity.
Treatment: to 50g soybean meal as-is basis, 1g citric acid was added, mixed, 10U phytase was added with 5, 10, 25 or 50ml of distilled water, then mixed quickly and thoroughly before the moisture was absorbed by the meal particles. Treatment with HCl: to 50g soybean meal, phytase10U added with 12.5ml of distilled water, mixed, 12.5ml of 1.2N-HCl added and mixed thoroughly. Incubated in a plastic bag, placed flat for rapid heat transfer. Incubation pH (measured after incubation) was 5.28-5.34 in citric acid group; 4.95 in HCl treatment; initial pH (before incubation) in the citric acid group was 5.14 (single determination).

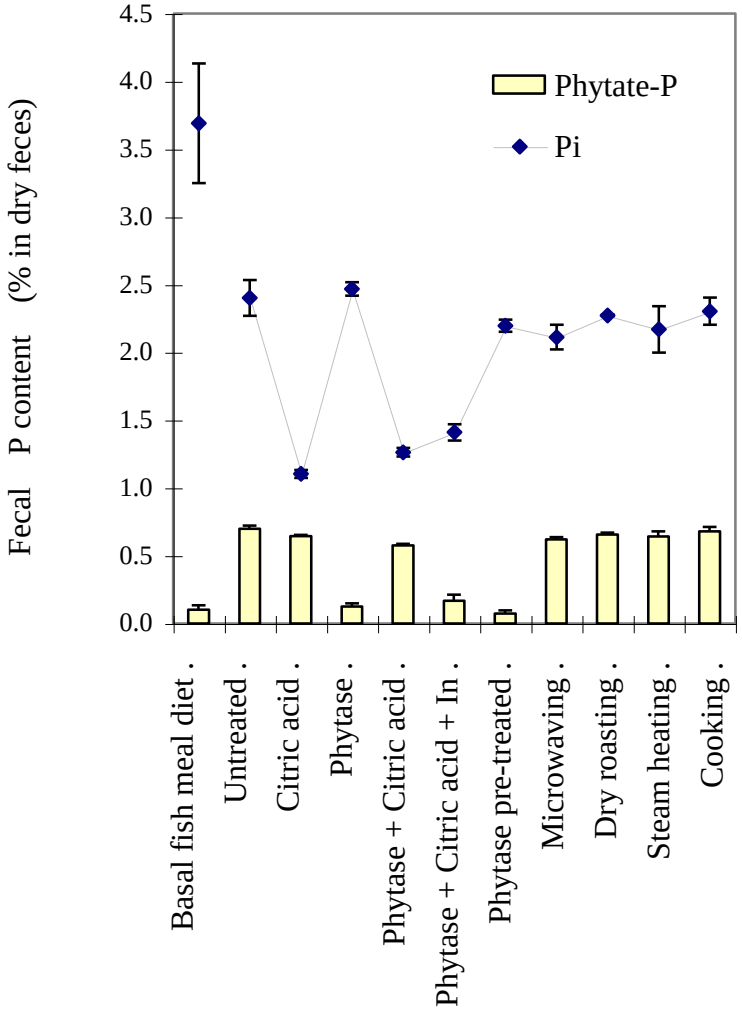


Figure 28. Phytate-P and inorganic-P (Pi) content in fish feces (experiments 1 and 3). The test diets contained the basal (fish meal) diet (70%, Table 55) and treated/untreated soybean meal (30%) on a dry basis with/without supplemental phytase (1000U per kg dry diet) or citric acid (5% per dry diet). Processing conditions were summarized in Table 56. Abbreviations: "Phytase + Citric acid + In" indicates that the "Phytase + Citric acid" diet was incubated at 50°C for 24h before feeding. The data values (in the figure, % in dry feces) for phytate-P (and Pi, shown in parentheses) are 0.102 (3.690) for the basal fish meal diet; 0.700 (2.403) for Untreated; 0.645 (1.104) for Citric acid; 0.126 (2.469) for Phytase; 0.575 (1.264) for Phytase + Citric acid; 0.168 (1.410) for Phytase + Citric acid + In; 0.074 (2.196) for Phytase pre-treated; 0.620 (2.113) for Microwaving; 0.655 (2.273) for Dry roasting; 0.642 (2.170) for Steam heating; 0.680 (2.304) for Cooking.

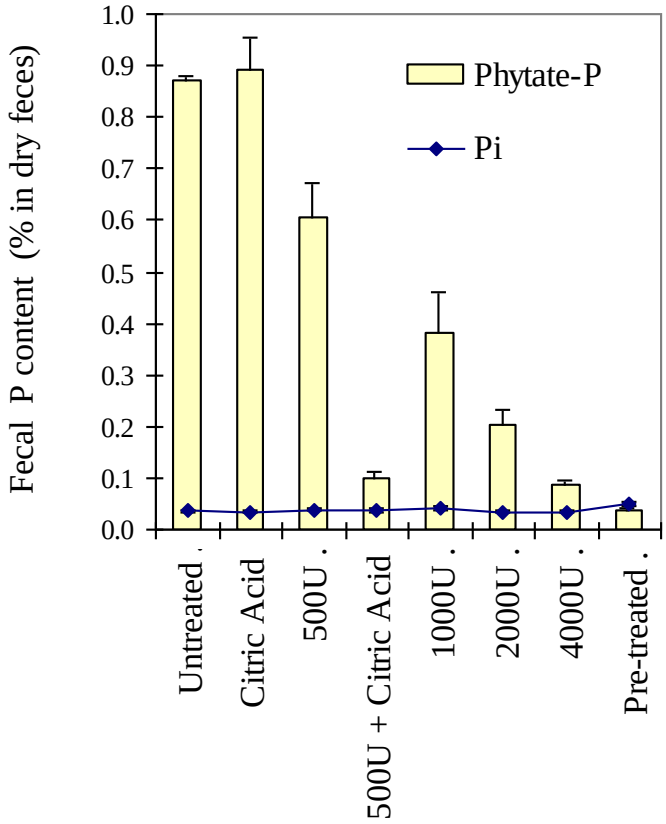


Figure 29. Phytate-P and inorganic-P (Pi) content in fish feces (experiments 2 and 3). The ingredient composition and analytical composition of the diets were given in Tables 57 and 58, respectively. The enzyme unit indicates the amount of phytase added to kg dry feed. "Pre-treated" indicates the treatment of soybean meal (200U/kg SBM; equivalent to 100U/ kg dry feed) for 24 h at 50 ± 2°C before mixing with other ingredients. The data values (in the figure, % in dry feces) for phytate-P (and Pi, shown in parentheses) are 0.871 (0.037) for Untreated; 0.890 (0.034) for Citric acid; 0.605 (0.037) for 500U; 0.098 (0.038) for 500U + Citric acid; 0.383 (0.041) for 1000U; 0.201 (0.035) for 2000U; 0.088 (0.035) for 4000U; 0.036 (0.050) for Pre-treated.

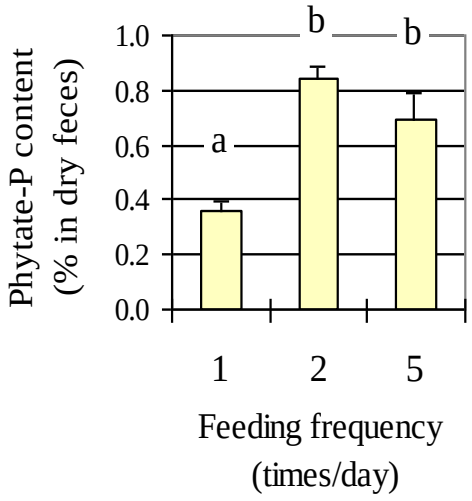


Figure 30. Effect of feeding frequency on the fecal phytate-P concentration in the phytase-supplemented diet. Each column represent the average (with SD indicated as the error bar) of 3 tanks containing 13 fish/tank. Low-ash soybean meal diet (the same formula given in Table 57) with supplemental phytase at 1000U per kg dry feed was formulated as moist pellets, stored frozen, and thawed when fed. The feeding rate was 1%BW (dry basis) per day for all treatments. Apparent availability of P was 76.4, 40.9, 59.5% (mean, n=3) in 1, 2 and 5 time feeding, respectively. Apparent digestibility of N (protein) was 94.0, 93.2, 93.9 (mean, n=3) in 1, 2 and 5 time feeding, respectively.

Chapter IV: Formulation of Low-Pollution Feeds

BACKGROUND

The preceding three chapters have provided a substantial amount of newer knowledge on the formulation of low-pollution (LP) feeds. There is, however, a considerable amount of valuable information currently available in the literature, e.g., protein/digestible energy (DE) ratio, optimum DE level, digestibility of carbohydrates, etc., which furthers this area of investigation. The purpose of this final chapter is to summarize essential knowledge in formulating LP-feeds. Also, some model LP-feeds were formulated and they were evaluated as described in the second half of this chapter for their effectiveness as low-polluting feeds using a mass balance method.

1. Principles Involved in the Formulation of Low-Pollution Feeds --- A Review

Availability of Dietary Nutrients

The apparent digestibility or availability coefficients indicate only a fractional absorption (% of intake) of dietary nutrients and totally overlook the actual amount of intake and absorption by fish. In formulating practical feeds, it is critical to pay attention to the limited scope of the coefficients. When diets contain nutrients in an amount higher than the requirement level, fish can regulate the absorption of the nutrient, depending on the nutrient. Conversely, when dietary intake is lower than the requirement, numerous biochemical or physiological mechanisms must be activated, in order to increase the efficiency of absorption or conservation of nutrients. Consequently, the actual availability of nutrients can not be correctly estimated. In the present study, all ingredients were incorporated in the diet at a 30% level; however, at levels lower or higher than 30%, there should be different digestibility values. This at least partly explains the discrepancy of some digestibility or availability values reported previously by other researchers. These interfering problems, however, were minimized in the present study to allow an accurate measurement of the inherent availability of nutrients in feed ingredients (for detail, see Iron section, p 61).

When a substantial portion of diets is comprised of plant ingredients, using phytase to increase P availability will be a critical measure to reduce P excretion or to increase P retention by the fish. While fungal phytase is now commercially available as a feed supplement, plant phytases inherent in wheat, barley and rye (Reddy et al., 1982; Nayini and Markakis, 1986; Lasztity and Lasztity, 1990) should be considered as a low-cost alternative when the cost of feeds is one of the limiting factors in feed manufacturing. Stone et al. (1984) reduced phytic acid content in canola meal by blending it with acidified fish silage and wheat bran (source of phytase) and keeping the mixture at room temperature for 5 weeks. During that period, the low pH protected the wet material from bacterial spoilage and also facilitated phytate hydrolysis. Also, if endogenous phytase, that is present in some feed ingredients, is

not heat-inactivated during feed processing, this active enzyme could hydrolyze phytate in the stomach after it is ingested by the animal (Pointillart et al., 1987; Sandberg et al., 1987; Sandberg and Andersson, 1988; Anon., 1989). The hydrolysis of phytate in this way, however, appears to be less efficient, due to the limited gastric retention time of the chyme and the low ambient temperature, particularly in coldwater fish.

The digestibility of carbohydrates (and thus that of energy) varies substantially depending on the processing temperature and the inclusion level in the feed (Singh and Nose, 1967; Pfeffer et al., 1991). If feeds are processed at relatively low temperatures to conserve endogenous phytase, the carbohydrate portion of feeds may not be well-utilized by fish. This also increases gastric passage rate of the chyme and reduces the time available for the digestion of phytate phosphorus as well as other dietary nutrients (Spannhof and Plantikow, 1983). Further consideration is therefore required to increase availability of both P and carbohydrates in plant ingredients such as wheat bran.

Interaction

The level of one nutrient (or anti-nutrient) in a diet always affects the absorption (availability) of other nutrients in the diet by antagonistic/synergistic interactions and dilution/concentration of dietary nutrients. This may happen during feed processing and storage as well as after ingestion. In addition, some dietary components such as soluble fibers may increase endogenous excretion of minerals by binding them or by blocking enterohepatic circulation of bile salts. It is therefore necessary to be aware of the general pattern of interactions among nutrients and dietary components having a specific property, since more than one ingredients are used in fish feeds. For this reason, using mathematical formulae or computer programming to formulate complex feeds is unjustified; nutrient interactions change the dietary requirement of nutrients due to the different bioavailability of nutrients from feed to feed by direct or indirect interactions in diets and in the GI tract that further involve minerals of endogenous origin. Tables 63 and 64 summarize the general correlation between dietary nutrient levels and the apparent availability of nutrients (% absorption/Intake) or the net absorption of nutrients (% absorption/feeds). Also, Fig. 31 indicates the general pattern of mineral availability in various dietary sources. Generally, bone mineral components (Ca, P, Mg, ash) are highly antagonistic to the absorption of many minerals in diets. The absorption of P from diets is significantly and negatively correlated to the concentrations of ash, Ca, P and Sr in the diet; i.e., the more the ash, Ca, P or Sr in diets, the less P is absorbed (as the fractional net absorption). The actual net absorption of bone minerals, however, remains fairly stable, which indicates that fish absorb these minerals as much as they require but not in excess. This also suggests that reducing bone minerals in diets is an essential approach in formulating LP-feeds. Once this requirement is met, then the interfering effect of other dietary components having binding properties with minerals, notably phytic acid in plant ingredients, will more likely reveal their nutritionally significant potentials.

Nutrient interactions are always present; however, they become obvious only when the degree of interactions is relatively high. For example, when casein basal diet contains 10mg of available nutrient-A per 100g diet, and this diet is to be mixed with test ingredient at a 7 to 3 ratio (casein basal diet to test ingredient), then the mixed diet should contain at least 7 mg of the available nutrient-A per 100g diet even if the same nutrient-A supplied from the test ingredient is totally unavailable. If the net absorption of nutrient-A from the diet is below this level (7 mg /100g), then this is a clear evidence of (antagonistic) interaction. In other words, the test ingredient reduced the absorption of nutrient-A supplied from the casein basal diet portion of the diet. The substances in the test ingredient that might be responsible for the lowered absorption of the nutrient-A could be other minerals (e.g., Ca, P, Fe), phytic acid, fiber or other dietary components having binding, precipitating, or oxidation/reduction properties. This type of interaction is summarized in Table 65. The absorption of many trace elements, especially that of Fe and Mn, is substantially reduced by many feed ingredients as compared with the absorption from the casein basal diet alone. Also, it is evident that some plant ingredients markedly reduce Ca and Sr absorption.

It was Edward Mellanby who, after more than 20 years of research on rickets, made the following statement: "Our view is that the phytic acid in a rachitogenic cereal like oatmeal immobilizes all, or almost all, of the Ca contained in the cereal by converting it into an insoluble Ca phytate which cannot be absorbed, and further, that the excess of phytic acid (over and above that required to precipitate the Ca of the cereal) can exert an additional anticalcifying effect by precipitating other Ca present in the non-cereal part of the diet (Harrison and Mellanby, 1939)." At present, it is also known that endogenous minerals, secreted into the intestinal lumen via bile and digestive fluids, are also involved in the interaction with dietary components which reduce the efficiency of enterohepatic circulation of bile and reabsorption of other minerals of endogenous origin, resulting in a negative balance and dispossession of minerals from the body pool or a rapid onset of clinical deficiency.

Mineral Availability or Disavailability

In formulating feeds, this interacting property of feed ingredients needs to be considered, especially for the antagonistic interactions as seen in many ingredients having negative availability values. Ca, Mn and Fe contained in many feed ingredients are not only unavailable (zero availability) but further those ingredients reduce the absorption of Ca, Mn and Fe supplied from the other part of the diet including inorganic mineral supplements. Thus, feed ingredients have two distinct and opposing effects; i.e., (1) supplying minerals and (2) supplying substances which reduce the absorption (availability) of minerals. Available mineral contents in feed ingredients could, therefore, be below zero due to this second effect of the feed ingredients in compound feeds (Tables 65, 67 and 68).

Levels of Phosphorus

When the total nutrient content in diets is higher than the requirement level, then fish might regulate the net absorption of the nutrient. For some minerals, the regulatory site is not at the gastrointestinal level, but the fish may excrete an excess amount via urine or gills. Apparently, there is an absorption plateau in P at around 6000-7000 mg/kg feed (Fig. 54), which is more or less the requirement level for fish. Also, the fish bone study (p 71) provides further evidence in that the fractional net absorption (apparent availability) of P was high in low-P (low-bone) diet and was low in high-P (high-bone) diet. This suggests that the absorption of P from the GI tract is regulated and is saturable when the P source is fish bone or hydroxyapatite (in fish meal). Conversely, the absorption of P appears to be unregulated when the source of P is K- or Na-salts (ref. Ch.1), or when citric acid is used in the diet (ref. Ch.3). When fish bone (fish meal) is the source of P in the diet, it can be digested by citric acid, and Ca can be sequestered as Ca-citrate, which then liberates HPO_4^{2-} and $H_2PO_4^-$ (Misra, 1996), thereby rendering P more available to fish.

Fish meal is a major source of P in commercial salmonid feeds, which normally supplies P well above the dietary requirement for the fish. The excess amount of P in the feed is then excreted by the fish into the environment. The use of fish meal, especially those of high P content, should therefore be substantially reduced in LP-feeds. The availability of P in fish meal is relatively high when dietary level (of P) is low, but it can be further increased by supplementing the diet with citric acid. The use of fish meal replacers that contain less P is essential to reduce overall P content in feeds. To replace fish meal with other protein sources, however, involves several practical problems, i.e., tends to reduce total protein (and some essential amino acid) content in the feed, cause amino acid imbalance, reduce palatability, increase carbohydrate and fiber contents (i.e., reduce dry matter digestibility). This is particularly the case when plant protein sources such as soybean meal is used in place of fish meal in the feed. When the dietary P source is phytic acid (in plant ingredients), the use of phytase enzyme will be essential to hydrolyze phytate-P. The supplementation of citric acid largely enhances the effect of phytase; however, the presence of soluble Ca (e.g., from fish bone) strongly inhibits phytate hydrolysis, presumably due to the binding of Ca^{2+} with phytic acid in the intestinal lumen to form an indigestible precipitate. Although availability of P in both soybean meal (with phytase) and fish meal can be increased by the supplemental citric acid, the combination of these 3 components strongly inhibits phytate hydrolysis in soybean meal. In a study with human subjects, the absorption of Ca from milk was significantly reduced when milk was consumed together with oatmeal (source of phytate), but when milk and oatmeal were consumed separately at different times of the day, Ca absorption was not impaired (original paper Cruickshank et al., 1943; reviewed in Mellanby, 1950). This observation was confirmed more recently with wheat bran (Weaver et al., 1991). Using two diets, which differ in the composition, may offer a solution for the above problem; namely, Diet-A may contain soybean meal and citric acid, while Diet-B contains fish

meal and citric acid. Then, these two diets should be fed separately at different time of a day or alternate days.

Digestible Protein / Digestible Energy Levels

Although protein digestibility is similar among ingredients, the contents of protein and essential amino acids (not studied) in feed ingredients are highly variable. Both need to be considered in order to meet the adequate digestible protein (or more correctly, available limiting essential amino acid) /digestible energy ratio in LP-feeds. The use of blood meal involves three practical problems; 1) low EAA content + EAA imbalance leading to increased deamination and N-excretion, 2) as a pro-oxidant, Fe catalyzes lipid oxidation during storage of feeds, and 3) excessive absorption of Fe may cause toxic effects (NRC, 1980; Lall, 1989). Consequently, blood meal can not be used as a major feed ingredient although its low-P and high protein contents, high availability of both P and protein, and the low level of interaction in diets are of definite advantage for LP-feeds. Other low-ash animal protein sources such as feather meal, low-ash poultry byproduct meal and low-ash fish meal can be considered the most useful ingredients for low-pollution feeds.

High fat content is generally preferable in LP-feeds since it provides high energy without significant supply of P. Increasing dietary fat level is, therefore, a simple yet very efficient approach to reduce P excretion by the fish. However, according to Sakamoto and Yone (1980), P-deficient red seabream increased retention of "dietary fats." This effect may be attributed to glucose intolerance induced by hypophosphatemia, chronic hyperinsulinemia analogous to non-insulin dependent diabetes mellitus, reduction of the growth hormone, insulin-induced lipogenesis, and reduced efficiency of oxidative (and also the substrate-level) phosphorylation which reduces overall catabolism of energy sources via TCA cycle (see Ch.1). This metabolic shift associated with the P-deficiency also strongly inhibits beta-oxidation of fatty acids and, therefore, provides an useful opportunity to efficiently alter fatty acid composition of fish (fillets) by the dietary lipid source. Feeding fish with P-deficient diets containing a large amount of saturated fats (as a finishing feed) for a few months before harvesting could offer another measure to improve storage stability of frozen fillets. Since high levels of dietary fat and low levels of dietary P both independently elevate the fat content of the fish, it appears to be essential to adjust the level of dietary P depending on the desired fatness of the final product.

One of the most important roles of carbohydrates in salmonid feeds may not be attributed to its nutritional part as an energy source, but instead to its effect as a binder in both compressed and extruded feeds, or to its effect in increasing insulin secretion to stimulate, upon absorption, anabolic pathways in the intermediary metabolism. In LP feeds, both effects seem to be important. Insulin, however, also stimulates lipogenesis, making fish more fat. Since hypophosphatemia or P-deficiency reduces glucose utilization (glucose intolerance), high levels of digestible carbohydrates in LP-feeds may even become toxic to fish. Increasing DE value by increasing carbohydrate digestibility may reduce feed intake and

reduce growth rate of the fish, although feed efficiency could be improved (Hilton et al., 1981; Pfeffer et al., 1991). Kaushik et al. (1985) noted a more efficient utilization of protein and energy in trout fed digestible starch (30% per diet) than in those fed raw starch. Kaushik et al. (1989) also noted that low-protein (38%) diets containing high levels (38%) of digestible carbohydrate did not adversely affect overall growth or nutrient retention efficiencies in rainbow trout. Kim and Kaushik (1992) reported efficient utilization of digestible carbohydrates as an energy source in rainbow trout fed a diet containing 38, 9 and 30% of digestible protein, digestible lipids and digestible carbohydrates, respectively, with protein retention of 41.4%. Pieper and Pfeffer (1980a, b) noted up to 40% of digestible carbohydrates in diets were efficiently utilized as an energy source and increased protein retention in rainbow trout. These findings indicate that where lipids are unavailable as an energy source for aquaculture feeds, high levels of digestible carbohydrates can be tolerated even for carnivorous species like rainbow trout. As mentioned above, if fish are hypophosphatemic in marginal P-deficiency, however, it might induce glucose intolerance, and the high level of digestible carbohydrates in diets could have pathological consequences.

Optimum digestible protein (DP) /digestible energy (DE) ratio in rainbow trout feeds has been reported to be 92 mg/kcal (Cho and Kaushik, 1985), 105 mg/kcal (Cho and Woodward, 1989) or 22.6 g/MJ DE (94.56 mg/kcal) (Cowey, 1995). Similar DP/DE values have been found in the literature with other fishes (NRC, 1993; Cowey, 1995). Medale et al. (1995), however, reported in rainbow trout that at high ration levels, N excretion was 39-40% of digestible N intake for the diet having a DP/DE ratio of 18 mg/kJ (75.31 mg/kcal) and 44% of digestible N intake for the diet having a DP/DE ratio of 23 mg/kJ (96.23 mg/kcal). Lanari et al. (1995) reported in rainbow trout that growth and feed utilization improved markedly as dietary DP/DE ratio increased from 16.35 to 18.23 g/MJ (68.41 to 76.27 mg/kcal) with no significant effect on nitrogen discharge per kg of weight gain (mean values 29.1 to 29.9 g N / kg weight gain). These results may suggest that rainbow trout require DP in an amount at around 95 mg/kcal DE to support maximum growth, while the requirement of DP to provide maximum nitrogen retention (minimum N excretion) may be around 75 mg/kcal DE.

The dietary protein requirement in fishes is higher than those in terrestrial homeotherms (e.g., rats chicks and cats). This difference is due to the greater requirement for energy in homeotherms, and that the net retention of dietary N by fish is similar or even higher than that of omnivorous birds and mammals (Smith et al., 1978; Lovell, 1979; Bowen, 1987). This indicates that fish farming is a practice to convert feeds to foods in a more efficient way than the other animal-producing sectors do.

Many factors have been shown to influence fish growth, e.g., environmental stresses, cultural conditions, fish strain, life stage, physiological and endocrine factors, nutrient balance, interactions, availabilities, deficiency of trace nutrients, presence of anti-nutritional factors in feeds and water, feeding frequency, feeding rate, feed processing conditions, etc. (Abbott and Dill, 1989; Iwama, 1996). This

indicates that expressing nutrient requirements per actual growth or net-N-retention of fish offers a definite advantage over expressing nutrient requirements in the conventional per diet basis. Unfortunately, neither fish growth nor N-retention cannot be accurately predicted from the feed composition or information currently available in the literature. It is therefore essential to feed the diet containing an excess amount of available P to measure the potency of the feed (N-gain /feed) before actually prescribing the minimum dietary inclusion level of P to that particular diet. Since fish consume diets to meet their energy demand as do other animals (Cho et al., 1976; Grove et al., 1978; Smith, 1989), expressing nutrient requirements per DE appears to be the least compromised way. The implicit assumption underlying the energy-based expression is that fish grow in proportion to the energy intake, which is correlated but inaccurate. In higher animals and humans, various factors are known to affect energy expenditure, including nutritional state (over- or under-feeding), thyroid function, activity of sympathetic nervous system (levels of catecholamines), physical activity, dietary composition (varied degrees of heat losses via the specific dynamic action), various futile cycles (e.g., the Cori cycle) in intermediary metabolism, and the intervention of uncoupling proteins in the mitochondrial oxidative phosphorylation to generate heat rather than ATP (Linder, 1991; Napoli and Horton, 1996). If fish is similar to higher animals in regard to these metabolic responses, various stress factors in practical aquaculture conditions (e.g., adverse water quality, high temperature, low oxygen, stocking density, handling, bacterial infection, overfeeding, etc.) may well affect the utilization of dietary energy by increasing the activity of various energy-wasting systems. The requirements of dietary nutrients when they are expressed as per energy basis may not remain constant for this reason. Also, nutrient requirements for the maintenance and those for the growth should be very different, especially those for the constructive nutrients such as N, P, Ca, and many other minerals. Dietary nutrients used for the maintenance can be largely recycled, while those used for the growth (retention or deposition) are not. Consequently, every time this maintenance/growth ratio changes, the overall dietary requirement should change. The factors which change the maintenance/growth ratio are the life stage (size or age) of the fish, feeding levels, various stresses, nutrition or any other factors that might affect the growth rate of the fish.

Finishing Feed

Hardy et al. (1993) proposed a periodic feeding of low- and high-phosphorus feeds to increase retention of dietary P and to reduce excretion of P into water. An extension of this method is the use of low-phosphorus feeds as a finishing diet. It is, of course, imperative to harvest fish before signs of P deficiency, such as growth depression and reduced feed efficiency arise. Eya and Lovell (1997a) noted that year-2 channel catfish fed commercial-type feeds containing only 0.2% available P did not reduce weight gain and feed efficiency in a 140 day feeding period compared with those fed diets containing higher concentration of P. On the other hand, rainbow trout reduced N-retention within 12 days on

low-P diets in the present study (expt.1 in Ch.1, Fig.35), indicating an impaired protein retention or growth of fish by dietary P restriction. The ultimate goal, however, is not to deplete P stores in the body of fish but to reduce P excretion into the water, suggesting there may be little benefit to reduce the dietary P level lower than the minimum requirement level for fish unless the fish increase absorption and retention efficiency of dietary P at sub-requirement levels of intake. The present study (Ch.1) may not support this possibility since the urinary P excretion increased linearly once the dietary P intake exceeded a certain level.

Dilution Feed

The dilution feed by itself is nutritionally incomplete. Usually, commercial trout production feeds contain phosphorus at a level higher than the dietary requirement. The dilution feeds, formulated with low phosphorus ingredients such as plant protein sources, are very low and deficient in phosphorus content. Phosphorus concentrations in the effluent water from the trout hatchery can be minimized by feeding commercial (phosphorus-excess) feeds and the dilution (phosphorus-deficient) feeds in an appropriate ratio. The ratio needs to be adjusted according to the size of fish (due to the different requirement of P at different life stages) in addition to the phosphorus and energy content of both feeds. This ratio, however, may be best determined by actually monitoring the amount of phosphorus excretion and the fish growth rather than by calculating the ratio from the feed composition.

Vitamins and Minerals

Although high-energy rations reduce feed intake of fish and therefore the fish require increased concentration of micronutrients in their diets, the amount of mineral supplements in the LP-diets should be reduced to a level of the minimum dietary requirement. Inorganic Cu and Fe are known to be strong pro-oxidants for unsaturated fatty acids and for certain labile vitamins, to increase rancidity of dietary lipids, and to cause a loss of vitamins in feeds during storage (Cummings and Mattill, 1931; Desjardins et al., 1987). Since the availability of these metals in many feed ingredients has been determined in the previous chapter (Ch.2), redundant supplementation of these pro-oxidant metals (as inorganic supplements) can be avoided. This will be important for LP-feeds containing high amounts of labile unsaturated fatty acids. The availability of Cu is relatively high in all ingredients, while the availability of Fe is high in blood meal and feather meal. Inorganic mineral supplements can be replaced with these feed ingredients containing highly available forms of metals. Feeding rancid fats causes undesirable effects; e.g., impairment of fish health, increase of vitamin E requirements, decrease in vitamin E stored in tissues and possibly reduction in frozen storage stability of fillets (Hashimoto et al., 1966; Watanabe et al., 1966; Smith, 1979; Hung et al., 1981; Cowey et al., 1984). Due to its chelating property, dietary citric acid may protect labile nutrients in feeds and possibly fish fillets from metal-catalyzed lipid oxidation during storage. The addition of citric acid also stabilizes ascorbic acid and possibly other labile vitamins in feeds during storage, not only by the chelation of pro-oxidant metals but by reducing

dietary pH where the stability of labile vitamins substantially increases. Lowered dietary pH by the addition of citric acid may also reduce bacterial growth during storage of moist pellets.

Feeds for Broodstock and Triploid Fish

Essentially no information is currently available in the literature regarding the minimum P requirement in broodstock fish. The P content in the ovary appears to be lower than that in the whole body (Shearer, 1984). This indicates that less P is required to increase the weight of ovary than to increase the weight of whole fish (somatic growth). The P requirement could therefore be lower in broodstock fish than in growing immature fish. It should be imperative, however, to determine the actual P requirement for brood fish at various stages such as at pre-spawning and during the post-spawning (recovery) period because various metabolic, physiological and endocrinological changes are associated with reproduction, e.g., reduced feed intake, reduced weight gain and altered hormonal concentrations. For these measurements, it is critical to find extremely sensitive and rapid indicators to estimate P status of large brood fish. Although the content of phosphorus in gametes, viability of eggs, performance of offspring and survival of post-spawning brood fish might be considered as the possible response criteria for studying the dietary adequacy of phosphorus (as have been used by other workers), the urinary content of P appears to be most satisfactory as the indicator of P adequacy based on the result of the present study (Ch.1). It is very rapid, non-invasive and provides a reasonable estimate for the requirement of P in broodstock fish.

Female triploid fish have been introduced at a commercial scale in various aquaculture species to avoid 1) reduced growth rate and deterioration of flesh quality associated with reproduction and 2) to prevent genetic effect of net pen escapees on the wild (indigenous) stocks. Oliva-Teles and Kaushik (1990) reported that the growth rate, food conversion, protein efficiency ratios, and apparent digestibility of protein and energy did not differ between triploid and diploid juvenile rainbow trout. In spite of the increasing use of triploid fish in commercial aquaculture, currently available data for its specific nutrient requirements, especially for large immature fish, are quite limited. Since triploid fish have definite physiological differences from their diploid counterpart, it should be considered a different species having different nutrient requirements. High incidence of deformity of the lower jaw of triploid Atlantic salmon has been suspected as a sign of nutrient deficiency (McGeachy et al., 1996).

Frequency of Feeding

Since high-energy (high-fat) feeds are typical for LP-feeds, fish could be fed a whole ration of a day in a single feeding, particularly in large fish. If low-energy feeds are the choice due to the limited availability of local feed ingredients, multiple feedings may be necessary to meet the energy demand of the fish and to cope with faster gastric emptying time with low-energy feeds (Lee and Putnam, 1973; Grove et al., 1978; Hilton et al., 1983). Free amino acid supplements, e.g., L-lysine-HCl and DL-methionine, have been commonly used in feeds deficient in those essential amino acids. This is

particularly the case for feeds for which plant ingredients are the primary protein source. Because the utilization of supplemental free amino acids substantially increases by increasing the feeding frequency (Yamada et al., 1981a, b), multiple feedings will be necessary for the diet supplemented with free amino acids. Since the effect of supplemental phytase is reduced by multiple feeding, phytase and free amino acids may not be used in the same ration for an economical reason.

Acceptability and Palatability of Feeds

Soybean meal is less palatable than fish meal or casein to both coho salmon and rainbow trout (Ch.2); however, no such pattern was noted in the phytase trial in which soybean meal was used at a 50% level in diets (Ch.3). The cause of the reduced acceptability of diets containing high levels of soybean meal needs to be further investigated if it is intended to be used as the major protein source in production feeds. It may be the heat treatment of soybean meal that reduces the soluble amino acid content and the palatability to fish. Also, the diet history of fish, particularly at earlier stages (normally fish meal-based commercial feeds), could be a major determinant for the preference of feeds (Refstie et al., 1997). Fish strain might also affect feed preference as well as the utilization of carbohydrates in diets.

Uneaten Feeds

It should be worth remembering that LP-feeds exert their effect only when they are consumed by fish. Uneaten feeds directly (100%) contribute to the pollution, thus minimizing uneaten feeds in aquaculture practices could be a single most effective procedure to reduce discharge of P and other wastes from aquaculture systems. There are four potential sources of uneaten feeds; 1) feeding by inexperienced staff, 2) overfeeding; 3) use of demand feeders, 4) fines. Feeding fish indeed requires months or years of feeding practice using good techniques. Since feeds are often the single largest variable cost in most aquaculture operations (especially in intensive sectors), minimizing feed wastage and increasing feed efficiency (reducing feed conversion ratio) make important differences in reducing operational cost as well as being critical to pollution reduction. To separate pellets and fines before feeding must be an essential practice, particularly in developing countries where the availability of appropriate feed ingredients, binder materials or manufacturing plants is limited (Wood, 1995). If powder or minced feeds are used for feeding, it may cause substantial loss of nutrients into water (Cuzon et al., 1982), which is both economically and environmentally unjustified.

Table 63. Correlations between nutrient concentration in diets (left column) and the apparent availability of nutrients (rows) in rainbow trout[1].

Dietary concentration	Apparent availability (% of Intake)												
	DM[4]	CP[3]	Ash	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
TDN[2]	1.00	0.29	0.28	**0.44**	0.21	0.26	0.12	**0.46**	0.05	**0.55**	0.32	**0.42**	0.20
CP[3]	**0.89**	0.03	-0.01	0.34	0.02	0.07	0.12	0.31	0.13	**0.48**	0.07	**0.44**	0.15
Ash	-0.32	**-0.70**	**-0.90**	**-0.43**	**-0.43**	**-0.72**	-0.40	**-0.84**	**-0.55**	-0.20	**-0.91**	-0.19	**-0.76**
Ca	-0.30	**-0.65**	**-0.91**	**-0.47**	-0.27	**-0.74**	0.16	**-0.79**	**-0.45**	-0.19	**-0.91**	-0.22	**-0.42**
Cu	-0.08	-0.28	0.28	-0.14	**-0.49**	-0.35	**0.50**	**-0.44**	**-0.45**	-0.38	-0.19	0.03	-0.37
Fe	0.29	-0.14	0.09	0.23	0.12	0.33	-0.08	0.19	0.25	0.18	0.16	0.31	0.12
K	0.05	0.20	0.07	-0.02	**0.48**	0.20	-0.25	0.27	0.09	**0.44**	0.18	-0.21	0.37
Mg	-0.18	0.12	-0.19	-0.14	**0.40**	0.07	**-0.42**	0.15	0.19	**0.49**	-0.03	-0.25	0.26
Mn	-0.34	-0.11	-0.08	-0.09	0.36	0.25	**-0.49**	0.16	0.34	0.21	0.05	-0.22	0.23
Na	0.13	**-0.46**	-0.36	-0.11	**-0.53**	**-0.48**	**0.57**	**-0.55**	**-0.59**	-0.26	**-0.40**	0.17	**-0.47**
P	-0.27	**-0.60**	**-0.90**	**-0.47**	-0.11	**-0.68**	0.07	**-0.71**	**-0.42**	-0.05	**-0.87**	-0.28	-0.31
Sr	-0.30	-0.42	**-0.74**	**-0.49**	**-0.45**	**-0.69**	0.25	**-0.71**	**-0.42**	-0.30	**-0.80**	-0.19	**-0.56**
Zn	0.12	**-0.72**	**-0.44**	0.33	**0.70**	0.27	-0.40	0.26	0.24	0.28	0.15	0.23	**0.69**

[1] Positive values indicate positive linear correlation (synergistic effect) and the negative values indicate negative linear correlation (antagonistic effect) (data from Study-1, 2 and 3 in Ch.2). Values with bold case are statistically significant ($P < 0.05$). All diets contained the casein diet and one of the following test ingredients at 7:3 ratio, respectively (except for deboned whitefish meal-C, capelin meal, herring meal-B and the casein diet per se). The ingredients were herring meal-A, herring meal-B, capelin meal, anchovy meal, Peruvian meal, menhaden meal-A, menhaden meal-B, deboned whitefish meal-A, deboned whitefish meal-B, whole whitefish meal, skin&bone whitefish meal, poultry byproduct meal, meat&bone meal, feather meal-A, feather meal-B, blood meal (ring-dried), casein diet (A), casein diet (B), deboned whitefish meal-C, deboned whitefish meal-C+2% fish bone, deboned whitefish meal-C+5% fish bone, deboned whitefish meal-C+10% fish bone, soybean meal, wheat gluten meal, corn gluten meal, wheat middling, wheat flour. Nutrient concentration in diets ranged 46.5-66.2% (crude protein), 3.1-14.5% (ash), 0.35-5.05% (Ca), 4.1-93.8 ppm (Cu), 78-967 ppm (Fe), 0.30-1.24% (K), 0.11-0.33% (Mg), 4.23-72.2 ppm (Mn), 0.23-0.83% (Na), 0.58-2.74% (P), 2.4-234 ppm (Sr), 31-161 ppm (Zn).

[2] Total digestible nutrients.

[3] Crude protein.

[4] Dry matter.

Table 64. Correlation coefficients[1] between dietary nutrient concentrations and the net absorption of dietary nutrients in rainbow trout.

	Net absorption[2]												
	DM	CP	Ash	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
TDN	**0.89**	0.28	0.11	0.17	0.20	0.33	0.10	0.08	0.14	0.47	0.15	-0.06	0.25
CP	1.00	**0.98**	0.02	0.27	0.14	0.40	-0.21	-0.15	-0.03	0.52	0.09	0.21	0.27
Ash	-0.05	-0.19	**0.58**	0.61	-0.43	-0.35	0.00	-0.35	-0.56	0.22	0.62	0.53	-0.58
Ca	0.00	-0.24	-0.01	**0.28**	-0.18	-0.36	-0.18	-0.39	-0.56	0.26	0.30	0.36	-0.42
Cu	0.12	-0.88	-0.81	0.13	**-0.29**	-0.17	-0.45	-0.69	-0.42	0.26	-0.20	0.69	-0.38
Fe	0.46	0.22	0.11	0.06	0.09	**0.90**	-0.08	-0.01	0.17	0.15	-0.04	-0.14	0.12
K	-0.21	0.56	0.64	0.05	0.39	-0.07	**0.97**	0.80	0.19	0.01	0.47	-0.47	0.35
Mg	-0.35	0.62	0.68	0.03	0.29	-0.10	0.74	**0.88**	0.20	0.01	0.45	-0.39	0.25
Mn	-0.54	0.60	0.59	-0.12	0.26	0.02	0.45	0.68	**0.38**	-0.41	0.17	-0.43	0.24
Na	0.35	-0.71	-0.45	0.30	-0.38	-0.14	-0.36	-0.65	-0.61	**0.67**	0.15	0.50	-0.41
P	-0.04	-0.03	0.20	0.30	-0.05	-0.37	0.04	-0.16	-0.50	0.27	**0.46**	0.20	-0.30
Sr	-0.04	-0.45	-0.37	-0.03	-0.33	-0.31	-0.38	-0.50	-0.53	0.13	-0.15	**0.55**	-0.55
Zn	0.13	0.58	0.68	0.63	0.65	0.02	0.29	0.42	0.18	0.00	0.64	0.04	**0.70**

[1] The correlation is on a diet basis (data from Study-1, 2 and 3 in Ch.2). All diets contained the casein diet and one of the following test ingredients at 7:3 ratio, respectively (except for deboned whitefish meal-C, capelin meal, herring meal-B and the casein diet per se). The ingredients were herring meal-A, anchovy meal, menhaden meal-A, deboned whitefish meal-A, poultry-byproduct meal, feather meal-A, casein basal diet-A, menhaden meal-B, Peruvian meal, meat&bone meal, feather meal-B, blood meal (ring-dried), deboned whitefish meal-B, whole whitefish meal, skin&bone whitefish meal, capelin meal, casein basal diet-B, herring meal-B, deboned whitefish meal-C, deboned whitefish meal-C+2% fish bone, deboned whitefish meal-C+5% fish bone, deboned whitefish meal-C+10% fish bone, soybean meal, wheat gluten meal, corn gluten meal, wheat middling and wheat flour.

[2] Values in bold case are the correlation between dietary concentration and the net absorption of the same nutrient. The net absorption is an actual amount of nutrients absorbed from diets. Positive values (positive correlation) indicate the synergistic effect; negative values (negative correlation) indicate the antagonistic effect.

Table 65. Inhibitory potential of feed ingredients on the absorption of dietary nutrients.

	Net absorption (%) of nutrients from the Casein diet[1]											
	CP	Ash	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn
FM (anchovy)	-----	-----	-----	-----	36.8	-----	-----	18.0	-----	-----	-----	-----
FM (herring)	-----	-----	-----	-----	21.5	-----	-----	4.3	-----	-----	-----	-----
FM (menhaden) A	-----	-----	-----	66.9	24.8	-----	-----	10.6	-----	-----	-----	70.6
FM (menhaden) B	-----	-----	-----	-----	7.1	-----	-----	14.9	-----	-----	-----	-----
FM (Peruvian)	-----	-----	-----	-----	27.3	-----	-----	15.5	-----	-----	-----	-----
FM (whitefish, deboned) A	-----	-----	-----	63.6	19.2	-----	-----	11.5	-----	-----	-----	75.4
FM (whitefish, deboned) B	-----	-----	-----	76.3	17.7	-----	-----	13.9	-----	-----	-----	-----
FM (whitefish, whole)	-----	-----	40.5	59.2	-49.5	-----	-----	-6.7	-----	-----	-----	49.5
FM (whitefish, skin&bone)	-----	-----	-----	69.8	-16.3	-----	85.2	9.8	-----	-----	-----	41.5
Meat&bone meal	-----	-----	-----	-----	-15.6	-----	85.9	14.0	-----	-----	-----	67.5
Poultry byproduct meal	-----	-----	-----	-----	38.0	-----	-----	8.4	-----	-----	-----	-----
Feather meal A	-----	-----	-----	-----	-----	-----	-----	39.6	-----	-----	-----	-----
Feather meal B	-----	-----	-----	-----	-----	-----	-----	19.0	-----	-----	-----	-----
Blood meal (ring-dried)	-----	-----	-----	-----	-----	98.7	-----	-----	-----	-----	-----	-----
Soybean meal	-----	-----	1.1	-----	-----	-----	-----	25.8	52.0	-----	-36.0	77.8
Wheat flour	-----	-----	33.5	-----	-----	-----	-----	46.8	63.1	-----	26.9	-----
Wheat middling	-----	-----	5.4	-----	28.3	-----	-----	32.9	48.9	-----	-18.9	69.3
Wheat gluten meal	-----	-----	-----	-----	-----	-----	-----	-----	78.2	-----	-----	-----
Corn gluten meal	-----	65.6	4.3	-----	14.5	-----	81.5	10.5	42.5	-----	-39.4	56.7
Casein diet A	97.9	80.0	41.3	79.3	45.4	99.0	90.8	53.7	87.0	84.5	40.5	79.6
Casein diet B	98.3	79.2	40.9	77.8	31.0	99.3	89.5	58.4	80.4	82.1	47.8	78.7

[1] Data extracted from Study 1 and 2 in Ch. 2 with rainbow trout. Values indicate the net absorption of dietary nutrients from the casein diet portion (70% of the whole diet) on the assumption that the nutrient contributed from the ingredient (30% in the diet) is totally unavailable (see Interaction section, p 152 for detail). Dash lines indicate that there is an absorption of nutrients from the test ingredient (null hypothesis invalid). See tables in the digestibility trials for the availability of nutrients in test ingredients. The concentrations of nutrients in the casein diets A and B (dry basis) were respectively as follows: 58.6, 61.3% (crude protein); 4.00, 4.14% (ash); 0.497, 0.493% (Ca); 4.93, 5.24 ppm (Cu); 98.0, 98.7 ppm (Fe); 0.743, 0.754% (K); 0.227, 0.228% (Mg); 34.9, 33.3 ppm (Mn); 0.412, 0.376% (Na); 0.741, 0.732% (P); 3.37, 3.25 ppm (Sr); 123, 121 ppm (Zn).

Table 66. Total and digestible (available) crude protein (CP) and phosphorus contents in feed ingredients[1].

Ingredient	Dry matter Digesti-bility	Protein Total CP	Protein Digesti-bility	Protein Digesti-ble CP	Phosphorus Total P	Phosphorus Availa-bility	Phosphorus Availa-ble P
				%			
Herring meal-A	89.2	73.6	94.6	69.7	2.05	44.4	0.91
Herring meal-B*	-----	-----	-----	-----	2.41	52.1 [3]	1.26
Anchovy meal	87.7	73.7	93.7	69.0	2.90	50.4	1.46
Menhaden meal-A	78.6	67.7	89.8	60.8	3.43	36.5	1.25
Menhaden meal-B	70.3	66.6	84.8	56.5	3.61	35.0	1.27
Peruvian fish meal	79.0	61.7	85.6	52.9	2.92	43.9	1.28
Whitefish meal, deboned meal-A	92.6	78.2	96.7	75.7	1.69	46.8	0.79
Whitefish meal, deboned meal-B	86.2	71.5	93.7	67.0	1.57	36.0	0.57
Whitefish meal, whole meal	74.0	71.7	88.4	63.4	3.50	17.2	0.60
Whitefish meal, skin & bone meal	50.5	46.9	76.1	35.7	7.41	11.8	0.87
Poultry byproduct meal-A	91.6	81.0	95.9	77.6	2.17	63.5	1.38
Poultry byproduct meal-B*	81.4	68.2	85.8	58.5	2.50	47.7	1.19
Poultry byproduct meal, low ash*	84.6	72.7	89.5	65.1	1.65	50.8	0.84
Poultry byproduct meal, deboned*	73.7	70.2	82.7	58.1	2.09	47.4	0.99
Meat meal*	59.5	63.0	80.9	50.9	2.76	2.5	0.07
Meat meal, low ash*	66.0	67.3	80.9	54.5	2.28	44.7	1.02
Meat & bone meal-A	55.9	58.5	79.8	46.6	5.59	26.9	1.51
Meat & bone meal-B*	61.6	58.9	79.0	46.5	2.68	21.8	0.58
Meat & bone meal, low ash*	56.5	59.8	78.3	46.8	2.49	35.0	0.87
Feather meal-A	83.8	77.3	85.9	66.4	0.75	61.7	0.46
Feather meal-B	77.7	75.6	83.3	63.0	1.26	79.4	1.00
Feather meal-C*	83.2	102.3	83.3	85.2	trace (< 0.05)	-----	0.39
Blood meal, ring-dried-A	93.5	93.0	94.1	87.5	0.12	107.4	0.13
Blood meal, ring-dried-B*	95.8	103.4	94.8	98.0	0.08	118.4	0.10
Blood meal, spray-dried*	88.3	94.9	91.2	86.6	0.72	103.5	0.74
Soybean meal, dehulled, solv-ext.-A	71.2	53.2	90.1	47.9	0.76	22.0	0.17
Soybean meal, dehulled, solv-ext.-B*	58.8	50.9	85.1	43.3	0.85	26.6 [3]	0.23
Soybean meal, dephytinized*	61.8	50.9	85.1	43.3	0.85	92.5 [3]	0.79
Wheat gluten meal	94.7	85.0	100.5	85.5	0.18	74.7	0.13
Corn gluten meal	87.7	72.3	97.3	70.4	0.54	8.5	0.05
Wheat middling	45.0	20.5	90.7	18.6	1.17	55.3	0.65
Wheat flour	43.0	15.5	100.4	15.6	0.32	47.0	0.15
Barley, grain*[2]	67.3	-----	-----	-----	0.33	47.1 [3]	0.16
Corn, dent yellow, grain*[2]	62.1	-----	-----	-----	0.26	36.7 [3]	0.10
Corn, flint yellow, grain*[2]	64.9	-----	-----	-----	0.34	36.3 [3]	0.12
Casein basal diet-A	83.7	58.6	97.9	57.3	0.74	84.5	0.63
Casein basal diet-B	86.8	61.3	98.3	60.3	0.73	82.1	0.60
Casein basal diet-C*	81.8	55.3	95.0	52.6	0.85	67.6	0.57

[1] Data extracted from Study 1, 2 and 5 (Ch. 2) and Study 5 (Ch.3) with rainbow trout. The digestibility or availability values were expressed as fractional net absorption of nutrients (% per intake). The dietary concentrations of protein and phosphorus (total, digestible or available) were expressed on a dry basis. The fecal samples were collected either by stripping (ingredients indicated with an asterisk) or settling (without asterisk). Dash line indicates that the value was not determined.

[2] Ingredients were heat-extruded.

[3] Determined in purified diets (diets low in phosphorus and calcium).

Table 67. Total and available Ash, Calcium, Magnesium, Potassium and Sodium contents in feed ingredients.[1]

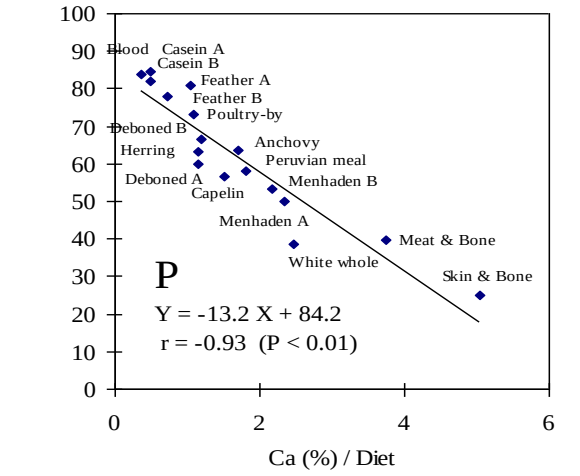
	Ash		Ca		Mg		K		Na	
	Total	Avail.	Total	Avail.	Total	Avail.	Total	Avail.	Total	Avail.
	% / dry ingredient									
Herring meal	9.05	2.63	2.743	0.051	0.203	0.101	0.70	0.69	0.57	0.46
Anchovy meal	16.56	8.42	4.709	1.381	0.352	0.174	1.07	1.07	1.35	1.22
Menhaden meal-A	20.25	6.01	6.353	1.016	0.292	0.077	1.04	1.02	0.69	0.51
Menhaden meal-B	20.37	5.35	6.674	0.970	0.271	0.100	0.89	0.85	0.65	0.53
Peruvian fish meal	15.91	6.69	4.844	1.103	0.385	0.223	0.84	0.83	1.26	1.19
Whitefish meal (deboned)-A	9.12	3.11	2.932	0.481	0.222	0.142	0.52	0.51	0.56	0.55
Whitefish meal (deboned)-B	9.34	3.48	2.671	0.324	0.241	0.173	0.61	0.59	0.54	0.44
Whitefish meal (whole)	18.51	1.96	7.084	-0.005	0.224	0.057	0.42	0.39	0.55	0.47
Whitefish meal (skin & bone)	38.51	3.48	15.674	0.766	0.286	-0.023	0.37	0.34	0.55	0.20
Poultry-byproduct meal	12.04	7.70	2.538	0.807	0.224	0.140	1.81	1.81	0.82	0.71
Meat & bone meal	31.07	6.96	11.317	1.077	0.242	-0.020	0.64	0.62	0.93	0.49
Feather meal-A	4.81	2.01	1.291	0.378	0.080	0.047	0.25	0.25	0.52	0.33
Feather meal-B	7.17	4.06	2.291	1.093	0.064	0.046	0.25	0.23	0.48	0.37
Blood meal (ring-dried)	1.97	1.58	0.039	0.005	0.029	0.036	0.00	-0.01	0.66	0.61
Soybean meal	7.94	3.99	0.419	-0.508	0.424	0.218	2.50	2.47	tr.	-0.37
Wheat gluten	0.77	0.74	0.117	0.093	0.036	0.062	0.27	0.27	tr.	-0.09
Corn gluten	0.68	-1.44	tr.	-0.459	0.057	-0.052	0.24	0.23	tr.	-0.46
Wheat middling	4.79	2.47	0.147	-0.459	0.595	0.315	1.33	1.31	tr.	-0.40
Wheat flour	1.00	0.50	0.031	-0.100	0.144	0.114	0.48	0.47	tr.	-0.25
Casein basal diet-A	4.00	3.20	0.497	0.205	0.227	0.206	0.74	0.74	0.41	0.36
Casein basal diet-B	4.14	3.28	0.493	0.202	0.228	0.204	0.75	0.75	0.38	0.30

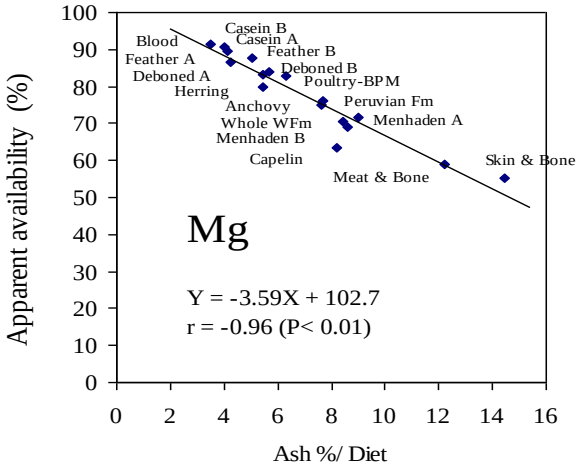
[1] Data extracted from Study 1 and 2 in Ch. 2 with rainbow trout. The available mineral content was calculated from the total mineral content of ingredients and the apparent availability coefficients. The available mineral content with the value lower than 0% indicates that the inherent mineral contained in the ingredient is unavailable and, in addition, the ingredient contains certain compounds which further reduce the availability of the mineral supplied from other ingredients (or from mineral supplements) in a complex diet. For example, soybean meal contains Ca at 0.419%, all of which is unavailable. In addition, soybean meal makes further 0.508% of Ca supplied from non-soybean meal portion of the diet unavailable. This property of soybean meal for making dietary Ca "disavailable" should be accounted for in formulating diets (rather than simply applying zero availability). See also Availability & Disavailability section (p 153) and Interaction section (p 152) for detail.

Table 68. Total and available Copper, Iron, Manganese, Strontium and Zinc contents in feed ingredients.[1]

	Cu		Fe		Mn		Sr		Zn	
	Total	Avail.	Total	Avail.	Total	Avail.	Total	Avail.	Total	Avail.
	ppm / dry ingredient									
Herring meal	4.39	1.10	153.3	-56.7	3.13	-41.75	45.72	3.56	146.3	4.3
Anchovy meal	6.32	2.66	308.0	-20.8	7.81	-30.88	66.70	16.59	129.8	22.3
Menhaden meal-A	2.54	-5.75	934.2	-50.8	70.35	-37.84	83.71	11.71	164.0	-27.7
Menhaden meal-B	3.72	1.77	966.5	-55.0	61.79	-33.79	85.85	13.99	236.3	6.9
Peruvian fish meal	5.11	2.67	303.5	-8.6	15.59	-33.32	91.45	23.72	155.3	26.2
Whitefish meal (deboned)-A	1.79	-3.86	218.6	-65.2	10.77	-37.48	156.81	58.08	130.2	-12.9
Whitefish meal (deboned)-B	2.03	-0.19	205.1	-30.6	14.19	-34.53	144.18	46.66	141.9	16.9
Whitefish meal (whole)	1.26	-2.27	182.8	-185.4	27.19	-50.56	351.75	22.05	133.6	-82.2
Whitefish meal (skin & bone)	1.40	-0.98	201.8	-109.1	67.99	-37.78	772.41	60.28	164.9	-104.9
Poultry-byproduct meal	3.68	0.67	197.5	-18.2	tr.	-39.63	56.79	19.63	119.0	18.7
Meat & bone meal	4.68	0.29	455.8	-107.3	15.46	-34.50	75.15	7.95	162.3	-31.5
Feather meal-A	19.10	8.81	768.0	404.7	74.78	-11.94	50.48	24.35	252.4	95.1
Feather meal-B	32.33	19.81	539.5	87.1	8.86	-30.57	24.01	10.85	209.9	50.6
Blood meal (ring-dried)	4.84	4.24	2993	1675	tr.	3.54	3.41	2.60	45.6	35.1
Soybean meal	16.95	8.61	362.6	-10.5	50.38	-24.73	19.49	-6.56	68.8	-68.9
Wheat gluten	5.85	4.76	59.2	28.7	25.76	29.52	3.27	2.31	56.9	57.2
Corn gluten	11.69	7.73	174.2	-75.4	1.04	-37.56	0.00	-6.71	23.8	-70.2
Wheat middling	9.92	3.91	126.4	-43.1	168.19	-18.67	7.40	-5.14	122.0	-32.4
Wheat flour	2.50	1.62	27.0	-13.3	21.63	-6.15	1.41	-1.17	13.0	2.1
Casein basal diet-A	4.93	3.91	98.0	44.4	34.89	18.72	3.37	1.36	123.0	97.9
Casein basal diet-B	5.24	4.08	98.7	30.6	33.26	19.44	3.25	1.55	120.6	95.0

[1] See footnote 1 in Table 67.





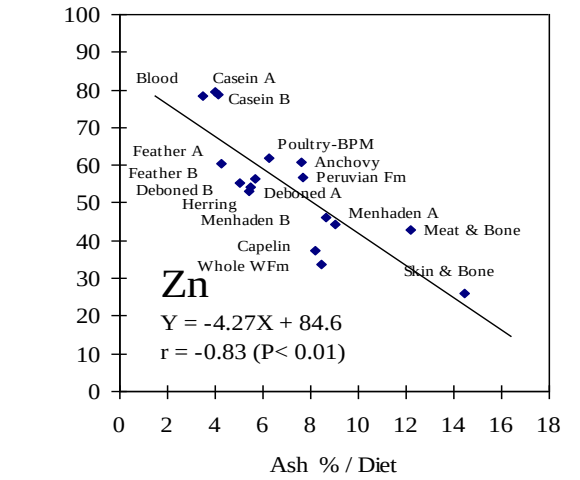


Figure 31. Correlation between dietary calcium or ash contents and the apparent availabilities of phosphorus, magnesium and zinc in whole diets containing 30-50% of one of the specified ingredients in the casein diet. Data extracted from Study 1 and 2 in Ch. 2 with rainbow trout. See also Fig. 57.

2. Formulation and Evaluation of Model Low-Pollution Feeds

MATERIALS AND METHODS

Four low-pollution (LP) feeds (standard, high-fish meal, high-soybean meal, and low-energy) were prepared using practical feed ingredients (Table 69). Two commercial feeds (compressed or extruded trout feeds) were also fed for comparison (control). A dilution feed, which contained P in an amount far below the dietary requirement (digestible energy basis), was introduced to "reduce" P content of commercial feeds by mixing together (1:1 ratio was used in this study) at the time of feeding. Dietary concentrations of P and minerals were determined to meet the minimum dietary requirement (digestible energy basis) of available minerals (Table 70). Feed ingredients of plant sources (soybean meal, wheat middling and flour) were pre-digested with fungal phytase (50°C-24h, 50% moisture, pH adjusted to 5.3 with citric acid), and steam-cooked (0.70 kg/cm^2, = ca.115°C) for 10 min before mixing with other ingredients. Diets were prepared as cold extruded moist-pellets, air-dried for 3 h at room temperature and stored at –20°C until fed.

Rainbow trout (initial body weight ca. 120g) were obtained from a commercial source. Fish were fed once to satiation and, ca. 1 h after feeding, all fish were anesthetized and the fasting fish were removed from the population by visual inspection. Thirteen fish were randomly stocked in each of eight fiberglass tanks receiving a continuous flow of spring water (15°C, oxygen near saturated) at 4L/min/tank. One tank was randomly assigned to each treatment. Fish were fed experimental feeds once daily as much as they would consume in one hour during the first week. In order to stabilize the daily feed intake before placing fish into the metabolic tank, feeding amount was reduced to 75% of the satiation level that was determined during the first week. For an unknown reason, fish became unable to consume this reduced size of a ration daily (75% of satiation) later during the second week. The ration size was, therefore, further reduced down to 50% of the satiation level for the third week. No mortality or abnormality of fish was observed at any time during the experiment. During the third week, fish were placed into the metabolic tanks, and the fecal and non-fecal wastes were collected as described previously (ref. Ch.1). This procedure was repeated four times (per treatment) on alternate days. Analytical procedures for feed, fecal and water samples were the same as previously described (ref. Ch.1).

RESULTS & DISCUSSION

Fecal excretion of P and protein, especially P, were markedly reduced in fish fed LP feeds (Fig. 32). The dilution feeds also were effective to reduce fecal excretion of P and protein. This effect of reduced fecal P excretion is attributed to the increased availability of P by preliminary treatment of plant feed ingredients with fungal phytase, and by the reduced intake of dietary P per se (Table 71). The

reduction of fecal P excretion over the commercial feeds was obvious either when it was expressed as per dietary intake (of P), per diet (dry matter) or per N-retention (fish growth).

In contrast to the remarkable difference in the fecal P excretion between LP feeds and commercial trout feeds, the excretion of P (and protein as N) into water was not largely different between LP and commercial feeds (Fig. 32). In addition, the amount of P excreted into water was relatively high in all treatments compared with the previous results (Ch.1 and 3). Phosphorus excreted by fish into the tank water is mostly of urinary origin, and this urinary P is an excess amount which was not required by fish for growth or maintenance. In this study, the dietary P was prescribed as per digestible energy basis (for its practical convenience). It is, however, the growth of fish which determines the actual P requirement in the feeds. The growth of fish fed LP feeds was not as high as had been expected. Consequently, fish received P in an amount more than they required for the growth, and this resulted in an increased excretion of P into the tank water. The potency of feeds is the direct factor that governs the requirement of P in diets (ref. expt. 5 and 6 in Ch.1). The potency of feeds, however, needs to be determined separately by an actual feeding trial, which makes the application of the growth-based expression (g/g-N-gain) more inconvenient than the other ways of expressing nutrient requirements (e.g., g /g diet, g /DE, g /day).

The retention of P by fish was ca. 20 g/100 g N-retention in commercial feeds, whereas that of LP feeds was apparently higher than that. In chapter 1 (expt.5), the retention of P reached a plateau of 0.277 (g P/g N-gain). This value falls between those of LP feeds and commercial feeds. The cause of this difference remains to be studied; however, as mentioned in the materials & methods section, the fish became anorexic over time (especially in the groups of fish fed LP feeds), the feed intake was relatively low, and the daily feeding level was not stabilized before fish were placed into the metabolic tanks. These variables (feeding level and daily feeding rate) needed to be strictly controlled to reduce experimental error.

In order to reduce dietary P, reducing fish meal (high in P content) is essential. Unfortunately, fish meal is the best protein source for fish feeds, especially for salmonid feeds. Use of plant protein sources to replace fish meal encounters several practical problems; e.g., reduction in palatability, reduction in protein content, tendency to cause amino acid imbalance, increase in carbohydrate content, and increase in price. When energy content in the diet is high, it may well cause protein/energy malnutrition due to the low essential amino acid content in many plant protein sources (limiting essential amino acid). LP feeds used in the present experiment contained higher levels of available carbohydrate and fats (≈ higher energy content) and much lower levels of protein (supplied mainly from soybean meal and blood meal ≈ lower essential amino acid content) than those of commercial fish meal-based feeds. These two factors could be responsible for the reduced feed intake and the reduced growth of the fish.

Since the supply of plant protein sources is more stable and feasible compared to animal byproduct materials, the use of plant ingredients should receive high priority, especially when considering the sustainability of aquaculture in terms of the future supply of ingredients for fish feeds. This is exactly what fish nutrition people have been working on since the days of Clive M. McCay & Abram V. Tunison (McCay, 1927). Because of this context, the present experiment utilized plant protein sources to replace fish meal rather than using low-phosphorus animal byproducts such as deboned fish meal, feather meal or meat meal. Although the use of plant protein sources is thus imperative, this requires extensive information on many aspects of nutrition, metabolism and feed engineering technologies, which have not been covered in the present dissertation. The ultimate goal of this project must be to minimize P excretion and the feed cost, while maximizing fish growth, feed efficiency and the final product quality. There is, of course, no end in this endeavor.

Table 69. Ingredient composition of low-pollution feeds[1].

Ingredients[2]	Std.	High-FM	High-Soy	Low-En	Comp	Extr	Dilution
			%				
Herring meal	12	22	---	---	C	C	---
Soybean meal[3]	20	---	50	20	O	O	20
Wheat middling[3]	28	---	20	45	M	M	35
Wheat flour[3]	---	38	---	20	M	M	---
Blood meal (ring-dried)	10	10	5	5	E	E	10
Fish oil	20	20	20	5	R	R	30
Vitamins[4] / Minerals*	4.5	4.5	4.5	4.5	C	C	4.5
SiO_2	0.5	0.5	0.5	0.5	I	I	0.5
Citric Acid	5	5	---	---	A	A	---
Total	100	100	100	100	L	L	100
Citric acid used for incubation[3]	0.68	0.076	1.2	0.89			0.75
phytase unit[3]	9.6	7.6	14	17	C	E	11
* Minerals added[5]					O	X	
Mg (g/kg)	0	0	0	0	M	T	0
Fe (mg/kg)	0	0	0	0	P	R	0
Zn (mg/kg)	53.9	28.3	74.1	49.5	R	U	61.0
Cu (mg/kg)	0.13	2.07	0	0	E	D	0.43
Mn (mg/kg)	30.0	25.7	31.0	24.5	S	E	28.2
K (g/kg)	0	4.5	0	0	S	D	0
Na (g/kg)	7.7	6.1	9.3	7.4	E		9.4
I (mg/kg)	1.29	1.23	1.28	0.86	D		1.45
Se (mg/kg)	0.35	0.33	0.35	0.23			0.39

[1] Abbreviations for the experimental diets: Std. (standard); High-FM (high-fish meal diet); High-Soy (high soybean meal diet); Low-En (low-energy diet); Comp (commercial compressed diet for trout); Extr (commercial extruded diet for trout); Dilution (dilution feed). See Materials and Methods section for detail.

[2] Total P content (ash content in parenthesis) of ingredients (%, as-is basis): herring meal, 2.276 (17.62); soybean meal, 0.702 (6.33); wheat middling, 0.501 (2.44); wheat flour, 0.092 (0.42); blood meal, 0.091 (2.23); fish oil, 0.002; #30 vitamin pre-mixture, 0.071; choline-60%, 0.065.

[3] Incubated 24h at 50°C using phytase, citric acid (to adjust pH of the mixture) and ca.50% water. Heated 10 min at 0.70 kg/cm^2 before mixing with other ingredients.

[4] L-ascorbyl polyphosphate 0.25 g; #30 vitamin pre-mixture 1.5 g; choline-60% 0.5 g per 100 g diet.

[5] Mineral supplements used: $ZnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4\ H_2O$, KCl, NaCl, KI, Na_2SeO_3. Inorganic supplements were added only when endogenous available minerals (inherent in feed ingredients) were not sufficient to meet the dietary requirements (calculated as per-digestible energy basis). See also Table 76 for the endogenous available mineral contents.

Table 70. Analytical composition of low-pollution feeds[1].

	Std.	High-FM	High-Soy	Low-En	Comp	Extr	Dilution
Calculated values							
Digestible Protein (%)	33.0	30.9	33.7	26.6	C	C	25.8
Total Phosphorus (%)	0.563	0.545	0.456	0.389	O	O	0.325
DE (Digestible Energy) (cal/g)	4205	4026	4191	2803	M	M	4731
Digestible Protein /DE (mg/kcal)	78.6	76.6	80.5	95.1	M	M	54.5
Total Phosphorus /DE (mg/kcal)	1.34	1.35	1.09	1.39	E	E	0.69
Endogenous available minerals[2]					R	R	
Mg (g/kg)	1.48	0.69	1.74	2.10	C	C	1.57
Fe (mg/kg)	147	150	70	60	I	I	150
Zn (mg/kg)	-18.8	5.3	-39.2	-26.2	A	A	-21.6
Cu (mg/kg)	3.37	1.28	5.30	4.02	L	L	3.51
Mn (mg/kg)	-14.8	-11.2	-15.9	-14.4			-11.1
K (g/kg)	9.4	3.3	15.0	11.8			9.5
Na (g/kg)	-0.7	0.7	-2.3	-2.7			-1.5
Analytical values							
Moisture	10.3	11.5	11.8	14.7	11.0	9.0	5.9
Crude Protein	33.2	30.3	34.1	25.7	47.0	49.7	25.4
Digestible Protein	31.2	28.5	32.0	23.8	42.4 (32.6)	45.5 (34.4)	----
Ash	7.23	7.60	7.09	5.96	10.15	8.74	5.74
Total Phosphorus	0.680	0.685	0.551	0.484	1.46 (0.915)	1.32 (0.848)	0.405
Total Calcium	0.621	0.997	0.193	0.126	2.095	1.998	0.123
Total Starch[3]	20.5	34.3	20.6	51.3	14.8	16.7	26.0
Digestible Starch[3]	14.5	33.1	14.2	41.4	7.2 (12.1)	14.8 (17.4)	----
Total Lipids[4]	23.0	19.0	21.8	5.1	14.5	16.3	34.3
Gross Energy (cal/g)	5521	5229	5535	4652	5174	5488	5756
DE (Digestible Energy) (cal/g)[5]	4834	4921	4725	3800	4204 (4469)	4922 (4924)	----
Digestible Protein /DE	64.6	57.8	67.8	62.7	100.8 (72.8)	92.5 (69.9)	----
Protein/ Starch (digestible) ratio	2.16	0.86	2.25	0.58	5.86 (2.70)	3.08 (1.98)	----
Total Phosphorus /DE	1.41	1.39	1.17	1.27	3.46 (2.05)	2.68 (1.72)	----

[1] Values are expressed on an dry basis (on an as-is basis for moisture content).

[2] The content of endogenous (inherent in feed ingredients) available minerals having a value less than 0 indicates the presence of antagonistic substances. This suggests the need for inorganic mineral supplementation in an amount greater than the minimum dietary requirements that were determined with purified diets (see Tables 65, 67, 68 for detail).

[3] The content of starch was determined by the direct acid-hydrolysis (1 N-sulfuric acid, 2 h) followed by Somogyi-Nelson's colorimetric determination (Oser, 1965) using a purified starch reagent (ICN biomedicals) as a standard. The content of digestible starch was determined from the content of total starch in feces and that in feeds (pooled samples of d 3, 4, 5 and 6; single determination) using SiO_2 as the inert marker to determine the apparent digestibility of starch. The digestibility of starch had the following values (%): 70.5 (Std.); 96.5 (High-FM); 69.1 (High-Soy); 80.8 (Low-En); 49.0 (Comp); 88.8 (Extr); 58.7 (Comp +Dilution); 81.0 (Extr +Dilution). Values in parentheses indicate the content of digestible starch in the mixed ration (commercial feed 50%, dilution feed 50%).

[4] Extracted by Goldfisch fat extractor with methylene chloride as a solvent.

[5] Determined from the gross energy contents in feeds and feces based on acid-insoluble ash as the inert marker. Values in parentheses indicate the content of digestible starch in the mixed ration.

Table 71. Apparent digestibility (availability) of dry matter, protein, phosphorus and energy of low-pollution feeds[1].

Diet[2]	Dry matter	Crude Protein	Phosphorus	Energy
Std.	85.0 ± 0.28	93.9 ± 0.22	96.5 ± 0.43	87.5
HF	91.9 ± 0.24	94.0 ± 0.21	90.7 ± 0.46	94.1
HS	80.9 ± 0.46	93.9 ± 0.12	89.9 ± 1.89	85.4
LEn	80.1 ± 0.47	92.7 ± 0.29	93.4 ± 1.92	81.7
Co	73.6 ± 0.66	90.2 ± 0.19	50.9 ± 0.73	81.3
Ex	84.4 ± 0.23	91.6 ± 0.11	59.9 ± 0.78	89.7
Co + Dil.	76.3 ± 0.23	90.7 ± 0.26	62.5 ± 0.44	81.7
Ex + Dil.	83.5 ± 0.33	92.2 ± 0.08	70.4 ± 0.44	87.6

[1] Net absorption (%) per intake. Each value represents the average (± sem) of day-16, 18, 20 and 22. Each value for energy is based on a single determination on a pooled sample of day-16, 18, 20 and 22.

[2] Diet abbreviations: Std. (standard); HF (high-fish meal); HS (high-soybean meal); LEn (low-energy); Co (commercial compressed trout feed); Ex (commercial extruded trout feed); Co + Dil. (compressed feed: Dilution feed, 1:1); Ex + Dil. (extruded feed: Dilution feed, 1:1).

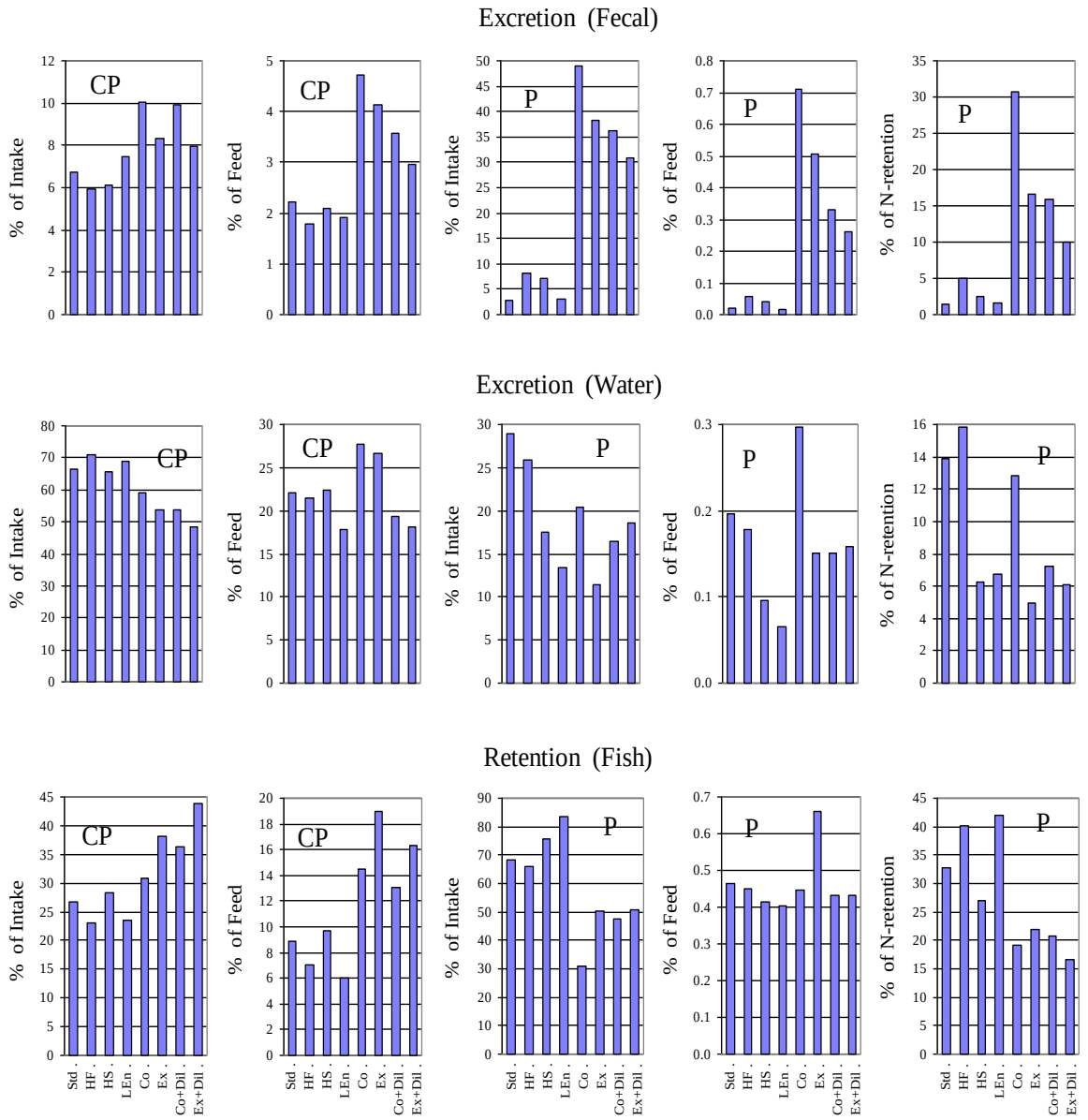


Figure 32. Excretion and retention of crude protein (CP) and phosphorus (P) by fish fed low-pollution or commercial feeds for 22 days (values are the data collected at day-22). Diet abbreviations in the x-axis: Std. (standard feed); HF (high-fish meal feed); HS (high-soybean meal feed); LEn (low-energy feed); Co (commercial compressed trout feed); Ex (commercial extruded trout feed); Co+Dil. (compressed feed 50%, Dilution feed 50%, mixed on an as-is basis); Ex+Dil. (extruded feed 50%, Dilution feed 50%, mixed on an as-is basis). Values for the retention (fish) were estimated by balance; i.e., Retention = amount fed – excretion (fecal) – excretion (water).

Literature Cited

Abbott, J.C., Dill, L.M., 1989. The relative growth of dominant and subordinate juvenile steelhead trout (Salmo gairdneri) fed equal rations. Behaviour, 108(1-2):104-111.

Ackefors, H., Enell, M., 1990. Discharge of nutrients from Swedish fish farming to adjacent sea areas. Ambio, 19(1):28-35.

Ackefors, H., Huner, J.V., Konikoff, M., 1994. Introduction to the General Principles of Aquaculture. Food Products Press. New York. pp.113-126.

Aikawa, J.K., 1976. Biochemistry and physiology of magnesium (Ch.28). In: A.S. Prasad, D. Oberleas (editors), Trace Elements in Human Health and Disease (Vol.2: Essential and Toxic Elements). Academic Press, Inc., New York, NY. pp.47-78.

Alcock, N., MacIntyre, I., 1962. Inter-relation of calcium and magnesium absorption. Clin. Sci., 22:185-193.

Aldor, T.A.M., Moore, E.W., 1970. Magnesium absorption by everted sacs of rat intestine and colon. Gastroenterology, 59:745-753.

Al-Masri, M.R., 1995. Absorption and endogenous excretion of phosphorus in growing broiler chicks, as influenced by calcium and phosphorus ratios in feed. Br. J. Nutr., 74:407-415.

American Public Health Association, American Water Works Association, Water Pollution Control Federation (editors), 1981. Standard Methods for the Examination of Water and Wastewater (15th ed.). American Public Health Association, Washington, DC., pp.417-420.

Anon., 1967. Zinc, calcium, and phytate. Nutr. Rev., 25:215-218.

Anon., 1984. Phosphorus bioavailability in poultry nutrition. Nutr. Rev., 42(11):387-389.

Anon., 1989. Phytase and phytate degradation in humans. Nutr. Rev., 47(5):155-157.

AOAC, 1984. Official Methods of Analysis of the Association of Official Analytical Chemists. Washington, D.C.

AOAC, 1990. Official Methods of Analysis. Association of Official Analytical Chemists, Inc., Arlington, VA, 1298p.

AOAC, 1995. Official Methods of Analysis of the Association of Official Analytical Chemist (16th ed.). AOAC International, Arlington, VA.

APHA (American Public Health Association), American Water Works Association, Water Pollution Control Federation, 1985. Standard Methods for the Examination of Water and Wastewater (16th ed.). American Public Health Association, Washington, DC., 1268p.

APHA (American Public Health Association), American Water Works Association, Water Pollution Control Federation, 1989. Standard Methods for the Examination of Water and Wastewater (17th ed.). APHA, Washington, DC.

Ashmead, H.D., Graff, D.J., Ashmead, H.H., 1985. Intestinal Absorption of Metal Ions and Chelates. C.C. Thomas, Springfield, IL. 251p.

Assous, E.F., Girard, M.L., 1962. Cited In: J. Bartos, M. Pesez (editors), 1976. Colorimetric and Fluorimetric Analysis of Steroids. Academic Press, London. pp.274.

Babbitt, J.K., Hardy, R.W., Reppond, K.D., Scott, T.M., 1994. Processes for improving the quality of whitefish meal. J. Aquat. Food Prod. Technol., 3(3):59-68.

Bales, C.W., Freeland-Graves, J.H., Lin, P-H., Stone, J., Dougherty, V., 1987. Plasma uptake of manganese: response to dose and dietary factors. In: C. Kies (editor), Nutritional Bioavailability of Manganese. American Chemical Society, Washington, DC., pp.112-122.

Barzel, U.S., Jowsey, J., 1969. The effects of chronic acid and alkali administration on bone turnover in adult rats. Clin. Sci., 36:517-524.

Berner, Y.N., Shike, M., 1988. Consequences of phosphate imbalance. Ann. Rev. Nutr., 8:121-148.

Berner, W., Kinne, R., Murer, H., 1976. Phosphate transport into brush-border membrane vesicles isolated from rat small intestine. Biochem. J., 160:467-474.

Bertinchamps, A.J., Miller, S.T., Cotzias, G.C., 1966. Interdependence of routes excreting manganese. Am. J. Physiol., 211:217-224.

Beutler, E, Yeh, M.K.Y., 1959. A simplified method for the determination of citric acid. J. Lab. Clin. Med., 54:125-131.

Beveridge, M.C.M., 1984. Cage and pen fish farming. Carrying capacity models and environmental impact. FAO Fisheries Technical Paper. No. 255. 131p.

Biehl, R.R., Baker, D.H., Deluca, H.F., 1995. 1-alpha-hydroxylated cholecalciferol compounds act additively with microbial phytase to improve phosphorus, zinc and manganese utilization in chicks fed soy-based diets. J. Nutr., 125(9):2407-2416.

Birge, S.J., Avioli, R.C., 1981. Intestinal phosphate transport and alkaline phosphatase activity in the chick. Am. J. Physiol., 204:E384-E390.

Bishop, C, Williams, C.B., 1958. Diminished blood ATP levels induced in the chick by dietary aluminum hydroxide gel. Fed. Proc., 17:15.

Bitar, K., Reinhold, J. G., 1972. Phytase and alkaline phosphatase activities in intestinal mucosa of rat, chicken, calf, and man. Biochim. Biophys. Acta., 268:442-452.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37:911-917.

Bonting, S.L., 1952. The Effect of a Prolonged Intake of Phosphoric Acid and Citric Acid in Rats. Thesis-University of Amsterdam., Netherlands. 97p.

Bour, N.J.S., Soullier, B.A., Zemel, M.B., 1984. Effect of level and form of phosphorus and level of calcium intake on zinc, iron and copper bioavailability in man. Nutr. Res., 4:371-379.

Bowen, S.H., 1987. Dietary protein requirements of fishes -- a reassessment. Can. J. Fish. Aquat. Sci., 44(11):1995-2001.

Boyd, C.E., 1990. Water Quality in Ponds for Aquaculture. Alabama Agricultural Experiment Station, Auburn University, Alabama. 482p.

Brautbar, N., Massry, S.G., 1984. The myopathy of phosphate depletion. Adv. Exp. Med. Biol., 178:363-375.

Brautbar, N., Baczynski, R., Carpenter, C., Massry, S.G., 1982b. Effects of phosphate depletion on the myocardium. Adv. Exp. Med. Biol., 151:199-207.

Brautbar, N, Tabernero-Romo, J., Coats, J.C., Massry, S.G., 1984. Impaired myocardial lipid metabolism in phosphate depletion. Kidney Int., 26:18-23.

Brautbar, N., Carpenter, C., Baczynski, R., Kohan, R., Massry, S.G., 1983. Impaired energy metabolism in skeletal muscle during phosphate depletion. Kidney Int., 24:53-57.

Brautbar, N., Baczynski, R., Carpenter, C., Moser, S., Geiger, P., Finander, P., Massry, S.G., 1982a. Impaired energy metabolism in rat myocardium during phosphate depletion. Am. J. Physiol., 242:F699-704.

Breves, G., Schröder, B., 1991. Comparative aspects of gastrointestinal phosphorus metabolism. Nutr. Res. Rev., 4:125-140.

Broz, J., Oldale, P., Perrin-Voltz, A.H., Rychen, G., Schulze, J., Nunes, C.S., 1994. Effects of supplemental phytase on performance and phosphorus utilization in broiler chickens fed a low phosphorus diet without addition of inorganic phosphates. Br. Poultry Sci., 35:273-280.

Cain, K.D., Garling, D.L., 1995. Pretreatment of soybean meal with phytase for salmonid diets to reduce phosphorus concentrations in hatchery effluents. Prog. Fish-Cult., 57:114-119.

Carroll, H.J., Oh, M.S., 1989. Calcium, phosphate, and magnesium metabolism (Ch.8). In: Water, Electrolyte and Acid-Base Metabolism (2nd ed.). J.B. Lippincott Company, Philadelphia, Pennsylvania. pp.287-348.

Champagne, E.T., 1989. Low gastric hydrochloric acid secretion and mineral bioavailability. In: F.R. Dintzis, J.A. Laszlo (editors), Mineral Absorption in the Monogastric GI Tract. Plenum Press, New York, NY. pp.173-184.

Cheryan, M., 1980. Phytic acid interactions in food systems. Crit. Rev. Food Sci. Nutr., 13:297-335.

Ching, S.V., Fettman, M.J., Hamar, D.W., Nagode, L.A., Smith, K.R., 1989. The effect of chronic dietary acidification using ammonium chloride on acid-base and mineral metabolism in the adult cat. J. Nutr., 119:902-915.

Cho, C.Y., Slinger, S.J., 1979. Apparent digestibility measurement in feedstuffs for rainbow trout. In: J.E. Halver, K. Tiews (editors), Finfish Nutrition and Fishfeed Technology (vol. II). Heenemann GmbH & Co., Berlin. pp.239-247.

Cho, C.Y., Kaushik, S.J., 1985. Effects of protein intake on metabolizable and net energy values of fish diets. In: C.B. Cowey, A.M. Mackie, J.G. Bell (editors), Nutrition and Feeding in Fish. Academic Press, London. pp.95-117.

Cho, C.Y., Woodward, B., 1989. Studies on the protein-to-energy ratio in diets for rainbow trout (Salmo gairdneri). In: Proceedings of the Eleventh Symposium on Energy Metabolism. European Association for Animal Production Publication 43. Wageningen, Netherlands. pp.37-40. (cited in NRC, 1993).

Cho, C.Y., Slinger, S.J., Bayley, H.S., 1976. Influence of level and type of dietary protein, and of level of feeding on feed utilization by rainbow trout. J. Nutr., 106(11):1547-1556.

Cotzias, G.C., Borg, D.C., Selleck, B., 1962. Specificity of zinc pathway through the body: turnover of Zn^{65} in the mouse. Am. J. Physiol., 202:359-363.

Cowey, C.B., 1995. Intermediary metabolism in fish with reference to output of end products of nitrogen and phosphorus. Water Sci. Technol., 31(10):21-28.

Cowey, C.B., Cho, C.Y. (editors), 1991. Nutritional Strategies and Aquaculture Waste. Proceedings of the First International Symposium on Nutritional Strategies in Management of Aquaculture Waste. University of Guelph, Ontario, Canada.

Cowey, C.B., Degener, E., Tacon, A.G., Youngson, A., Bell, J.G., 1984. The effect of vitamin E and oxidized fish oil on the nutrition of rainbow trout (Salmo gairdneri) grown at natural, varying water temperatures. Br. J. Nutr., 51(3):443-451.

Crawford, R.D., 1995. Proposed role for a combination of citric acid and ascorbic acid in the production of dietary iron overload: a fundamental cause of disease. Biochem. Mol. Med., 54:1-11.

Cromwell, G.L., Coffey, R.D., Monegue, H.J., Randolph, J.H., 1995a. Efficacy of low-activity, microbial phytase in improving the bioavailability of phosphorus in corn-soybean meal diets for pigs. J. Animal Sci., 73(2):449-456.

Cromwell, G.L., Stahly, T.S., Coffey, R.D., Monegue, H.J., Randolph, J.H., 1993. Efficacy of phytase in improving the bioavailability of phosphorus in soybean meal and corn-soybean meal diets for pigs. J. Animal Sci., 71:1831-1840.

Cromwell, G.L., Coffey, R.D., Parker, G.R., Monegue, H.J., Randolph, J.H., 1995b. Efficacy of a recombinant-derived phytase in improving the bioavailability of phosphorus in corn-soybean meal diets for pigs. J. Nutr., 73(7):2000-2008.

Cross, H.S., Debiec, H., Peterlik, M., 1990. Mechanism and regulation of intestinal phosphate absorption. Miner. Electrolyte Metab., 16:115-124.

Cruickshank, E.W.H., Duckworth, J., Kosterlitz, H.W., Warnock, G.M., 1945. The digestibility of the phytate-P of oatmeal in adult man. J. Physiol., 104:41-46.

Cuche, J.L., Ott, C.E., Marchand, G.R., Knox, F.G., 1976. Lack of effect of hypocalcemia on renal phosphate handling. J. Lab. Clin. Med., 88:271-275.

Cummings, M.J., Mattill, H.A., 1931. The auto-oxidation of fats with reference to their destructive effect on vitamin E. J. Nutr., 3(4):421-432.

Cuzon, G., Hew, M., Cognie, D., Soletchnik, P., 1982. Time lag effect of feeding on growth of juvenile shrimp, Penaeus japonicus Bate. Aquaculture, 29(1-2):33-44.

Dabrowski, K., Leray, C., Nonnotte, G., Colin, D.A., 1986. Protein digestion and ion concentrations in rainbow trout (Salmo gairdnerii Rich.) digestive tract in sea- and fresh water. Comp. Biochem. Physiol., 83A:27-39.

Dabrowski, K.R., Schwartz, F.J., 1986. Minerals content in the digestive tract of stomachless fish. Zool. Jb. Physiol., 90:193-200.

Danisi, G., Murer, H., 1991. Inorganic phosphate absorption in small intestine. In: Handbook of Physiology, The Gastrointestinal System (vol.4). American Physiological Society, Bethesda, MD., pp.323-336.

Danisi, G., Murer, H., Straub, R.W., 1984. Effect of pH on phosphate transport into intestinal brush-border membrane vesicles. Am. J. Physiol., 246:G180-G186.

Davidsson, L., Cederblad, A., Lonnerdal, B., Sandstrom, B., 1991. The effect of individual dietary components on manganese absorption in humans. Am. J. Clin. Nutr., 54:1065-1070.

Davies, M.I., Ritcey, G.M., Motzok, I., 1970. Intestinal phytase and alkaline phosphatase of chicks: Influence of dietary calcium, inorganic and phytate phosphorus and vitamin D3. Poultry Sci., 49:1280-1286.

Davis, G.K., Mertz, W., 1987. Copper (Ch.10). In: W. Mertz (editor), Trace Elements in Human and Animal Nutrition (5th ed., vol.1). Academic Press, Inc., San Diego, CA. pp.301-364.

Davis, J.L., Lewis, S.B., Schultz, T.A., Kaplan, R.A., Wallin, J.D., 1979. Acute and chronic phosphate depletion as a modulator of glucose uptake in rat skeletal muscle. Life Sci., 24:629-632.

Day, H.G., McCollum, E.V., 1939. Mineral metabolism, growth and symptomatology of rats on diet extremely deficient in phosphorus. J. Biol. Chem., 130:269-283.

De Boland, A.R., Garner, G.B., O'Dell, B.L., 1975. Identification and properties of "phytate" in cereal grains and oilseed products. J. Agric. Food Chem., 23:1186-1189.

De Leacy, E.A., Cowley, D.M., Brown, J.M., McWhinney, B.C., Chalmers, A.H., 1989. Effect of oral citrate on calcium absorption after an oral load of calcium phosphate. Clin. Chem., 35:1541(36:1530).

De Silva, S.S., Deng, D.F., Rajendram, V., 1997. Digestibility in goldfish fed diets with and without chromic oxide and exposed to sublethal concentrations of cadmium. Aquacult. Nutr., 3:109-114.

DeFronzo, R.A., Lang, R., 1980. Hypophosphatemia and glucose intolerance: evidence for tissue insensitivity to insulin. N. Engl. J. Med., 303:1259-1263.

Dempsey, E.F., Carroll, E.L., Albright, F., Henneman, F.H., 1958. A study of factors determining fecal electrolyte excretion. Metabolism, 7:108-118.

Denbow, D.M., Ravindran, V., Kornegay, E.T., Yi, Z., Hulet, R.M., 1995. Improving phosphorus availability in soybean meal for broilers by supplemental phytase. Poultry Sci., 74(11):1831-1842.

Dennis, V.W., 1992. Phosphate homeostasis(Ch.37). In: Handbook of Physiology (Section 8): Renal Physiology (vol. II), (ed. by E.E. Windhager). American Physiological Society, Oxford University Press, New York, USA. pp.1785-1815.

Desjardins, I.M., Hicks, B.D., Hilton, J.W., 1987. Iron catalyzed oxidation of trout diets and its effect on the growth and physiological response of rainbow trout. Fish Physiol. Biochem., 3(4):173-182.

Donaldson, W.E., 1979. Regulation of fatty acid synthesis. Fed. Proc., 38:2617-2621.

Edwards, H.E., Jr., 1993. Dietary 1 25 dihydroxycholecalciferol supplementation increases natural phytate phosphorus utilization in chickens. J. Nutr., 123(3):567-577.

Engelen, A.J., Van Der Heeft, F.C., Randsdorp, P.H.G., Smit, E.L.C., 1994. Simple and rapid determination of phytase activity. J. AOAC Int., 77:760-764.

Ertl, D.S., Young, K.A., Raboy, V., 1998. Plant genetic approaches to phosphorus management in agricultural production. J. Envir. Quality, 27(2):299-304.

Eya, J.C., Lovell, R.T., 1997a. Available phosphorus requirements of food-size channel catfish (Ictalurus punctatus) fed practical diets in ponds. Aquaculture, 154(3-4):283-291.

Eya, J.C., Lovell, R.T., 1997b. Net absorption of dietary phosphorus from various inorganic sources and effect of fungal phytase on net absorption of plant phosphorus by channel catfish Ictalurus punctatus. J. World Aquacult. Soc., 28(4):386-391.

Fairgrieve, W.T., Myers, M.S., Hardy, R.W., Dong, F.M., 1994. Gastric abnormalities in rainbow trout (Oncorhynchus mykiss) fed amine-supplemented diets or chicken gizzard-erosion-positive fish meal. Aquaculture, 127:219-232.

Falls, W.W., 1980. Economical, automatic, dry-food fish feeder. Prog. Fish-Cult., 42:240.

FAO. 1992. Aquaculture Production (1984-1990). FAO Fisheries Circular No. 815, Revision 4. FAO, Rome, Italy.

FAO. 1993. Fishery Statistics- Catches and Landings. FAO Yearbook Vol. 76. FAO, Rome, Italy.

FAO, 1996. Fishery Statistics. FAO Yearbook 1994, Vol. 79. FAO, Rome, Italy.

FAO, 1997. The State of World Fisheries and Aquaculture, 1996. FAO fisheries department. FAO, Rome, Italy. 125p.

Feldman, M., Walsh, J.H., Wong, H.C., Richardson, C.T., 1978. Role of gastrin heptadecapeptide in the acid secretory response to amino acids in man. J. Clin. Invest., 61:308-313.

Forbes, E.B., Keith, M.H., 1914. Phosphorus compounds in animal metabolism. Ohio Agric. Exp. Stn. Tech. Ser. Bull., 5:1-748.

Fox, J., Care, A.D., 1978a. Effect of low calcium and low phosphorus diets on the intestinal absorption of phosphate in intact and parathyroidectomized pigs. J. Endocrinol., 77:225-231.

Fox, J., Care, A.D., 1978b. Stimulation of duodenal and ileal absorption of phosphate in the chick by low-calcium and low-phosphorus diets. Calcif. Tissue Res., 26:243-245.

Foy, R.H., Rosell, R., 1991. Fractionation of phosphorus and nitrogen loadings from a Northern Ireland fish farm. Aquaculture, 96(1):31-42.

Furia, T.E., 1972. Sequestrants in food. In: T.E. Furia, (editor), Handbook of Food Additives (2nd ed.). CRC Press, Cleveland, OH. pp.271-294.

Furukawa, A., Tsukahara, H., 1966. On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feed. Bull. Jpn. Soc. Sci. Fish., 32:502-506.

Gatlin, III, D.M., Wilson, R.P., 1984. Zinc supplementation of practical channel catfish diets. Aquaculture, 41:31-36.

Gentesse, N., Lefrancois, M.R., Bernier, J.F., 1993. Dietary addition of citric acid and carcass fat deposition in broilers (Abstract). Poultry Sci., 72(suppl. 1):34.

Gentesse, N., Bernier, J.F., LeFrancois, M.R., 1994. Effect of dietary citric acid on diet metabolizability, blood acid-base balance and abdominal fat deposition in broilers (Abstract). Poultry Sci., 73(suppl.1):34.

Gibson, R.S., 1994. Content and bioavailability of trace elements in vegetarian diets. Am. J. Clin. Nutr., 59:1223s-1232s.

Golterman, H.L., 1970. Methods for Chemical Analysis of Fresh Waters (IBP handbook 8). Blackwell Scientific, Oxford. 172p.

Goodridge, A.G., 1973. Regulation of fatty acid synthesis in isolated hepatocytes. Evidence for a physiological role for long chain fatty acyl coenzyme A and citrate. J. Biol. Chem., 248:4318-4326.

Graf, E., 1986. Phytic Acid: chemistry & applications. Pilatus Press, Minneapolis. 344p.

Greger, J.L., Snedecor, S.M., 1980. Effect of dietary protein and phosphorus levels on the utilization of zinc, copper and manganese by adult males. J. Nutr., 110:2243-2253.

Grove, D.J., Loizides, L.G., Nott, J., 1978. Satiation amount, frequency of feeding and gastric emptying rate in Salmo gairdneri. J. Fish. Biol., 12(5):507-516.

Grover, J.H., 1996. Aquacultural research needs. In: R.A. Neal (editor), International Development: proceedings of the World Fisheries Congress, Theme 4 (World Fisheries Congress, 1992, Athens, Greece). Science Publishers, Inc., Lebanon, NH. USA. pp.58-61.

Grynspan, F., Cheryan, M., 1983. Calcium phytate: Effect of pH and molar ratio on in vitro solubility. JAOCS, 60:1761-1764.

Hafez, Y.S., Mohammed, A.I., Perera, P.A., Singh, G., Hussein, A.S., 1989. Effects of microwave heating and gamma irradiation on phytate and phospholipid contents of soybean (Glycine max. L.). J. Food Sci., 54:958-962.

Hajen, W.E., Beames, R.M., Higgs, D.A., Dosanjh, B.S., 1993. Digestibility of various feedstuffs by post-juvenile chinook salmon (Oncorhynchus tshawytscha) in sea water. 1. Validation of technique. Aquaculture, 112(4):321-332.

Halver, J.E., 1972. The vitamins. In: J.E. Halver (editor), Fish Nutrition. Academic Press, New York. pp.29-103.

Hambidge, K.M., Casey, C.E., Krebs, N.F., 1986. Zinc (Ch.1). In: W. Mertz (editor), Trace Elements in Human and Animal Nutrition (5th ed., vol. 2). Academic Press, Inc., Orlando, FL. pp.1-137.

Han, Y.M., Yang, F., Zhou, A.G., Miller, E.R., Ku, P.K., Hogberg, M.G., Lei, X.G., 1997. Supplemental phytases of microbial and cereal sources improve dietary phytate phosphorus utilization by pigs from weaning through finishing. J. Animal Sci., 75(4):1017-1025.

Hardy, R.W., 1995. Current issues in salmonid nutrition. In: C. Lim, D.J. Sessa (editors), Nutrition and Utilization Technology in Aquaculture. AOCS Press, Campaign, Illinois. pp.26-35.

Hardy, R.W., Shearer, K.D., 1985. Effect of dietary calcium phosphate and zinc supplementation on whole body zinc concentration of rainbow trout (Salmo gairdneri). Can. J. Fish. Aquat. Sci., 42:181-184.

Hardy, R.W., Sullivan, C.V., Koziol, A.M., 1987. Absorption, body distribution, and excretion of dietary zinc by rainbow trout (Salmo gairdneri). Fish Physiol. Biochem., 3:133-143.

Hardy, R.W., Fairgrieve, W.T., Scott, T.M., 1993. Periodic feeding of low-phosphorus diet and phosphorus retention in rainbow trout (Oncorhynchus mykiss). In: S.J. Kanshik, P. Luquet (editors), Fish Nutrition in Practice. 4th Int. Symp. Fish Nutrition and Feeding, Biarritz (France), 24-27 June 1991, Colloq.INRA no.61, pp.403-412.

Harland, B.F., Oberleas, D., 1986. Anion-exchange method for determination of phytate in foods: Collaborative study. J. AOAC., 69:667-670.

Harper, A.F., Kornegay, E.T., Schell, T.C., 1997. Phytase supplementation of low-phosphorus growing- finishing pig diets improves performance, phosphorus digestibility, and bone mineralization and reduces phosphorus excretion. J. Animal Sci., 75(12):3174-3186.

Harris, R.C., Hultman, E., 1992. Muscle phosphagen status studied by needle biopsy. In: J.M. Kinney, H.N. Tucker (editors), Energy Metabolism: Tissue determinants and cellular corollaries. Raven Press, New York. pp.367-379.

Harrison, D.C., Mellanby, E., 1939. Phytic acid and the rickets-producing action of cereals. Biochem. J., 33:1660-1680.

Harrison, H.E., Harrison, H.C., 1961. Intestinal transport of phosphate: action of vitamin D, calcium, and potassium. Am. J. Physiol., 201:1007-1012.

Hart, E.B., McCollum, E.V., Fuller, J.G., 1909. The rôle of inorganic phosphorus in the nutrition of animals. Am. J. Physiol., 23:246-277.

Harter, H.R., Santiago, J.V., Rutherford, W.E., Slatopolsky, E., Klahr, S., 1976. The relative roles of calcium, phosphorus, and parathyroid hormone in glucose- and tolbutamide-mediated insulin release. J. Clin. Invest., 58:359-367.

Hashimoto, Y., Okaichi, T., Watanabe, T., Furukawa, A., 1966. Muscular dystrophy of carp due to oxidized oil and the preventive effect of vitamin E. Bull. Jpn. Soc. Sci. Fish., 32:64-69.

He, E., Wurtsbaugh, W.A., 1993. An empirical model of gastric evacuation rates for fish and an analysis of digestion in piscivorous brown trout. Trans. Am. Fish. Soc., 122:717-730.

Heaton, F.W., Hodgkinson, A., Rose, G.A., 1964. Observations on the relation between calcium and magnesium metabolism in man. Clin. Sci., 27:31-40.

Hegsted, D.M., 1976. Balance studies. J. Nutr., 106:307-311.

Hegsted, D.M., Finch, C.A., Kinney, T.D., 1949. The influence of diet on iron absorption II. The interrelation of iron and phosphorus. J. Exp. Med., 90:147-156.

Helander, H.F., Keeling, D.J., 1993. Cell biology of gastric acid secretion. Baillieres Clin. Gastroenterol., 7:1-21.

Heth, D.A., Becker, W.M., Hoekstra, W.G., 1966. Effect of calcium, phosphorus and zinc on zinc-65 absorption and turnover in rats fed semipurified diets. J. Nutr., 88:331-337.

Hettleman, B.D., Sabina, R.L., Drezner, M.K., Holmes, E.W., Swain, J.L., 1983. Defective adenosine triphosphate synthesis. An explanation for skeletal muscle dysfunction in phosphate-deficient mice. J. Clin. Invest., 72:582-589.

Hildmann, B., Storelli, C., Danisi, G., Murer, H., 1982. Regulation of Na+-Pi cotransport by 1,25-dihydroxyvitamin D3 in rabbit duodenal brush-border membrane. Am. J. Physiol., 242:G533-G539.

Hilton, J.W., 1989. The interaction of vitamins, minerals and diet composition in the diet of fish. Aquaculture, 79:223-244.

Hilton, J.W., Cho, C.Y., Slinger, S.J., 1981. Effect of extrusion processing and steam pelleting diets on pellet durability, pellet water absorption, and the physiological response of rainbow trout (Salmo gairdneri R.). Aquaculture, 25(2-3):185-194.

Hilton, J.W., Atkinson, J.L., Slinger, S.J., 1983. Effect of increased dietary fiber on the growth of rainbow trout (Salmo gairdneri). Can. J. Fish. Aquat. Sci., 40(1):81-85.

Hiramatsu, Y., Okabe, S., 1994. Effect of intraduodenally administered histamine on gastric acid secretion in rats and guinea pigs. Dig. Dis. Sci., 39:689-697.

Hodgson, S.F., Hurley, D.L., 1993. Acquired hypophosphatemia. Endocrinol. Metab. Clin. North Am., 22:397-409.

Hoek, A.C., Lemmens, A.G., Mullink, J.W., Beynen, A.C., 1988. Influence of dietary calcium: phosphorus ratio on mineral excretion and nephrocalcinosis in female rats. J. Nutr., 118:1210-1216.

Hoff-Jorgensen, E., 1946. The influence of phytic acid on the absorption of calcium and phosphorus. 1. In dogs. Biochem. J., 40:189-192.

Holby, O., Hall, P.O.J., 1991. Chemical fluxes and mass balances in a marine fish cage farm. 2. Phosphorus. Mar. Ecol. Prog. Ser., 70(3):263-272.

Holstein, B., 1975. Gastric acid secretion in a teleostean fish: a method for the continous collection of gastric effluence from a swimming fish and its response to histamine and pentagastrin. Acta Physiol. Scand., 95:417-423.

Holstein, B., 1976. Effect of the H2-receptor antagonist metiamide on carbachol- and histamine-induced gastric acid secretion in the Atlantic cod, Gadus morhua. Acta Physiol. Scand., 97:189-195.

Hommes, F.A., 1980. The energy requirement for growth. A reevaluation. Nutr. Metab., 24:110-113.

Horl, W.H., Kreusser, W., Rambausek, M., Heidland, A., Ritz, E., 1983. Glycogen metabolism in phosphorus-depleted rats. Miner. Electrolyte Metab., 9:113-118.

Horrobin, D.F., 1990. The philosophical basis of peer review and the suppression of innovation. JAMA, 263(10):1438-1441.

Hung, S.S., Cho, C.Y., Slinger, S.J., 1981. Effect of oxidized fish oil, DL-alpha-tocopheryl acetate and ethoxyquin supplementation on the vitamin E nutrition of rainbow trout (Salmo gairdneri) fed practical diets. J-Nutr., 111(4):648-657.

Hunt, J.N., Knox, M.T., 1973. The effect of citric acid and its sodium salts in test meals on the gastric outputs of acid and of chloride. J. Physiol., 230:171-184.

Hurley, L.S., Keen, C.L., 1987. Manganese (Ch.6). In: W. Mertz (editor), Trace Elements in Human and Animal Nutrition (5th ed., vol. 1). Academic Press, Inc., San Diego, CA, pp.185-223.

Hurwitz, S., Bar, A., 1965. Absorption of calcium and phosphorus along the gastrointestinal tract of the laying fowl as influenced by dietary calcium and egg shell formation. J. Nutr., 86:433-438.

Hurwitz, S., Bar, A., 1966. Rate of passage of calcium-45 and yttrium-91 along the intestine, and calcium absorption in the laying fowl. J. Nutr., 89:311-316.

Hyvarinen, A, Nikkila, E.A., 1962. Specific determination of blood glucose with o-toluidine. Clin. Chim. Acta, 7:140-143.

IOM (Institute of Medicine), 1997. Dietary Reference Intakes: Calcium, Magnesium, Phosphorus, Vitamin D, and Fluoride. National Academy Press, Food and Nutrition Board. Washington, DC., 250p.

Irving, J.T., 1973. Intestinal absorption of calcium and phosphorus (Ch.3). In: Calcium and Phosphorus Metabolism. Academic Press, Inc., New York, NY. pp.21-28.

Iwama, G.K., 1996. Growth of salmonids (Ch.7). In: W. Pennell, B.A. Barton (editors), Principles of Salmonid Culture. Elsevier, New York.

Jackson, L.S., Li, M.H., Robinson, E.H., 1996. Use of microbial phytase in channel catfish Ictalurus punctatus diets to improve utilization of phytate phosphorus. J. World Aquac. Soc., 27:309-313.

Jackson, M.J., Jones, D.A., Edwards, R.H.T., Swainbank, I.G., Coleman, M.L., 1984. Zinc homeostasis in man: Studies using a new stable isotope-dilution technique. Br. J. Nutr., 51:199-208.

Jobling, M., 1985. Growth. In: P.Tytler, P.Calow (editors), Fish Energetics. Johns Hopkins University Press, Baltimore, Maryland. pp.213-230.

Johnson, M.A., 1986. Interaction of dietary carbohydrate, ascorbic acid and copper with the development of copper deficiency in rats. J. Nutr., 116:802-815.

Jongbloed, A.W., Mroz, Z., Kemme, P.A., 1992. The effect of supplementary Aspergillus niger phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. J. Animal Sci., 70:1159-1168.

Karl, D.M., Tien, G., 1992. MAGIC: A sensitive and precise method for measuring dissolved phosphorus in aquatic environments. Limnol. Oceangr., 37:105-116.

Kates, M., 1986. Techniques of Lipidology (2nd ed.). Elsevier Science Publishers, Amsterdam, The Netherlands. 451p.

Kaufman, H.W., 1986. Interactions of inositol phosphates with mineralized tissues. In: E. Graf (editor), Phytic Acid: Chemistry and applications. Pilatus Press. Minneapolis. pp.303-320.

Kaushik, S.J., de-Oliva-Teles, A., 1985. Effect of digestible energy on nitrogen and energy balance in rainbow trout. Aquaculture, 50(1-2):89-101.

Kaushik, S.J., Medale, F., Fauconneau, B., Blanc, D., 1989. Effect of digestible carbohydrates on protein/energy utilization and on glucose metabolism in rainbow trout (Salmo gairdneri R.). Aquaculture, 79(1-4):63-74.

Kempson, S., Kim, J., Northrup, T., Know, F., Dousa, T., 1979. Alkaline phosphatase in adaptation to low dietary phosphate intake. Am. J. Physiol., 237:E465-E473.

Ketaren, P.P., Batterham, E.S., Dettmann, E.B., Farrell, D.J., 1993. Phosphorus studies in pigs 3. Effect of phytase supplementation on the digestibility and availability of phosphorus in soya-bean meal for grower pigs. Br. J. Nutr., 70(1):289-311.

Ketola, H.G., 1979. Influence of dietary zinc on cataracts in rainbow trout (Salmo gairdneri). J. Nutr., 109:965-969.

Ketola, H.G., 1982. Effect of phosphorus in trout diets on water pollution. Salmonid, 6(2):12-15.

Ketola, H.G., 1985. Mineral nutrition: Effects of phosphorus in trout and salmon feeds on water pollution. In: C.B. Cowey, A.M. Mackie, J.G. Bell, (editors), Nutrition and Feeding in Fish. Academic Press, London. pp.465-473.

Ketola, H.G., Harland, B.F., 1993. Influence of phosphorus in rainbow trout diets on phosphorus discharges in effluent water. Trans. Am. Fish. Soc., 122:1120-1126.

Ketola, H.G., Richmond, M.E., 1994. Requirement of rainbow trout for dietary phosphorus and its relationship to the amount discharged in hatchery effluent. Trans, Am. Fish. Soc., 123:587-594.

Kim, J.D., Kaushik, S.J., 1992. Contribution of digestible energy from carbohydrates and estimation of protein/energy requirements for growth of rainbow trout (Oncorhynchus mykiss). Aquaculture, 106(2):161-169.

Kirchgessner, M., Weigand, E., 1983. Zinc absorption and excretion in relation to nutrition. Metal Ions in Biological Systems, 15:319-361.

Kleiber, M., Goss, H., Guilbert, H.R., 1936. Phosphorus deficiency metabolism and food utilization in beef heifers. J. Nutr., 12:121-153.

Knochel, J.P., 1977. The pathophysiology and clinical characteristics of severe hypophosphatemia. Arch. Intern. Med., 137:203-220.

Knochel, J.P., 1981. Hypophosphatemia. West. J. Med., 134:15-26.

Knochel, J.P., Haller, R., Ferguson, E., 1980. Selective phosphorus deficiency in the hyperalimented hypophosphatemic dog and phosphorylation potentials in the muscle cell. Adv. Exp. Med. Biol., 128:323-334.

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

Kornegay, E.T., Qian, H., 1996. Replacement of inorganic phosphorus by microbial phytase for young pigs fed on a maize-soyabean-meal diet. Br. J. Nutr., 76(4):563-578.

Kornegay, E.T., Denbow, D.M., Yi, Z., Ravindran, V., 1996. Response of broilers to graded levels of microbial phytase added to maize-soyabean-meal-based diets containing three levels of non-phytate phosphorus. Brit. J. Nutr., 75(6):839-852.

Kossmann, H., 1990. Present status and problems of aquaculture in the Nordic countries with special reference to fish feed. In: M. Takeda, T. Watanabe (editors), The Current Status of Fish Nutrition in Aquaculture. Proceedings of the Third International Symposium on Feeding and Nutrition in Fish, 28 August-1 September 1989, Toba, Japan. pp.27-39.

Kreisberg, R.A., 1977. Phosphorus deficiency and hypophosphatemia. Hosp. Pract., 12:121-128.

Kreusser, W.J., Kurokawa, K., Azner, E., Massry, S.G., 1978. Phosphate depletion: effect on inorganic phosphorus and adenine nucleotides, urinary phosphate and calcium balance. Miner. Electrolyte Metab., 1:30-42.

Kreusser, W., Haberbosch, W., Gnasso, A., Ritz, E., Augustin, J., 1984. Hyperlipoproteinemia in phosphate depletion. Adv. Exp. Med. Biol., 178:427-433.

Krieger, C.H., Steenbock, H., 1940. Cereals and rickets. XII. The effect of calcium and vitamin D on the availability of phosphorus. J. Nutr., 20:125-132.

Lall, S.P., 1989. The minerals (Ch.5). In: J.E. Halver (editor), Fish Nutrition (2nd ed.). Academic Press, San Diego, CA. pp.219-257.

Lall, S.P., 1991. Digestibility, metabolism and excretion of dietary phosphorus in fish. In: C.B. Cowey, C.Y. Cho (editors), Nutritional Strategies and Aquaculture Waste. Proceedings of the First International Symposium on Nutritional Strategies in Management of Aquaculture Waste. University of Guelph, Guelph, Ontario, Canada, pp.21-36.

Lanari, D., D'-Agaro, E.D., Ballestrazzi, R., 1995. Effect of dietary DP/DE ratio on apparent digestibility, growth and nitrogen and phosphorus retention in rainbow trout, Oncorhynchus mykiss (Walbaum). Aquacult. Nutr., 1(2):105-110.

Langhans, W., Damaske, U., Scharrer, E., 1985. Different metabolites might reduce food intake by the mitochondrial generation of reducing equivalents. Appetite, 6:143-152.

Lasztity, R., Lasztity, L., 1990. Phytic acid in cereal technology (Ch.5). In: Y. Pomerantz (editor), Advances in Cereal Science and Technology (vol.X). American Association of Cereal Chemists, Incorporated. St. Paul, Minnesota, USA. pp.309-371.

Latta, M., Eskin, M., 1980. A simple and rapid colorimetric method for phytate determination. J. Agric. Food Chem., 28:1313-1315.

Lee, D.B., Walling, M.W., Corry, D.B., 1986. Phosphate transport across rat jejunum: influence of sodium, pH, and 1,25-dihydroxyvitamin D3. Am. J. Physiol., 251:G90-G95.

Lee, D.B.N., Brautbar, N, Kleeman, C.R., 1981. Disorders of phosphorus metabolism (Ch.6). In: F. Bronner, J.W. Coburn (editors), Disorders of Mineral Metabolism (vol. III), Academic Press, New York. pp.283-421.

Lee, D.J., Putnam, G.B., 1973. The response of rainbow trout of varying protein-energy ratios in a test diet. J. Nutr., 103(6):916-922.

Lei, X., Ku, P.K., Miller, E.R., Ullrey, D.E., Yokoyama, M.T., 1993. Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. J. Nutr., 123(6):1117-1123.

Lei, X.G., Ku, P.K., Miller, E.R., Yokoyama, M.T., Ullrey, D.E., 1994. Calcium level affects the efficacy of supplemental microbial phytase in corn-soybean meal diets of weanling pigs. J. Animal Sci., 72(1):139-143.

Lemann, J., Jr., Litzow, J.R., Lennon, E.J., 1966. The effects of chronic acid loads in normal man: Further evidence for the participation of bone mineral in the defense against chronic metabolic acidosis. J. Clin. Invest., 45:1608-1614.

Lemann, J., Jr., Litzow, J.R., Lennon, E.J., 1967. Studies of the mechanisms by which chronic metabolic acidosis augments calcium excretion in man. J. Clin. Invest., 46:1318-1328.

Lemann, J., Jr., Lennon, E.J., Goodman, A.D., Litzow, J.R., Relman, A.S., 1965. Net balance of acid in subjects given large loads of acid or alkali. J. Clin. Invest., 44:507-517.

Lessard, P., Lefrancois, M.R., Bernier, J.F., 1993. Dietary addition of cellular metabolic intermediates and carcass fat deposition in broilers. Poultry Sci., 72:535-545.

Li, M.H., Robinson, E.H., 1997. Microbial phytase can replace inorganic phosphorus supplements in channel catfish Ictalurus punctatus diets. J. World Aquacult. Soc., 28(4):402-406.

Lichtman, M.A., Miller, D.R., Freeman, R.B., 1969. Erythrocyte adenosine triphosphate depletion during hypophosphatemia in a uremic subject. N. Engl. J. Med., 280:240-244.

Lichtman, M.A., Millaer, D.R., Cohen, J., Waterhouse, C., 1971. Reduced red cell glycolysis, 2,3-diphosphoglycerate and adenosine triphosphate concentration, and increased hemoglobin-oxygen affinity caused by hypophosphatemia. Ann. Int. Med., 74:562-568.

Linder, M.C., 1991. Energy metabolism, intake, and expenditure (Ch.8). In: M.C. Linder (editor), Nutritional Biochemistry and Metabolism (2nd ed.), Elsevier, New York. pp.277-304.

Liu, J., Bollinger, D.W., Ledoux, D.R., Ellersieck, M.R., Veum, T.L., 1997. Soaking increases the efficacy of supplemental microbial phytase in a low-phosphorus corn-soybean meal diet for growing pigs. J. Animal Sci., 75(5):1292-1298.

Lonnerdal, B., 1989. Dietary factors affecting trace element absorption in infants. Acta Paediatr. Scand., suppl. 351:109-113.

Lotz, M., Zisman, E., Bartter, F.C., 1968. Evidence for a phosphorus-depletion syndrome in man. N. Engl. J. Med., 278:409-415.

Lovell, R.T., 1979. Fish culture in the United States. Science, 206(4425):1368-1372.

Lowe, J.T., Steenbock, H., 1936. Cereals and rickets. VIII. The hydrolysis of phytin in the intestine. Biochem. J., 30:1991-1995.

Lyon, D.B., 1984. Studies on the solubility of Ca, Mg, Zn, and Cu in cereal products. Am. J. Clin. Nutr., 39:190-195.

Mäkinen, T., 1993. Effect of feeding schedule on growth of rainbow trout. Aquaculture Int., 1(2):124-136.

Marcus, C.S., Lengemann, F.W., 1962. Use of radioyttrium to study food movement in the small intestine of the rat. J. Nutr., 76:179-182.

Marshall, W.P., Banasiak, M.F., Kalkhoff, R.K., 1978. Effects of phosphate deprivation on carbohydrate metabolism. Horm. Metab. Res., 10:369-373.

McAleese, D.M., Forbes, R.M., 1961. The requirement and tissue distribution of magnesium in the rat as influenced by environmental temperature and dietary calcium. J. Nutr., 73:94-106.

McCance, R.A., Widdowson, E.M., 1942a. Mineral metabolism of healthy adults on white and brown bread dietaries. J. Physiol., 101:44-85.

McCance, R.A., Widdowson, E.M., 1942b. Mineral metabolism on dephytinized bread. J. Physiol., 101:304-313.

McCann, J.P., Loo, S.C., Aalseth, D.L., Abribat, T., 1997. Differential effects of GH stimulation on fasting and prandial metabolism and plasma IGFs and IGF-binding proteins in lean and obese sheep. J. Endocrinol., 154:329-346.

McCartney, T.H., 1969a. The effect of dietary inorganic phosphate and vitamin D supplementation on the liver glycogen of fingerling brown trout. Fisheries Research Bulletin, 31:5-8.

McCartney, T.H., 1969b. The effect of dietary carbohydrate level and supplemental phosphorus on the liver glycogen of fingerling brown trout. Fisheries Research Bulletin, 32:26-31.

McCay, C.M., 1927. Goals in nutrition. Trans. Am. Fish. Soc., 57:261-265.

McCollum, E.V., 1957. A History of Nutrition; the sequence of ideas in nutrition investigations. Houghton Mifflin, Boston. 451p.

McCollum, E.V., Orent-Keiles, E., Day, H.G., 1939. The Newer Knowledge of Nutrition, 5th ed. (Ch. XV). Macmillan Company, New York. pp.363-397.

McGeachy, S.A., O'-Flynn, F.M., Benfey, T.J., Friars, G.W., 1996. Seawater performance of triploid Atlantic salmon in New Brunswick aquaculture. Bull. Aquacult. Assoc. Can., 96(2):24-28.

Medale, F., Brauge, C., Vallee, F., Kaushik, S.J., 1995. Effects of dietary protein/energy ratio, ration size, dietary energy source and water temperature on nitrogen excretion in rainbow trout. Water Sci. Technol., 31(10):185-194.

Mellanby, E., 1919. An experimental investigation on rickets. Lancet, 1:407-412.

Mellanby, E., 1949. The rickets-producing and anticalcifying action of phytate. J. Physiol., 109:488-533.

Mellanby, E., 1950. A Story of Nutritional Research; the effect of some dietary factors on bones and the nervous system (Ch.16, Phytate and Ca absorption in man). Williams & Wilkins, Baltimore. pp.395-410.

Mertz, W., 1987. Use and misuse of balance studies. J. Nutr., 117:1811-1813.

Miller, W.E., Maloney, T.E., Greene, J.C., 1974. Algal productivity in 49 lake waters as determined by algal assays. Water Res., 8:667-679.

Misra, D.N., 1996. Interaction of citric acid with hydroxyapatite: surface exchange of ions and precipitation of calcium citrate. J. Dent. Res., 75(6):1418-1425.

Mitchell, R.D., Edwards, H.M., 1996a. Effects of phytase and 1,25-dihydroxycholecalciferol on phytate utilization and the quantitative requirement for calcium and phosphorus in young broiler chickens. Poultry Sci., 75(1):95-110.

Mitchell, R.D., Edwards, H.M., 1996b. Additive effects of 1,25-dihydroxycholecalciferol and phytase on phytate phosphorus utilization and related parameters in broiler chickens. Poultry Sci., 75(1):111-119.

Møllgaard, H., 1946. On phytic acid, its importance in metabolism and its enzymatic cleavage in bread supplemented with calcium. Biochem. J., 40:589-603.

Mommsen, T.P., 1998. Growth and metabolism. In: D.H. Evans (editor), The Physiology of Fishes (2nd ed.), CRC Press, Boca Raton, FL. pp.65-97.

Monsen, E.R., 1988. Iron nutrition and absorption: dietary factors which impact iron bioavailability. J. Am. Diet. Assoc., 88:786-790.

Morris, E.R., 1986. Phytate and dietary mineral bioavailability. In: E. Graf (editor), Phytic Acid: Chemistry and applications. Pilatus Press. Minneapolis. pp.57-76.

Morris, E.R., 1987. Iron (Ch.4). In: W. Mertz (editor), Trace Elements in Human and Animal Nutrition (5th ed., vol. 1). Academic Press, Inc., San Diego, CA. pp.79-142.

Morris, S., Ray, S.C., 1939. The effect of a phosphorus deficiency on the protein and mineral metabolism of sheep. Biochem. J., 33(8):1209-1216.

Moser, S., Brautbar, N., Gura, V., Kleeman, C.R., Massry, S.G., 1981. On the mechanism of hypophosphaturia during acute and chronic dietary phosphate restriction. Mineral Electrolyte Metab., 5:39-48.

Motulsky, H., 1995. Intuitive Biostatistics. Oxford University Press, New York.

Mroz, Z., Jongbloed, A.W., Kemme, P.A., 1994. Apparent digestibility and retention of nutrients bound to phytate complexes as influenced by microbial phytase and feeding regimen in pigs. J. Animal Sci., 72(1):126-132.

Murai, T., Andrews, J.W., 1975. Pantothenic acid supplementation of diets for catfish fry. Trans. Am. Fish. Soc., 104(2):313-316.

Murry, A.C., Lewis, R.D., Amos, H.E., 1997. The effect of microbial phytase in a pearl millet-soybean meal diet on apparent digestibility and retention of nutrients, serum mineral concentration, and bone mineral density of nursery pigs. J. Animal Sci., 75(5):1284-1291.

Nakamura, Y., 1982. Effects of dietary phosphorus and calcium contents on the absorption of phosphorus in the digestive tract of carp. Bull. Jpn. Soc. Sci. Fish., 48:409-413.

Nakamura, Y., 1985. In vitro absorption of inorganic phosphate and other electrolytes in the carp intestine. Comp. Biochem. Physiol., 80A:17-20.

Napoli, R., Horton, E.S., 1996. Energy requirements (Ch.1). In: E.E. Ziegler, L.J. Filer, Jr. (editors), Present Knowledge in Nutrition (7th ed.). ILSI Press, Washington, DC. pp.1-6.

Nayini, N.R., Markakis, P., 1986. Phytases. In: E. Graf (editor), Phytic Acid: Chemistry and applications. Pilatus Press. Minneapolis. pp.101-118.

Nelson, T.S., 1967. The utilization of phytate phosphorus by poultry – A review., Poultry Sci., 46(4):862-871.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J., Ware, J.H., 1968. The availability of phytate phosphorus in soybean meal before and after treatment with a mold phytase. Poultry Sci., 47:1842-1848.

Nemere, I., Norman, A.W., 1991. Transport of calcium (Ch.13). In: Handbook of Physiology: The Gastrointestinal System (Section 6, Volume 4). American Physiological Society, Bethesda, MD. pp.337-360.

New, M.B., 1997. Aquaculture and the capture fisheries - balancing the scales. World Aquaculture, 28(2):11-30.

New, M.B., Tacon, A.G.J., Csavas, I. (editors), 1995. Farm-Made Aquafeeds. FAO, Rome, Italy. 434p.

Nielsen, F.H., 1986. Other elements (Ch.10). In: W. Mertz (editor), Trace Elements in Human and Animal Nutrition (5th ed., vol. 2). Academic Press, Inc., Orlando, FL, pp.415-463.

Nielsen, F.H., Sunde, M.L., Hoekstra, W.G., 1966. Effect of some dietary synthetic and natural chelating agents on the zinc-deficiency syndrome in the chick. J. Nutr., 89:35-42.

Nolan, K.B., Duffin, P.A., McWeeny, D.J., 1987. Effects of phytate on mineral bioavailability. In vitro studies on Mg2+, Ca2+, Fe3+, Cu2+ and Zn2+ (also Cd2+) solubilities in the presence of phytate. J. Sci. Food Agric., 40:79-85.

NRC (National Research Council), 1980. Mineral Tolerance of Domestic Animals. National Academy of Sciences, National Academy Press. Washington, D.C. 577p.

NRC (National Research Council), 1984. Nutrient Requirements of Poultry (8th rev. ed.). National Academy Press, Washington, D.C. 71p.

NRC (National Research Council), 1988. Nutrient Requirements of Swine (9th rev. ed.). National Academy Press, Washington, D.C. 93p.

NRC (National Research Council), 1993. Nutrient Requirements of Fish., National Academy Press, Washington, D.C. 114p.

Nugura, D., Edwards, Jr., H.M., 1963. Influence of dietary Ca and P levels on the Mg requirement of the chick. J. Nutr., 80:181-184.

O'Dell, B.L., Morris, E.R., Regan, W.O., 1960. Magnesium requirement of guinea pigs and rats. J. Nutr., 70:103-111.

Oberleas, D., 1983. The role of phytate in zinc bioavailability and homeostasis (Ch.10). In: G.E. Inglett (editor), Nutritional Bioavailability of Zinc. American Chemical Society, Washington, DC. pp.145-158.

Ogino, C., Takeda, H., 1976. Mineral requirements in fish. III. Calcium and phosphorus requirements of carp. Bull. Jpn. Soc. Sci. Fish., 42:793-799.

Ogino, C., Takeda, H., 1978. Requirements of rainbow trout for dietary calcium and phosphorus. Bull. Jpn. Soc. Sci. Fish., 44:1019-1022.

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

Oliva-Teles, A., Kaushik, S.J., 1990. Growth and nutrient utilization by 0+ and 1+ triploid rainbow trout, Oncorhynchus mykiss. J. Fish Biol., 37(1):125-133.

Omaye, S.T., Turnbull, J.D., Sauberlich, H.E., 1979. Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. Method in Enzymology, 62:3-11.

O'Quinn, P.R., Knabe, D.A., Gregg, E.J., 1997. Efficacy of Natuphos in sorghum-based diets of finishing swine. J. Animal Sci., 75(5):1299-1307.

Oser, B.L. (editor), 1965. Carbohydrate metabolism (Ch.22). In: B.L.Oser (editor), Hawk's Physiological Chemistry (14th ed.). McGraw-Hill, Inc., New York. pp.759-785.

Pak,C.Y., Harvey, J.A., Hsu, M.C., 1987. Enhanced calcium bioavailability from a solubilized form of calcium citrate. J. Clin. Endocrinol. Metab., 65:801-805.

Papavasiliou, P.S., Miller, S.T., Cotzias, G.C., 1966. Role of liver in regulating distribution and excretion of manganese. Am. J. Physiol., 211:211-216.

Papoutsoglou, S.E., Papaparaskeva-Papoutsoglou, E.G., 1978. Comparative studies on body composition of rainbow trout (Salmo gairdneri R.) in relation to type of diet and growth rate. Aquaculture, 13:235-243.

Paripatananont, T., Lovell, R.T., 1997. Comparative net absorption of chelated and inorganic trace minerals in channel catfish Ictalurus punctatus diets. J. World Aquac. Soc., 28:62-67.

Passonneau, J.V., Lowry, O.H., 1993. Enzymatic Analysis. Humana Press, Totowa, NJ. 403p.

Perney, K.M., Cantor, A.H., Straw, M.L., Herkelman, K.L., 1993. The effect of dietary phytase on growth performance and phosphorus utilization of broiler chicks. Poultry Sci., 72(11):2106-2114.

Peterlik, M., Wasserman, R.H., 1978. Effect of vitamin D on transepithelial phosphate transport in chick intestine. Am. J. Physiol., 234:E379-E388.

Petito, S.L., Evans, J.L., 1984. Calcium status of the growing rat as affected by diet acidity from ammonium chloride, phosphate and protein. J. Nutr., 114:1049-1059.

Pfeffer, E., Beckmann-Toussaint, J., Henrichfreise, B., Jansen, H.D., 1991. Effect of extrusion on efficiency of utilization of maize starch by rainbow trout (Oncorhynchus mykiss). Aquaculture, 96(3-4):293-303.

Philips, M., Beveridge, M., 1986. Cages and the effect on water condition. Fish Farmer, 9(3):17-19.

Phillips, A.M., Jr., Podoliak, H.A., Brockway, D.R., Vaughn, R.R., 1958. Metabolism of water and food phosphorus by brook trout. Fisheries Research Bulletin, 21:39-67.

Phillips, A.M., Jr., Podoliak, H.A., Dumas, R.F., Thoesen, R.W., 1959. Metabolism of dissolved phosphorus by trout. Fisheries Research Bulletin, 22:16-37.

Phillips, A.M., Jr., Podoliak, H.A., Dumas, R.F., Thoesen, R.W., 1959. Metabolism of dietary phosphorus by brook trout. Fisheries Research Bulletin, 22:37-57.

Phillips, A.M., Jr., Podoliak, H.A., Poston, H.A., Livingston, D.L., Booke, H.E., Pyle, E.A., Hammer, G.L., 1964. Distribution of dietary phosphate to the muscles and skeleton of brook trout. Fisheries Research Bulletin, 27:91-104.

Pieper, A., Pfeffer, E., 1980a. Studies on the comparative efficiency of utilization of gross energy from some carbohydrates, proteins and fats by rainbow trout (Salmo gairdneri, R.). Aquaculture, 20(4):323-332.

Pieper, A., Pfeffer, E., 1980b., Studies on the effect of increasing proportions of sucrose or gelatinized maize starch in diets for rainbow trout (Salmo gairdneri, R.) on the utilization of dietary energy and protein. Aquaculture, 20(4):333-342.

Pileggi, V.J., De Luca, H.F., Steenbock, H., 1955. The role of vitamin D and intestinal phytase in the prevention of rickets in rats on cereal diets. Arch. Biochem. Biophys., 58:194-204.

Pillay, T.V.R., 1992. Aquaculture and the Environment. Fishing News, Oxford, England. 189p.

Pinchasov, Y., Elmaliah, S., 1995. Broiler chick responses to anorectic agents: dietary acetic and propionic acids and the blood metabolites. Ann. Nutr. Metab., 39:107-116.

Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G., Leonard, J.R. (editors), 1982. Fish Hatchery Management. United States Department of the Interior, Fish and Wildlife Service, Washington, DC. 517p.

Podoliak, H.A., Smigielski, A.S., 1971. Metabolism of dietary phosphate by brown and rainbow trout. Fisheries Research Bulletin, 33:43-55.

Pointillart, A., Fourdin, A., Fontaine, N., 1987. Importance of cereal phytase activity for phytate phosphorus utilization by growing pigs fed diets containing triticale or corn. J. Nutr., 117(5):907-913.

Porn-Ngam, N., Satoh, S., Takeuchi, T., Watanabe, T., 1993. Effect of the ratio of phosphorus to calcium on zinc availability to rainbow trout in high phosphorus diet. Nippon Suisan Gakkaishi, 59:2065-2070.

Post, G., Shanks, W.E., Smith, R.R., 1965. A method for collecting metabolic excretions from fish. Prog. Fish-Cult., 27:108-111.

Qian, H., Kornegay, E.T., Denbow, D.M., 1997. Utilization of phytate phosphorus and calcium as influenced by microbial phytase, cholecalciferol, and the calcium:total phosphorus ratio in broiler diets. Poultry Sci., 76(1):37-46.

Qian, H., Veit, H.P., Kornegay, E.T., Ravindran, V., Denbow, D.M., 1996. Effects of supplemental phytase and phosphorus an histological and other tibial bone characteristics and performances of broilers fed semi-purified diets. Poultry Sci., 75(5):618-626.

Quamme, G.A., 1985. Phosphate transport in intestinal brush-border membrane vesicles: effect of pH and dietary phosphate. Am. J. Physiol., 249:G168-G176.

Quamme, G.A., Dirks, J.H., 1994. Magnesium metabolism (Ch.13). In: R.G. Narins (editor), Maxwell and Kleeman's Clinical Disorders of Fluid and Electrolyte Metabolism (5th ed.). McGraw-Hill, Inc., New York, NY. pp.373-397.

Raboy, V. 1990. Biochemistry and genetics of phytic acid synthesis. In: D.J. Morre, W.F. Boss, F.A. Loewus (editors), Plant Biology (vol. 9, Inositol Metabolism in Plants). Wiley-Liss, New York. pp. 55-76.

Raboy, V., 1997. Accumulation and storage of phosphate and minerals. In: B.A. Larkins, I.K. Vasil (editors), Cellular and Molecular Biology of Plant Seed Development (vol. 4), Kluwer Academic Publishers, Dordrecht, Boston. pp. 441-477.

Raboy, V., Dickinson, D.B., Below, F.E., 1984. Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of Glycine max and G. soja. Crop Sci., 24:431-434.

Raboy, V., Below, F.E., Dickinson, D.B., 1989. Alteration of maize kernel phytic acid levels by recurrent selection for protein and oil. J. Heredity, 80:311-315.

Rackis, J.J., 1974. Biological and physiological factors in soybeans. J. Am. Oil Chem. Soc., 51:161A-174A.

Rapoport, S., Guest, G.M., 1938. Changes of organic acid-soluble phosphorus, diphosphoglycerate, adenosinetriphosphate, and inorganic phosphorus in the blood cells of rats during the development and healing of rickets. J. Biol. Chem., 126:749-761.

Ravindran, V., Kornegay, E.T., 1993. Acidification of weaner pig diets: A review. J. Sci. Food Agric., 62, 313-322.

Reddy, N.R., Sathe, S.K., Salunkhe, D.K., 1982. Phytates in legumes and cereals Nutritional significance of phytic acid. Adv. Food Res., 28:1-92.

Refstie, S., Helland, S.J., Storebakken, T., 1997. Adaptation to soybean meal in diets for rainbow trout, Oncorhynchus mykiss. Aquaculture, 153(3-4):263-272.

Reynolds, T.M., Smith, S.C., 1994. Poor reproducibility of strontium absorption test [letter]. Clin. Chem., 40:1789-1790.

Richardson, N.L., Higgs, D.A., Beames, R.M., McBride, J.R., 1985. Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile chinook salmon (Oncorhynchus tshawytscha). J. Nutr., 115:553-567.

Riche, M., Brown, P.B., 1996. Availability of phosphorus from feedstuffs fed to rainbow trout, Oncorhynchus mykiss. Aquaculture, 142:269-282.

Roberts, A.H., Yudkin, J., 1961. Effect of phytate and other dietary factors on intestinal phytase and bone calcification in the rat. Br. J. Nutr., 15:457-471.

Rodehutscord, M., 1996. Response of rainbow trout (Oncorhynchus mykiss) growing from 50 to 200g to supplements of dibasic sodium phosphate in a semipurified diet. J. Nutr., 126:324-331.

Rodehutscord, M., Pfeffer, E., 1995. Effects of supplemental microbial phyatse on phosphorus digestibility and utilization in rainbow trout (Oncorhynchus mykiss). Water Sci. Technol., 31(10):143-147.

Rolfe, D.F.S., Brown, G.C., 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev., 77(3):731-758.

Rose, I.A., Warms, J.V.B., O'Connell, E.L., 1964. Role of inorganic phosphate in stimulating the glucose utilization of human red blood cells. Biochem. Biophys. Res. Comm., 15:33-37.

Rudman, D., Millikan, W.J., Richardson, T.J., Bixler, T.J. 2d., Stackhouse, J., McGarrity, W.C., 1975. Elemental balances during intravenous hyperalimentation of underweight adult subjects. J. Clin. Invest., 55:94-104.

Sakamoto, S., Yone, Y., 1978. Effect of dietary phosphorus level on chemical composition of red seabream. Bull. Jpn. Soc. Sci. Fish., 44:227-229.

Sakamoto, S., Yone, Y., 1980. A principal source of deposited lipid in phosphorus deficient red sea bream. Bull. Jpn. Soc. Sci. Fish., 46(10):1227-1230.

Sakhaee, K., Alpern, R., Poindexter, J., Pak, C.Y., 1992. Citraturic response to oral citric acid load. J. Urol., 147:975-976.

Saltman, P., Hegenauer, J., Strause, L., 1984. For the want of a nail. Trace elements in health and disease. In: O.M. Rennert, W. Chan (editors), Metabolism of Trace Metals in Man. Vol.I: Developmental Aspects. CRC Press, Inc., Boca Raton, FL, pp.1-16.

Sandberg, A.S., Andersson, H., 1988. Effect of dietary phytase on the digestion of phytate in the stomach and small intestine of humans. J. Nutr., 118(4):469-473.

Sandberg, A.S., Andersson, H., Carlsson, N.G., Sandstrom, B., 1987. Degradation products of bran phytate formed during digestion in the human small intestine: effect of extrusion cooking on digestibility. J. Nutr., 117(12):2061-2065.

Sandstrom, B., 1988. Factors influencing the uptake of trace elements from the digestive tract. Proc. Nutr. Soc., 47:161-167.

Sandstrom, B., 1992. Dose dependence of zinc and manganese absorption in man. Proc. Nutr. Soc., 51:211-218.

Sandstrom, B., Andersson, H., Kivisto, B., Sandberg, A-S., 1986. Apparent small intestinal absorption of nitrogen and minerals from soy and meat-protein-based diets. A study on human ileostomy subjects. J. Nutr., 116:2209-2218.

Satoh, S., Takeuchi, T., Watanabe, T., 1987a. Availability to rainbow trout of zinc in white fish meal and of various zinc compounds. Nippon Suisan Gakkaishi, 53:595-599.

Satoh, S., Poe, W.E., Wilson, R.P., 1989. Effect of supplemental phytate and /or tricalcium phosphate on weight gain, feed efficiency and zinc content in vertebrae of channel catfish. Aquaculture, 80:155-161.

Satoh, S., Takeuchi, T., Watanabe, T., 1991. Availability of manganese and magnesium contained in white fish meal to rainbow trout Oncorhynchus mykiss. Nippon Suisan Gakkaishi,. 57:99-104.

Satoh, S. Porn-Ngam, N., Takeuchi, T., Watanabe, T., 1993a. Effect of various types of phosphates on zinc availability to rainbow trout. Nippon Suisan Gakkaishi, 59:1395-1400.

Satoh, S., Takeuchi, T., Watanabe, T., Porn-Ngam, N., 1993b. Effect of the ratio of phosphorus to calcium on zinc availability to rainbow trout in high phosphorus diet. Nippon Suisan Gakkaishi, 59:2065-2070.

Satoh, S., Tabata, K., Izume, K., Takeuchi, T., Watanabe, T., 1987b. Effect of tri-calcium phosphate on availability of zinc to rainbow trout. Bull. Jpn.. Soc. Sci. Fish., 53:1199-1205.

Satoh, S., Viyakarn, V., Yamazaki, Y., Takeuchi, T., Watanabe, T., 1992. A simple method for determination of available phosphorus content in fish diet. Nippon Suisan Gakkaishi, 58:2095-2100.

Scarpa, J., Gatlin, D.M.,III, 1992. Dietary zinc requirements of channel catfish, Ictalurus punctatus , swim-up fry in soft and hard water. Aquaculture, 106(3-4):311-322.

Schaefer, A., Koppe, W.M., Meyer-Burgdorff, K.H., Guenther, K.D., 1995. Effects of microbial phytase on the utilization of native phosphorus by carp in a diet based on soybean meal. Water Sci. Technol., 31(10):149-155.

Schaible, P.J., Bandemer, S.L., 1942. The effect of mineral supplements on the availability of manganese. Poultry Sci., 21:8-14.

Schilke, R.E., Johnson, R.E., 1965. A colorimetric method for estimating acetoacetate. Am. J. Clin. Path., 43:539-543.

Schwartz, R., Apgar, B.J., Wien, E.M., 1986. Apparent absorption and retention of Ca, Cu, Mg, Mn, and Zn from a diet containing bran. Am. J. Clin. Nutr., 43:444-455.

Schwarz, F.J., Kirchgessner, M., Roth, H.P., 1983. Influence of picolinic acid and citric acid on intestinal absorption of zinc in vitro and in vivo. Res. Exp. Med., 182:39-48.

Scott, M.L., Nesheim, M.C., Young, R.J., 1982. Essential inorganic elements. In: Nutrition of the Chicken (3rd ed.). M.L. Scott and Associates, Publishers, New York, NY. pp.277-382.

Sebastian, S., Touchburn, S.P., Chavez, E.R., Lague, P.C., 1996a. The effects of supplemental microbial phytase on the performance and utilization of dietary calcium, phosphorus, copper, and zinc in broiler chickens fed corn-soybean diets. Poultry Sci., 75(6):729-736.

Sebastian, S., Touchburn, S.P., Chavez, E.R., Lague, P.C., 1996b. Efficacy of supplemental microbial phytase at different dietary calcium levels on growth performance and mineral utilization of broiler chickens. Poultry Sci., 75(12):1516-1523.

Segal, M.A., 1955. A rapid electrotitrimetric method for determining CO2 combining power in plasma or serum. Am. J. Clin. Pathol., 25:1212-1216.

Shaw, H.M., Saunders, R.L., Hall, H.C., Henderson, E.B., 1975. Effect of dietary sodium chloride on growth of Atlantic salmon (Salmo salar). J. Fish. Res. Bd. Can., 32:1813-1819.

Shearer, K.D., 1984. Changes in elemental composition of hatchery-reared rainbow trout, Salmo gairdneri , associated with growth and reproduction. Can. J. Fish. Aquat. Sci., 41(11):1592-1600.

Shearer, K.D., 1994. Factors affecting the proximate composition of cultured fishes with emphasis on salmonids. Aquaculture, 119:63-88.

Shearer, K.D., Hardy, R.W., 1987. Phosphorus deficiency in rainbow trout fed a diet containing deboned fillet scrap. Prog. Fish-Cult., 49:192-197.

Shearer, K.D., Maage, A., Opstvedt, J., Mundheim, H., 1992. Effects of high-ash diets on growth, feed efficiency, and zinc status of juvenile Atlantic salmon (Salmo salar). Aquaculture, 106:345-355.

Shell, E.W., 1983. Fish Farming Research. Alabama Agricultural Experiment Station, Auburn University. 108p.

Shell, E.W., 1993. The Development of Aquaculture : an ecosystems perspective. Alabama Agricultural Experiment Station, Auburn University. 265p.

Sherman, C.C., Mendel, L.B., Smith, A.H., 1936. The metabolism of orally administered citric acid. J. Biol. Chem., 113:265-271.

Shiau, S.Y., Chen, M.J., 1993. Carbohydrate utilization by tilapia (Oreochromis niloticus x O. aureus) as influenced by different chromium sources. J. Nutr., 123:1747-1753.

Simons, P.C., Versteegh, H.A., Jongbloed, A.W., Kemme, P.A., Slump, P., Bos, K.D., Wolters, M.G., Beudeker, R.F., Verschoor, G.J., 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr., 64:525-540.

Singh, R.P, Nose, T., 1967. Digestibility of carbohydrates in young rainbow trout. Bull. Freshwater Fish. Res. Lab., 17:21-25.

Sips, A.J.A.M., van der Vijgh, W.J.F., Barto, R., Netelenbos, J.C., 1995. Intestinal strontium absorption: From bioavailability to validation of a simple test representative for intestinal calcium absorption. Clin. Chem., 41:1446-1450.

Sklan, D., Dubrov, D., Eisner, U., Hurwitz, S., 1975. ^{51}Cr-EDTA, ^{91}Y and ^{141}Ce as nonabsorbed reference substance in the gastrointestinal tract of the chicken. J. Nutr., 105:1549-1552.

Slinger, S.J., Razzaque, A., Cho, C.Y., 1979. Effect of feed processing and leaching on the losses of certain vitamins in fish diets. In: J.E. Halver, K. Tiews (editors), Finfish Nutrition and Fish Feed Technology (vol. II). pp.425-434.

Smit, G.L., Hattingh, J., 1981. The effect of hypoxia on haemoglobins and ATP levels in three freshwater fish species. Comp. Biochem. Physiol., 68A:519-521.

Smith, C.E., 1979. The prevention of liver lipoid degeneration (ceroidosis) and microcytic anaemia in rainbow trout (Salmo gairdneri) Richardson fed rancid diets: a preliminary report. J. Fish. Dis., 2:429-437.

Smith, K.T., Rotruck, J.T., 1988. Trace mineral and calcium interactions. In: L.S. Hurley (editor), Trace Elements in Man and Animals 6. Plenum Press, New York, NY. pp.221-228.

Smith, R.R., 1967. Evaluation of Carbohydrates in the Diet of Rainbow Trout. Thesis, Master of Science, Utah State University, Logan, UT. 44p.

Smith, R.R., 1970. Urine flow. Prog. Sport Fish. Res., 1969:103.

Smith, R.R., 1971. A method for measuring digestibility and metabolizable energy of fish feeds. Prog. Fish-Cult., 33:132-134.

Smith, R.R., 1977. Recent research involving full-fat soybean meal in salmonid diets. Salmonid, 1(4):8-18.

Smith, R.R., 1989. Nutritional energetics (Ch.1). In: J.E. Halver (editor), Fish Nutrition (2nd ed.). Academic Press, San Diego, CA. pp.1-29.

Smith, R.R., Rumsey, G.L., Scott, M.L., 1978. Heat increment associated with dietary protein, fat, carbohydrate and complete diets in salmonids: comparative energetic efficiency. J-Nutr., 108(6):1025-32.

Smith, R.R., Peterson, M.C., Allred, A.C., 1980. Effect of leaching on apparent digestion coefficients of feedstuffs for salmonids. Prog. Fish-Cult., 42:195-199.

Smith, R.R., Winfree, R.A., Rumsey, G.W., Allred, A., Peterson, M., 1995. Apparent digestion coefficients and metabolizable energy of feed ingredients for rainbow trout Oncorhynchus mykiss. J. World Aquac. Soc., 26:432-437.

Snedecor, G.W., Cochran, W.G., 1980. Statistical Methods (7th ed.). The Iowa State University Press, Ames, Iowa. 507p.

Sobel, C., 1964. Determination of fecal lipids. In: R.J. Henry (editor), Clinical chemistry, principles and technics. Hoeber Medical Division, Harper & Row Publishers, New York. pp.881-883.

Söderlund, K., Hultman, E., 1986. Effects of delayed freezing on content of phosphagens in human skeletal muscle biopsy samples. J. Appl. Physiol., 61:832-835.

Spannhof, L., Plantikow, H., 1983. Studies on carbohydrate digestion in rainbow trout. Aquaculture, 30(1-4):95-108.

Spencer, R.P., 1959. Potassium metabolism and gastrointestinal function: A review. Am. J. Dig. Dis., 4:145-158.

Spinelli, J., Houle, C.R., Wekell, J.C., 1983. The effect of phytates on the growth of rainbow trout (Salmo gairdneri) fed purified diets containing varying quantities of magnesium. Aquaculture, 30:71-83.

Steffens, W., 1989. Principles of Fish Nutrition (translator, B.D. Hemmings ; translation editor, L.M. Laird). Halsted Press, E. Horwood, New York. 384p.

Stickney, R.R., 1994. Principles of Aquaculture (Ch.1, Introduction). Wiley, New York. pp.1-44.

Stone, F.E., Hardy, R.W., Spinelli, J., 1984. Autolysis of phytic acid and protein in canola meal (Brassica spp.), wheat bran (Triticum spp.) and fish silage blends. J. Sci. Food Agric., 35(5):513-519.

Strunz, U.T., Walsh, J. H., Grossman, M.I., 1978. Stimulation of gastrin release in dogs by individual amino acids. Proc. Soc. Exp. Biol. Med., 157:440-441.

Tacon, A.G.J., 1994. Feed ingredients for carnivorous fish species; Alternatives to fishmeal and other fishery resources. FAO Fisheries Circular, No. 881., 35p.

Tacon, A.G.J., 1996. Feeding tomorrow's fish. World Aquacult., 27(3):20-32.

Tacon, A.G.J., 1997. Global trends in aquaculture and aquafeed production 1984-1995. International Aquafeed Directory. Turret RAI PLC. pp.5-37.

Tangkongchitr, U, Seib, P.A., Hoseney, R.C., 1982. Phytic acid. III. Two barriers to the loss of phytate during breadmaking. Cereal Chem., 59:216-221.

Taussky, H.H., Shorr, E., 1953. A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem., 202:675-685.

Taylor, I.L., Byrne, W.J., Christie, D.L., Ament, M.E., Walsh, J.H., 1982. Effect of individual l-amino acids on gastric acid secretion and serum gastrin and pancreatic polypeptide release in humans. Gastroenterology, 83:273-278.

Taylor, T.G., 1965. The availability of calcium and phosphorus of plant materials for animals. Proc. Nutr. Soc., 24:105-112.

Thillart, G.van den, Kesbeke, F, Waarde, A.van, 1980. Anaerobic energy-metabolism of goldfish, Carassius auratus (L.). J. Comp. Physiol., 136:45-52.

Toma, R.B., Tabekhia, M.M., 1979. Changes in mineral elements and phytic acid contents during cooking of three California rice varieties. J. Food Sci., 44:629-632.

Toothill, J., 1963. The effect of certain dietary factors on the apparent absorption of magnesium by the rat. Br. J. Nutr., 17:125-134.

Travis, S.F., Sugerman, H.J., Ruberg, R.L., Dudrick, S.J., Delivoria-Papadopoulos, M., Miller, L.D., Oski, F.A., 1971. Alterations of red-cell glycolytic intermediates and oxygen transport as a consequence of hypophosphatemia in patients receiving intravenous hyperalimentation. N. Engl. J. Med., 285:763-768.

Turnlund, J.R., King, J.C., 1983. Assessment of the bioavailability of dietary zinc in humans using the stable isotopes ^{70}Zn and ^{67}Zn. In: G.E. Inglett (editor), Nutritional Bioavailability of Zinc. American Chemical Society, Washington, DC. pp.31-40.

Turnlund, J.R., Durkin, N., Costa, F., Margen, S., 1986. Stable isotope studies of zinc absorption and retention in young and elderly men. J. Nutr., 116:1239-1247.

Van Slyke, D.D., Hiller, A., 1947. Application of Sendroy's iodometric chloride titration to protein-containing fluids. J. Biol. Chem., 167:107-124.

Van-Der-Klis, J.D., Versteegh, H.A.J., Simons, P.C.M., Kies, A.K., 1997. The efficacy of phytase in corn-soybean meal-based diets for laying hens. Poultry Sci., 76(11):1535-1542.

Vohra, P., Kratzer, F.H., 1964. Influence of various chelating agents on the availability of zinc. J. Nutr., 82:249-256.

Walker, A.R.P., Fox, F.W., Irving, J.T., 1948. Studies in human mineral metabolism 1. The effect of bread rich in phytate phosphorus on the metabolism of certain mineral salts with special reference to calcium. Biochem. J., 42:452-462.

Wasserman, R.H., Fullmer, C.S., 1989. On the molecular mechanism of intestinal calcium transport. In: F.R. Dintzis, J.A. Laszlo (editors), Mineral Absorption in the Monogastric GI Tract. Plenum Press, New York, NY. pp.45-65.

Watanabe, T., Matsuura, Y., Hashimoto, Y., 1966. Effect of natural and synthetic antioxidants on the incidence of muscle dystrophy of carp induced by oxidized saury oil. Bull. Jpn. Soc. Sci. Fish., 32:887-891.

Watanabe, T., Takeuchi, T., Satoh, S., Kiron, V., 1996. Digestible crude protein contents in various feedstuffs determined with four freshwater fish species. Fisheries Sci., 62:278-282.

Weaver, C.M., Heaney, R.P., Martin, B.R., Fitzsimmons, M.L., 1991. Human calcium absorption from whole wheat products. J. Nutr., 121(11):1769-1775.

Wedekind, K.J., Baker, D.H., 1990a. Manganese utilization in the chicks as affected by excess calcium and phosphorus ingestion. Poultry Sci., 69:977-984.

Wedekind, K.J., Baker, D.H., 1990b. Effect of varying calcium and phosphorus level on manganese utilization. Poultry Sci., 69:1156-1164.

Weigand, E., Kirchgessner, M., 1980. Total true efficiency of zinc utilization: Determination and homeostatic dependence upon the zinc supply status in young rats. J. Nutr., 110:469-480.

Wilgus, Jr, H.S., Patton, A.R., 1939. Factors affecting manganese utilization in the chicken. J. Nutr., 18:35-45.

Wilkinson, R., 1976. Absorption of calcium, phosphorus and magnesium. In: B.E.C. Nordin (editor), Calcium, Phosphate and Magnesium Metabolism. Churchill Livingstone, Longman Group Limited, Edinburgh, UK, pp.36-112.

Wilson, R.P., 1989. Amino acids and proteins (Ch.3). In: J.E. Halver (editor), Fish Nutrition (2nd ed.). Academic Press, San Diego, CA. pp.111-151.

Wood, J., 1995. Selecting equipment for producing farm-made aquafeeds. In: M.B. New, A.G.J. Tacon, I. Csavas (editors), Farm-Made Aquafeeds. FAO Fish. Tech. Paper 343. pp.135-147.

Wright, E., Hughes, R.E., 1976. Some effects of dietary citric acid in small animals. Fd. Cosmet. Toxicol., 14:561-564.

Yamada, S., Tanaka, Y., Katayama, T., 1981a. Feeding experiments with carp fry fed an amino acid diet by increasing the number of feedings per day. Bull. Jpn. Soc. Sci. Fish., 47(9):1247.

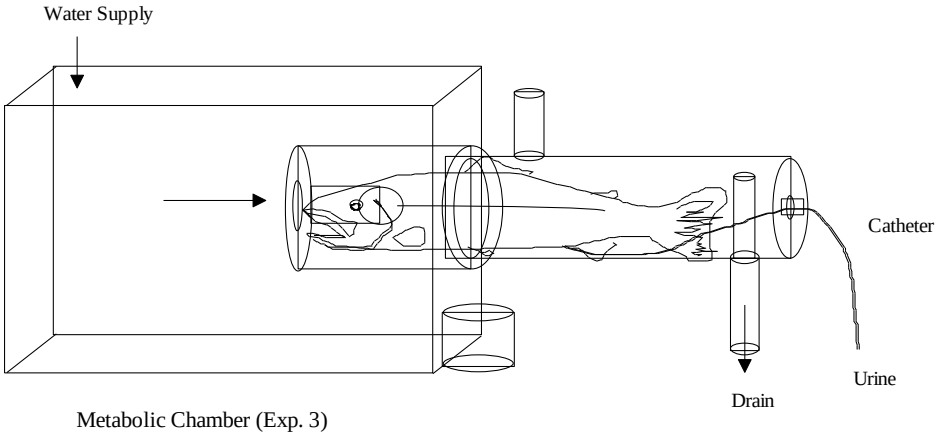
Yamada, S., Simpson, K.L., Tanaka, Y., Katayama, T., 1981b. Plasma amino acid changes in rainbow trout Salmo gairdneri force-fed casein and a corresponding amino acid mixture. Bull. Jpn. Soc. Sci. Fish., 47(8):1035-1040.

Yi, Z., Kornegay, E.T., Ravindran, V., Denbow, D.M., 1996a. Improving phytate phosphorus availability in corn and soybean meal for broilers using microbial phytase and calculation of phosphorus equivalency values for phytase. Poultry Sci., 75(2):240-249.

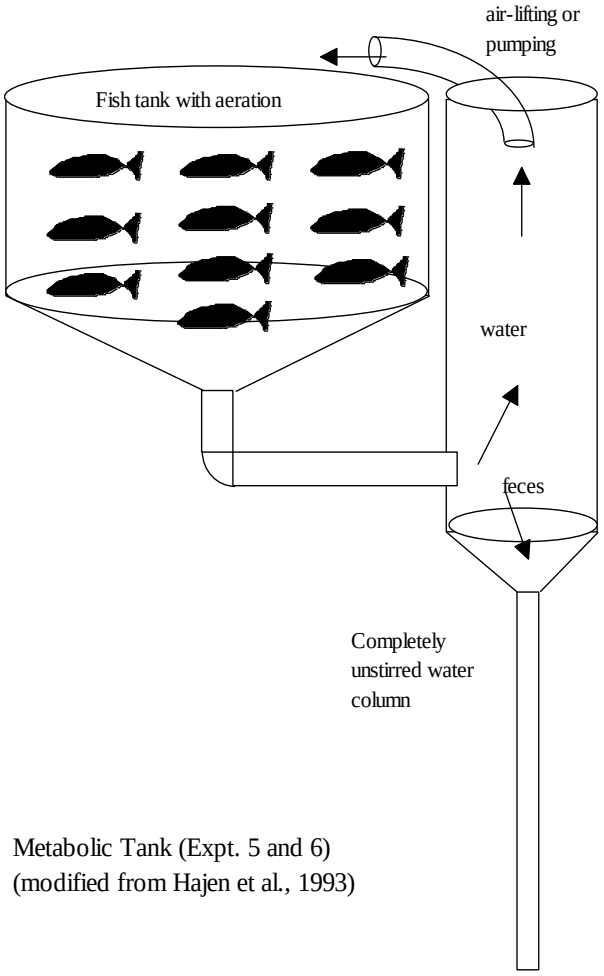
Yi, Z., Kornegay, E.T., Ravindran, V., Lindemann, M.D., Wilson, J.H., 1996b. Effectiveness of Natuphos(R) phytase in improving the bioavailabilities of phosphorus and other nutrients in soybean meal-based semipurified diets for young pigs. J. Animal Sci., 74(7):1601-1611.

Young, L.G., Leunissen, M., Atkinson, J.L., 1993. Addition of microbial phytase to diets of young pigs. J. Animal Sci., 71(8):2147-2150.

Appendix I. Data Figures for Chapter I



Metabolic Chamber (Exp. 3)
(see Smith, 1967 or Smith, 1971 for detail)



Metabolic Tank (Expt. 5 and 6)
(modified from Hajen et al., 1993)

Figure 33. Two systems used in the requirement study.

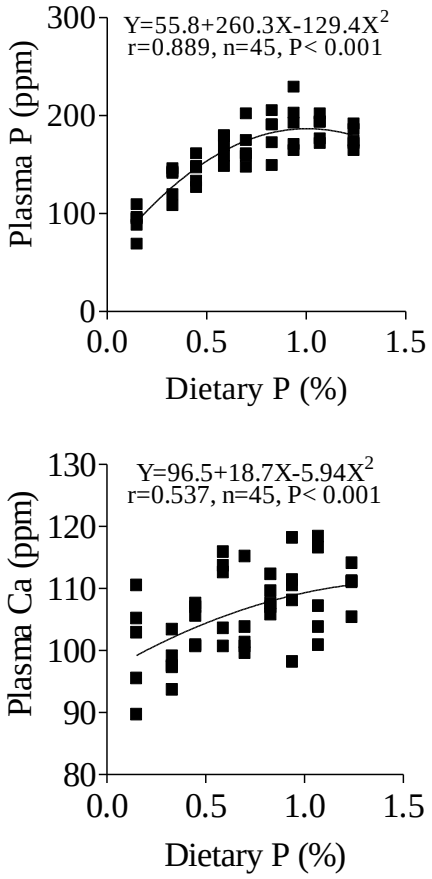


Figure 34. Plasma phosphorus and calcium concentrations of fish fed diets of various phosphorus content (Expt. 5). Fish were fed experimental diets for 12 days; blood samples were collected 24 h after feeding. Dietary P 1.006% for the maximum plasma P concentration, 0.737% for 95% of the maximum level. See also Table 4 in Chapter I.

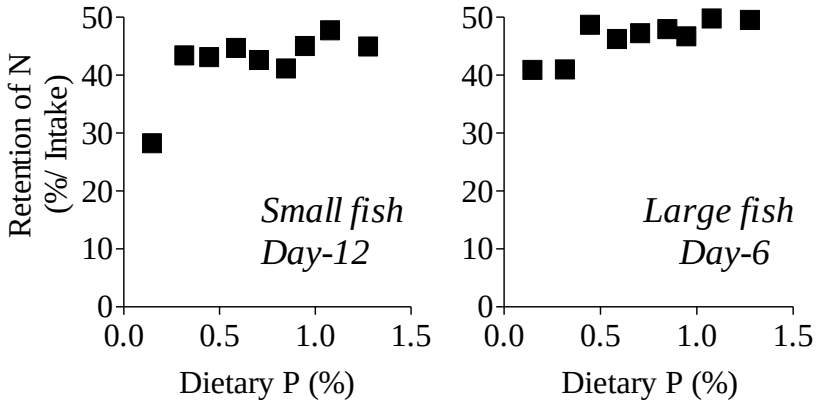


Figure 35. Retention of dietary N at various dietary P intakes (Expt. 5).

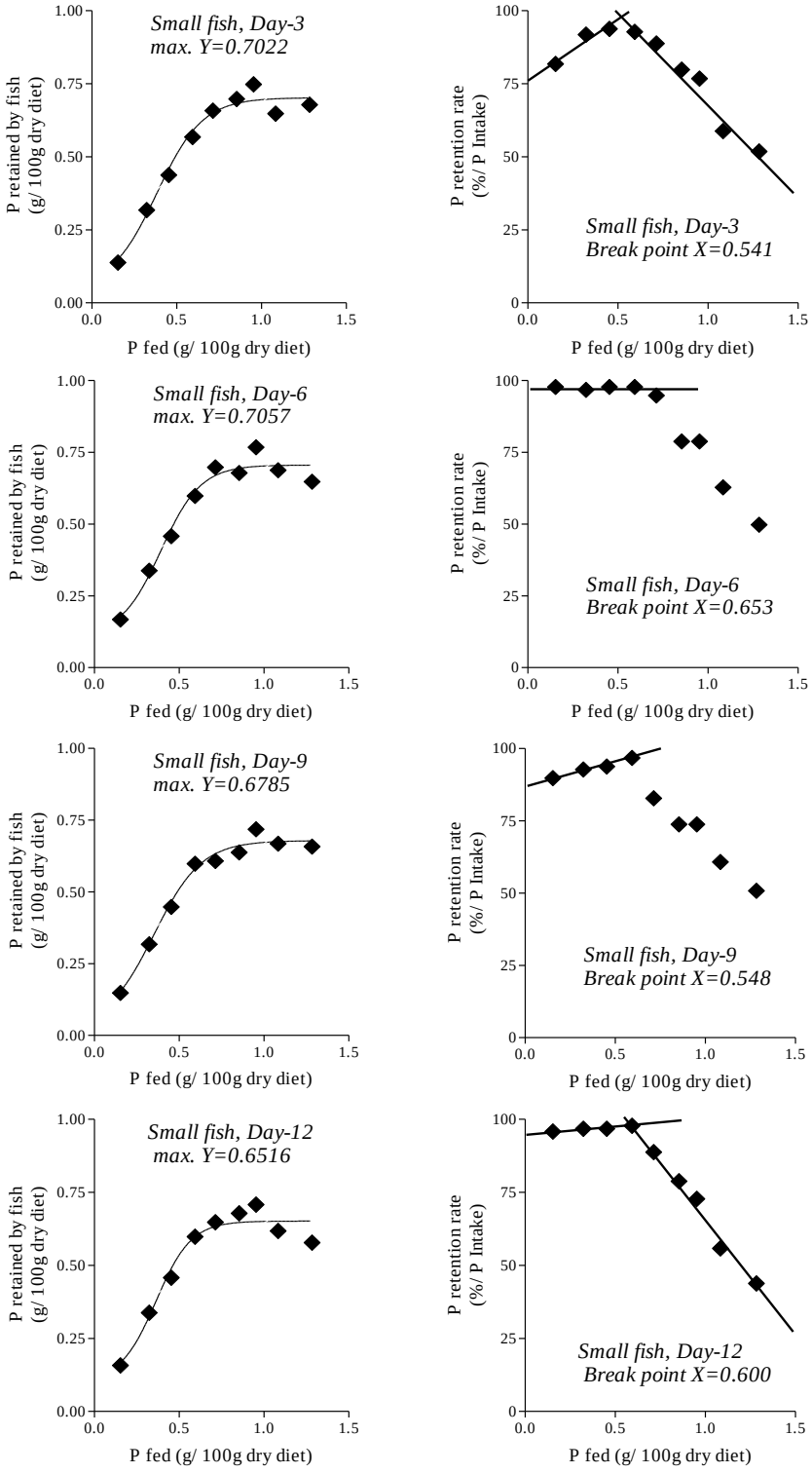


Figure 36. Retention plateau (Balance plateau) and Retention rate of dietary phosphorus at different intakes in small fish (Expt. 5).

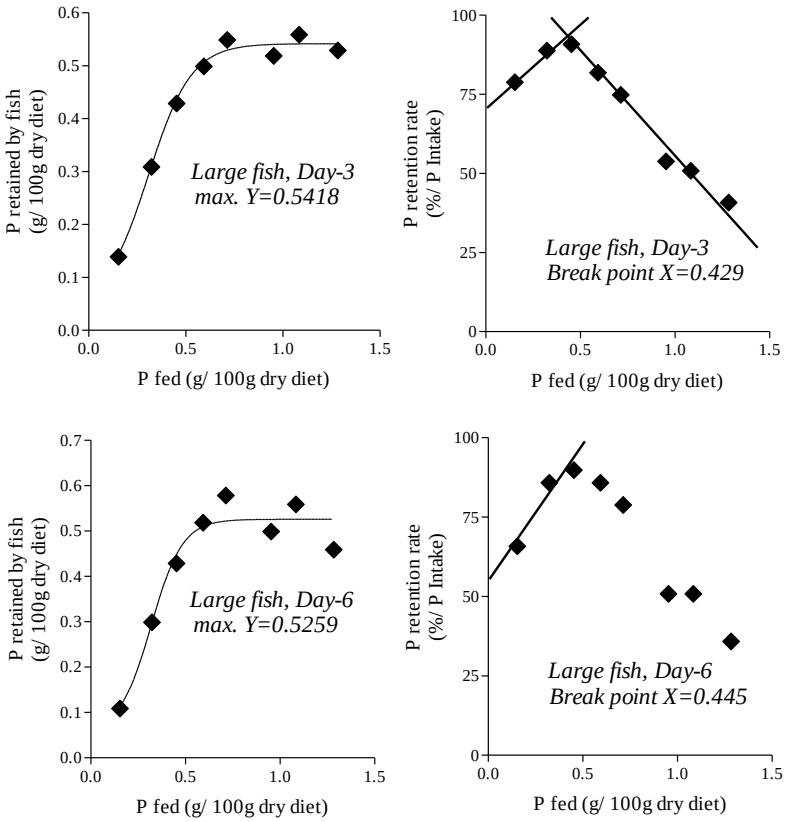


Figure 37. Retention plateau (Balance plateau) and Retention rate of dietary phosphorus at different intakes in Large fish (Expt. 5).

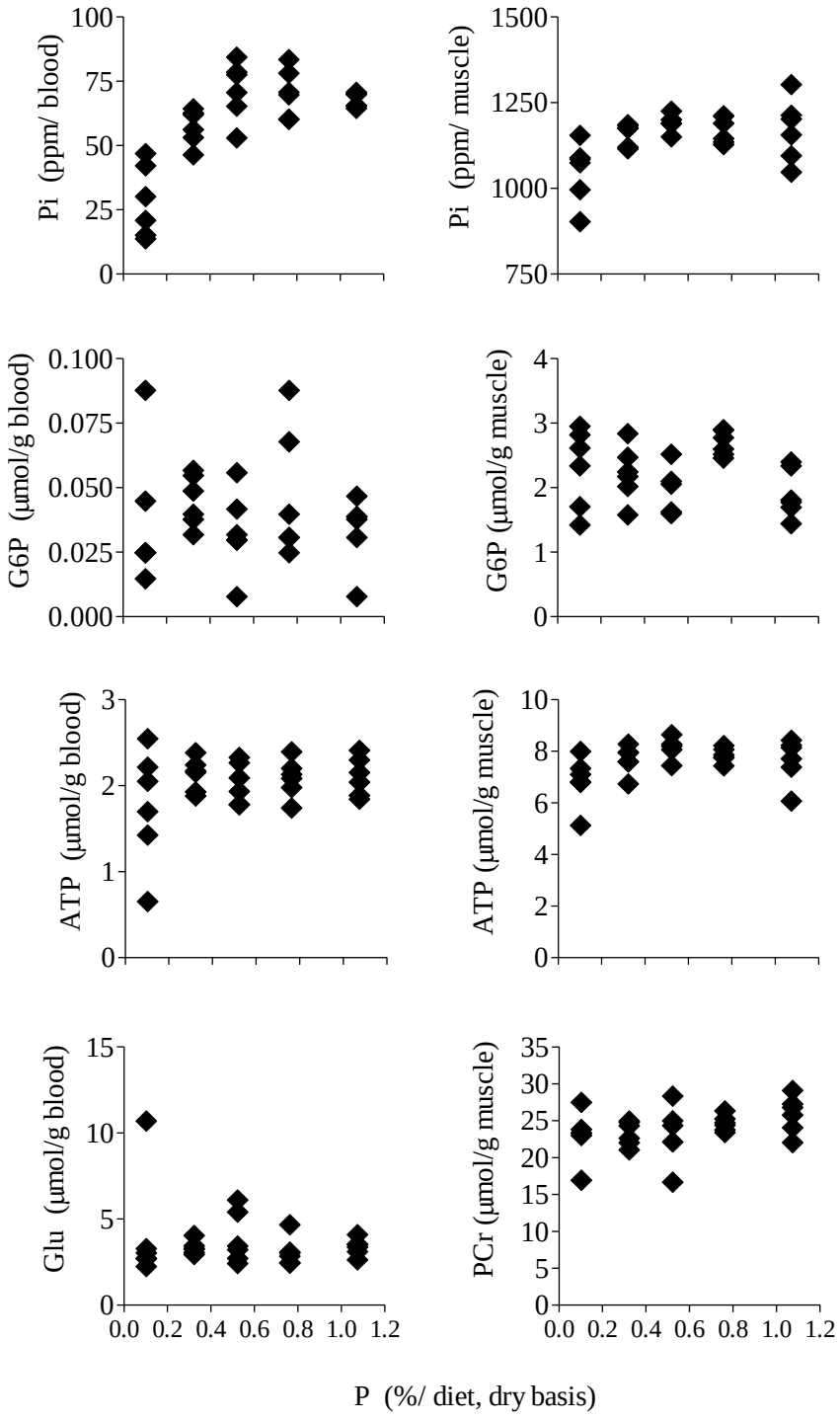


Figure 38. Effects of dietary phosphorus concentrations on the phosphorus compounds in blood and muscle (Expt. 1). Fish were fed experimental diets for 24 days; specimens were collected 12 h after feeding. Each point represents one fish. See also Table 3.

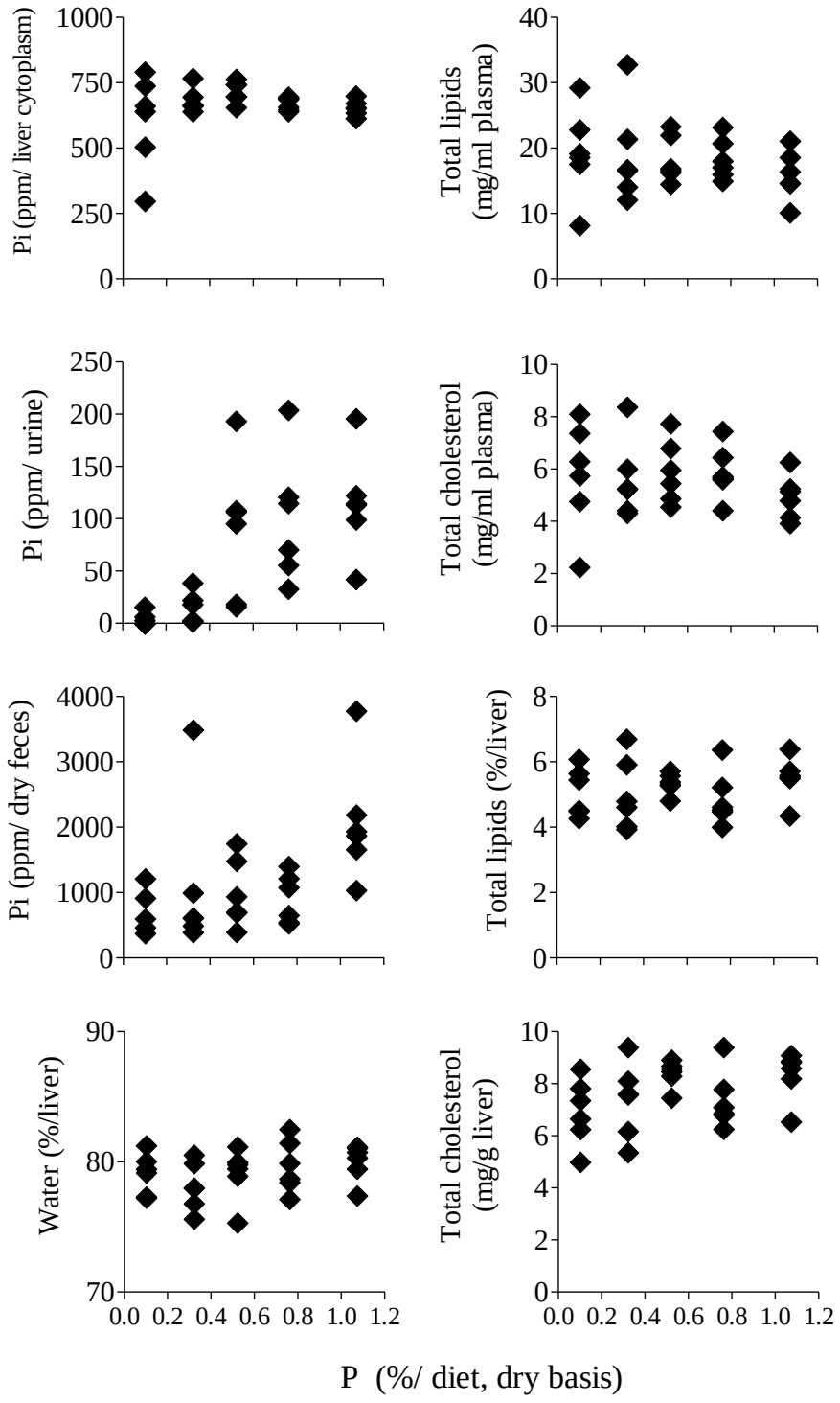


Figure 39. Effects of dietary phosphorus concentrations on the phosphorus and lipid levels in plasma, liver, urine and feces (Expt. 1). Fish were fed experimental diets for 24 days; specimens were collected 12 h after feeding. See also Table 3.

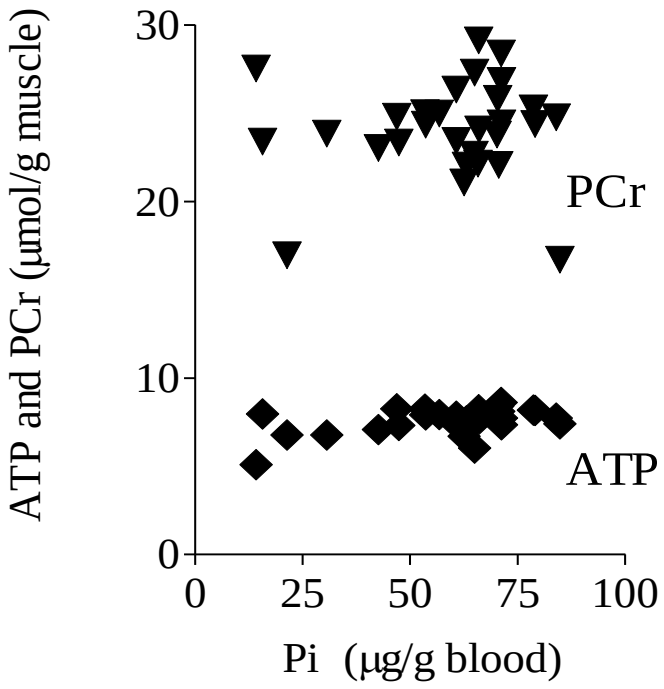


Figure 40. Relationship between blood inorganic phosphorus (Pi) concentrations and muscle ATP and creatine phosphate (PCr) concentrations (Expt. 1). Fish were fed experimental diets of varied phosphorus concentrations for 24 days; specimens were collected 12 h after feeding.

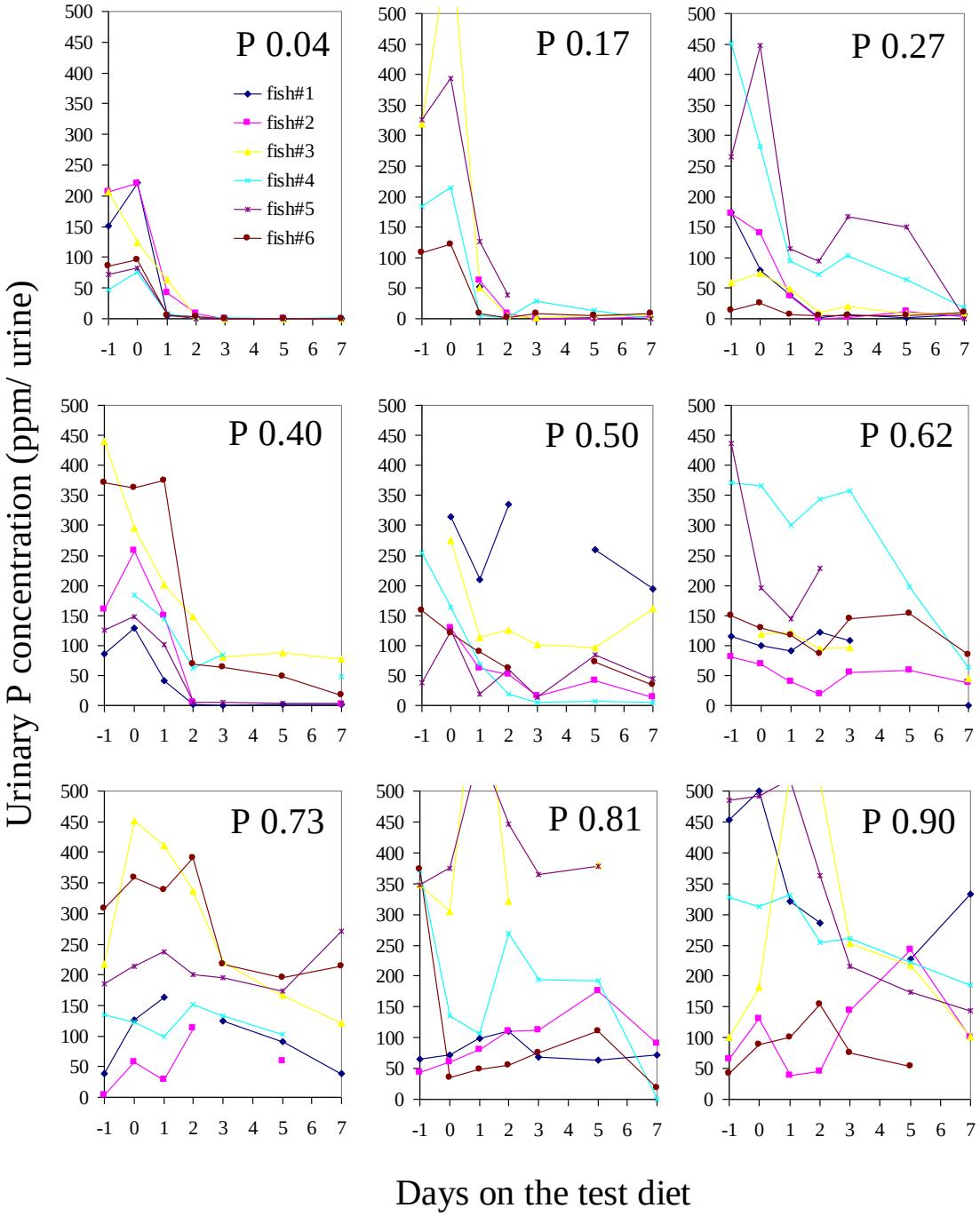


Figure 41. Change of urinary phosphorus concentrations over time when fish were fed various concentrations of phosphorus in diets (Expt. 2). Each fish was fed one of the experimental diets with a specific phosphorus content once daily at a level of 0.9% BW (dry basis). Urine samples were collected by stripping 24 h after feeding.

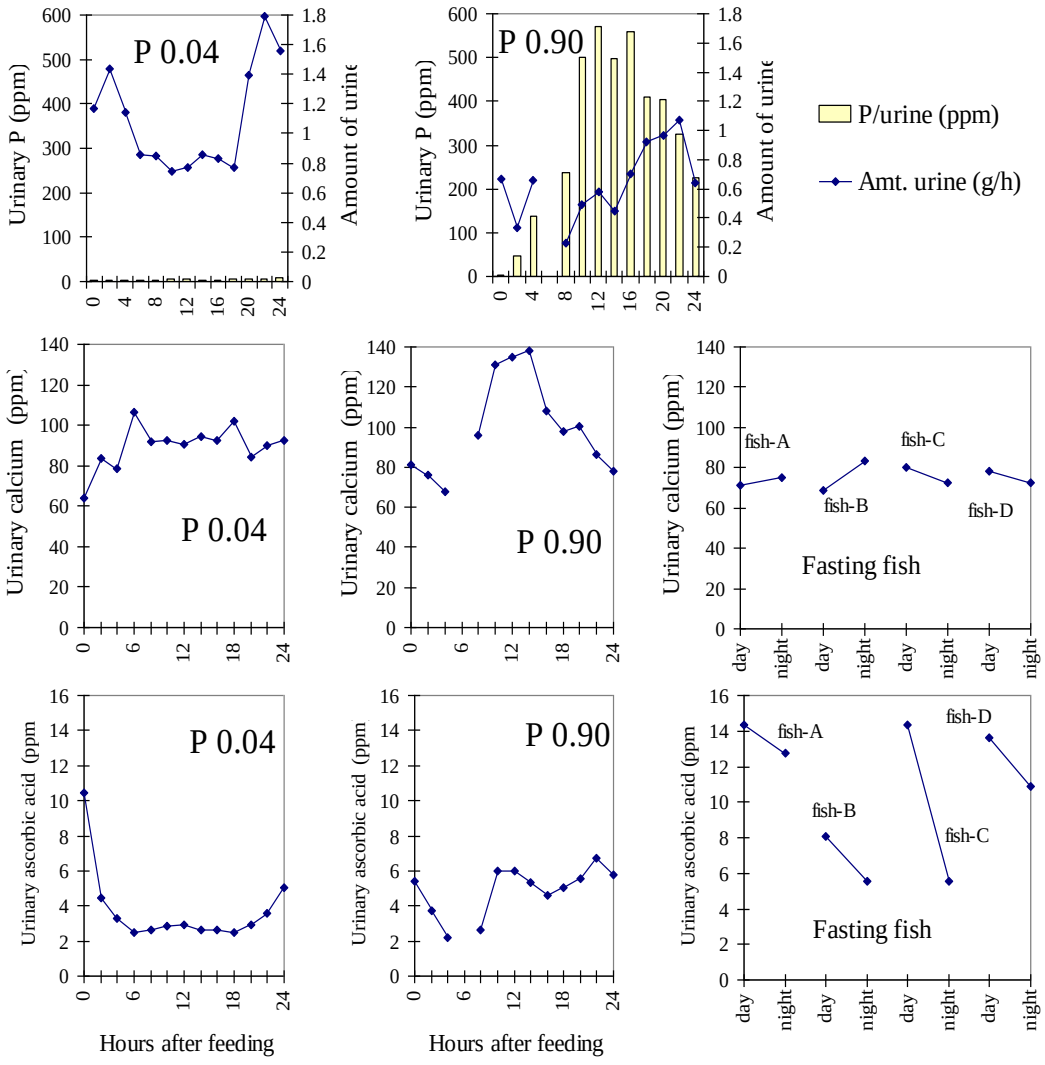


Figure 42. Phosphorus, calcium and ascorbic acid excretion of fish in a metabolic chamber: Preliminary data.

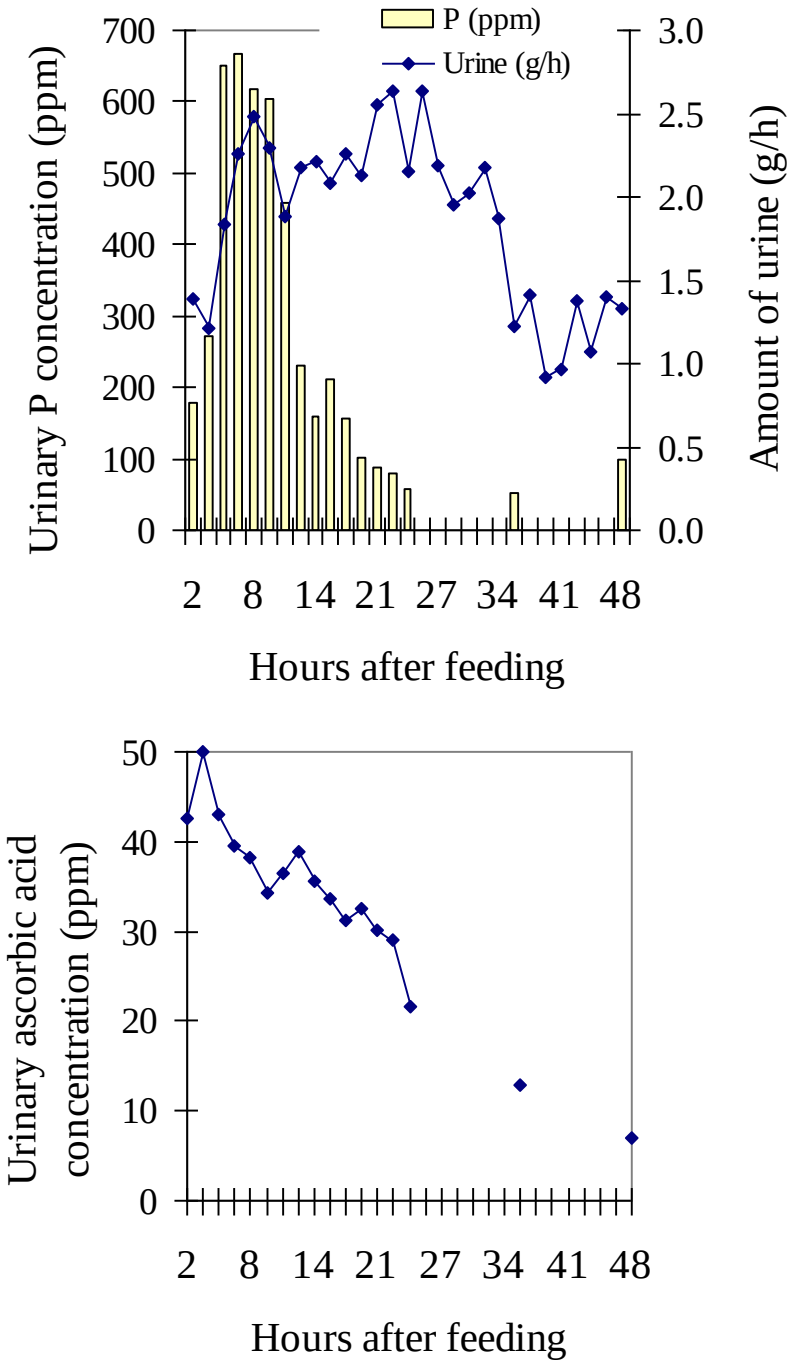


Figure 43. Collection of fish urine using metabolic chamber and catheter: preliminary data. The fish (BW 455g) received a purified diet containing 0.90% P at 0.9%BW (dry basis) once every day. After 7 days of feeding with the test diet, the fish was confined to the metabolic chamber. The collection of urine samples using a catheter was started one day after the confinement of the fish in the chamber. Urine samples collected between 24 and 34h or 36 and 46h were not analyzed (missing columns and dots in the figures).

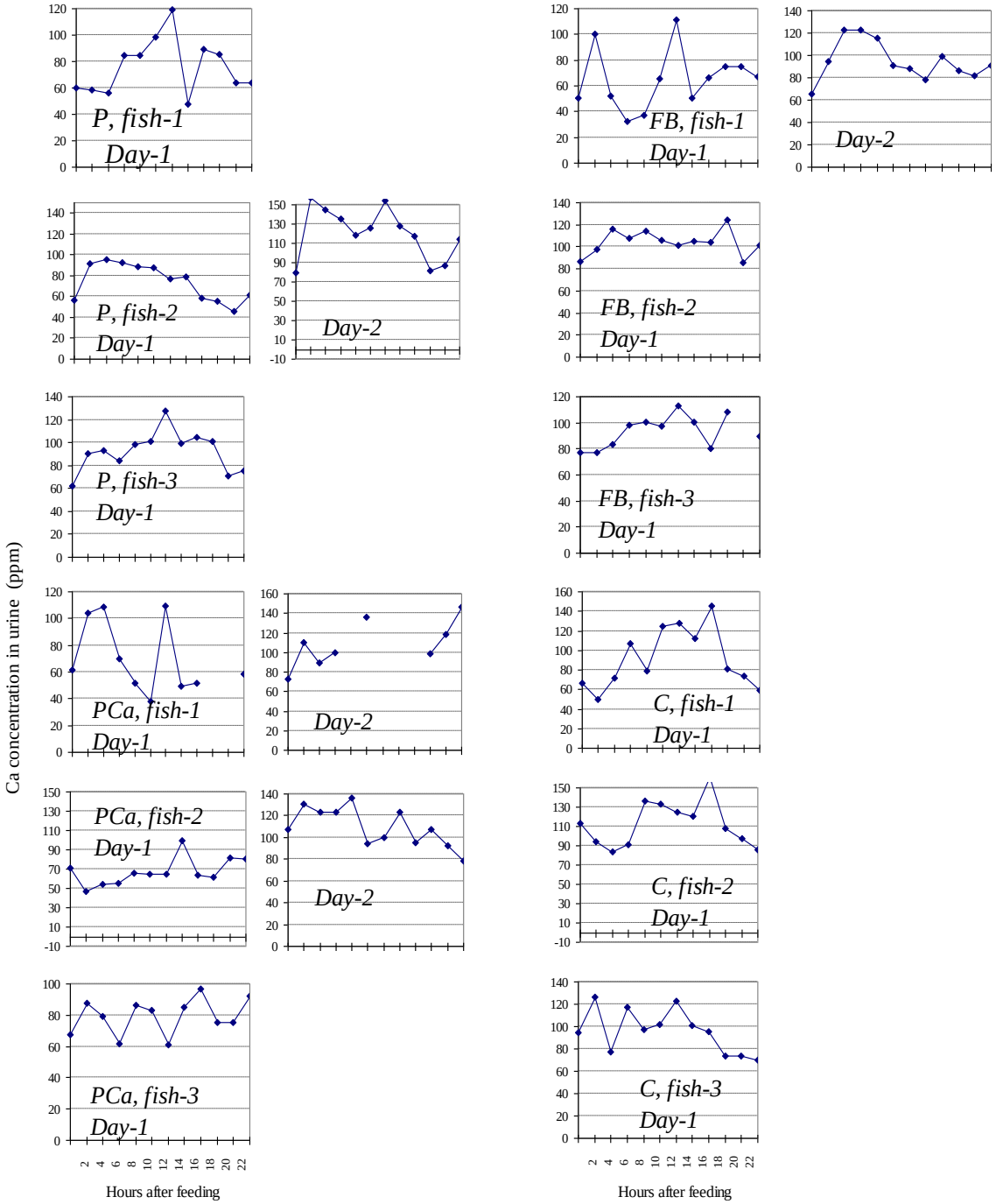
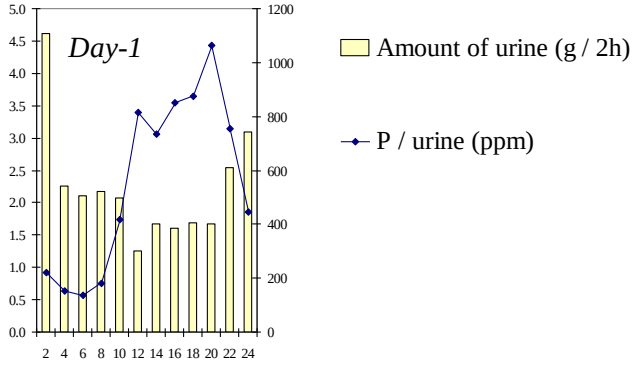
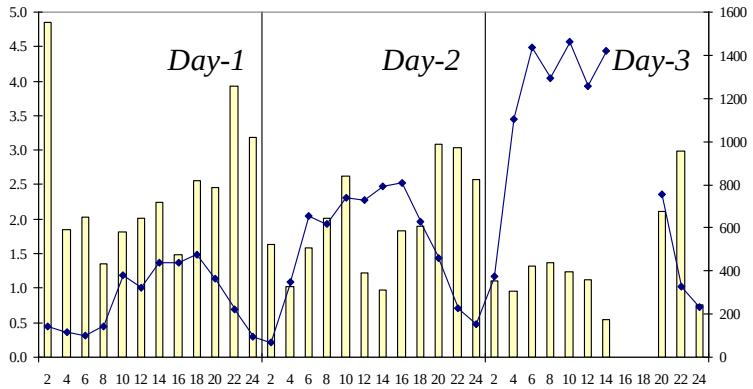


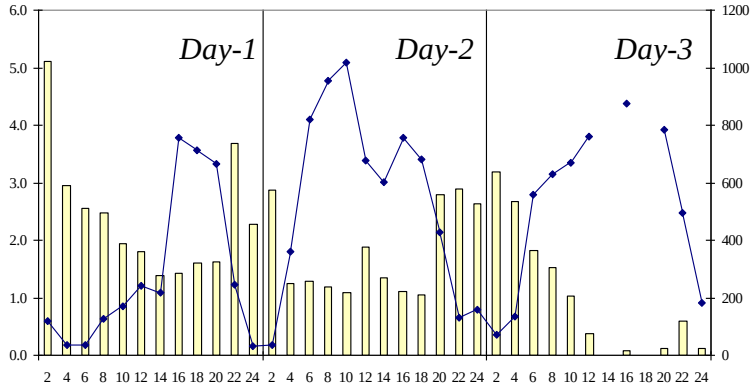
Figure 44. Urinary calcium concentrations of fish fed 4 kinds of diets (Expt. 3). P (potassium phosphate as the source of P), PCa (Diet P plus calcium carbonate), FB (fish bone as the source of P), C (commercial trout feed). Adjacent figures are the samples collected from the same fish at different days. Fish were placed in the metabolism chambers at Day-1, and urine samples were collected continuously using a catheter. Fish were fed at 7 a.m. each day. The urine collection vials were changed every 2 hours.



Balance Summary: Fish BW (g) 307.1; Total P fed (mg/ 24h) 25.68; Total P excreted in feces (mg/ 24h) 1.19; Urine amount (%BW/ 24h) 8.71; Total P excreted in urine (mg/ 24h) 13.08; Total P retained by fish (mg/ 24h) 11.42 (%/ dry diet) 0.395.

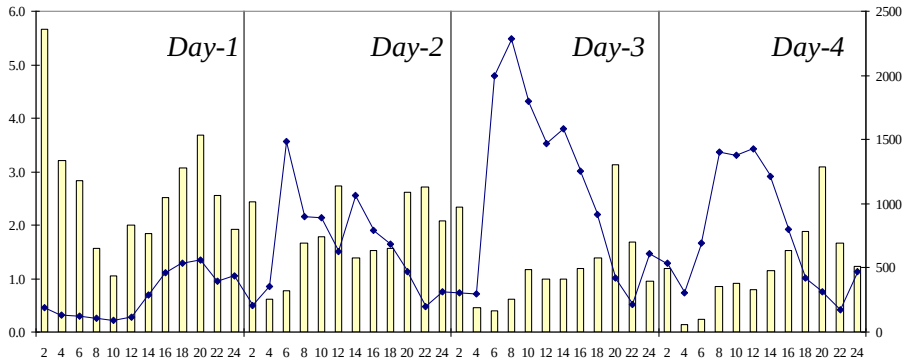


Balance Summary: Fish BW (g) 303.3; Total P fed (mg/ 24h) 25.3; Total P excreted in feces (mg/ 24h) 0.88; Urine amount (%BW/ 24h) 9.82; Total P excreted in urine (mg/ 24h) 7.57; Total P retained by fish (mg/ 24h) 16.84 (%/ dry diet) 0.591.



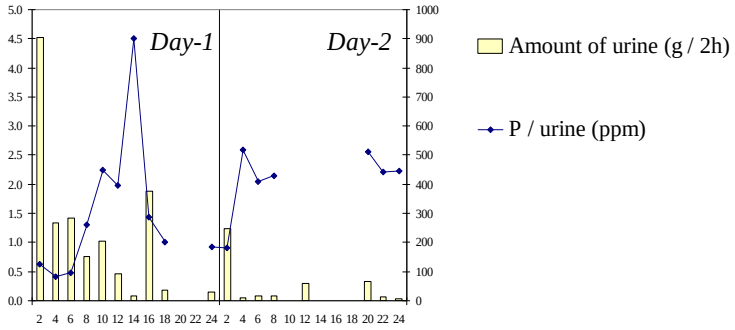
Balance Summary: Fish BW (g) 313.6; Total P fed (mg/ 24h) 26.19; Total P excreted in feces (mg/ 24h) 0.25; Urine amount (%BW/ 24h) 9.19; Total P excreted in urine (mg/ 24h) 6.46; Total P retained by fish (mg/ 24h) 19.48 (%/ dry diet) 0.660.

(Figure 45, continued)

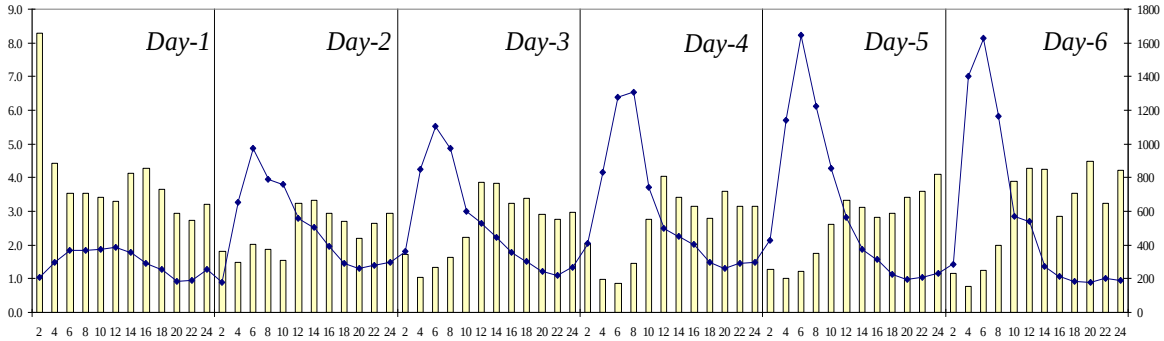


Balance Summary: Fish BW (g) 299.4; Total P fed (mg/ 24h) 24.97; Total P excreted in feces (mg/ 24h) 0.93; Urine amount (%BW/ 24h) 10.65; Total P excreted in urine (mg/24h) 9.61; Total P retained by fish (mg/ 24h) 14.43 (%/ dry diet) 0.513.

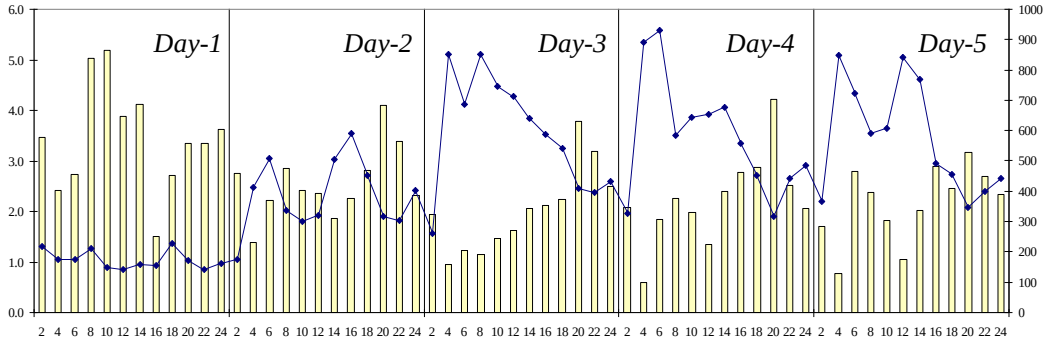
Figure 45. Urinary phosphorus excretion of rainbow trout fed diet "P" in the metabolic chamber (Expt. 3). The diet P contained KH_2PO_4 as the source of phosphorus. Values in the X-axis show the hours after feeding experimental diets. Fish were fed every 24 h. The scales in the primary and secondary Y-axis show the amount of urine (g/2h) and the urinary phosphorus concentrations (ppm), respectively.



Balance Summary: Fish BW (g) 305.0; Total P fed (mg/ 24h) 24.40; Total P excreted in feces (mg/ 24h) 5.50; Urine amount (%BW/ 24h) 3.87; Total P excreted in urine (mg/ 24h) 2.33; Total P retained by fish (mg/ 24h) 16.57 (%/ dry diet) 0.564.

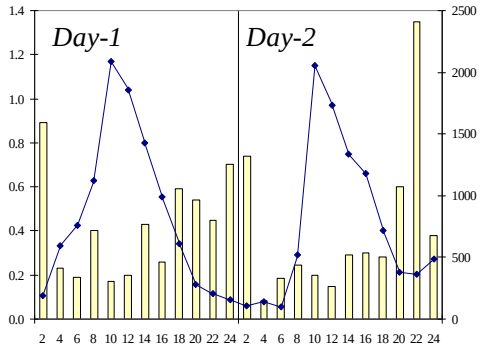


Balance Summary: Fish BW (g) 366.0; Total P fed (mg/ 24h) 29.11; Total P excreted in feces (mg/ 24h) 2.29; Urine amount (%BW/ 24h) 12.97; Total P excreted in urine (mg/ 24h) 13.72; Total P retained by fish (mg/ 24h) 13.10 (%/ dry diet) 0.374.



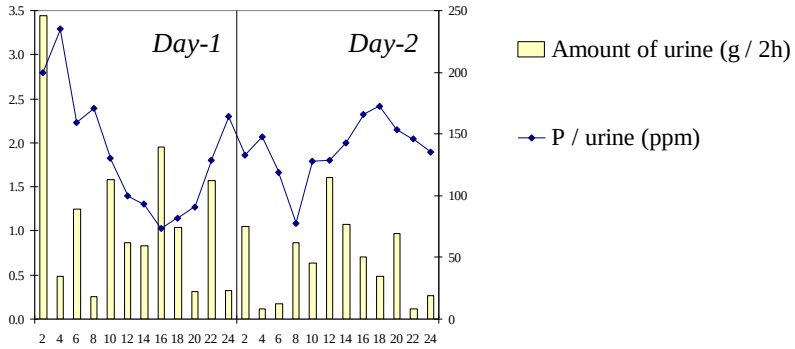
Balance Summary: Fish BW (g) 324.8; Total P fed (mg/ 24h) 25.81; Total P excreted in feces (mg/ 24h) 12.24; Urine amount (%BW/ 24h) 12.74; Total P excreted in urine (mg/ 24h) 7.18; Total P retained by fish (mg/ 24h) 6.40 (%/ dry diet) 0.206.

(Figure 46, continued)

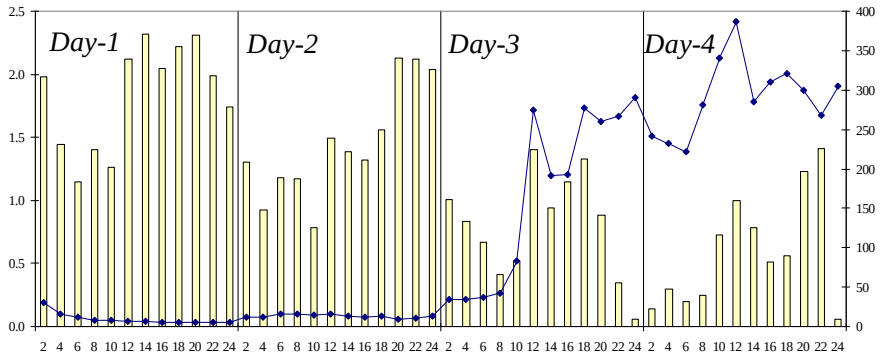


Balance Summary: Fish BW (g) 292.9; Total P fed (mg/ 24h) 23.30; Total P excreted in feces (mg/ 24h) 6.15; Urine amount (%BW/ 24h) 1.72; Total P excreted in urine (mg/ 24h) 3.21; Total P retained by fish (mg/ 24h) 13.94 (%/ dry diet) 0.497.

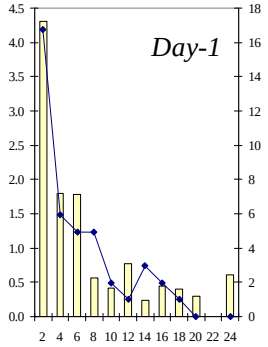
Figure 46. Urinary phosphorus excretion of rainbow trout fed diet "PCa" in the metabolic chamber (Expt. 3). The diet PCa contained KH_2PO_4 and $CaCO_3$ at the P/Ca ratio equivalent to fish bone. Values in the X-axis show the hours after feeding experimental diets. Fish were fed every 24 h. The scales in the primary and secondary Y-axis show the amount of urine (g/2h) and the urinary phosphorus concentrations (ppm), respectively.



Balance Summary: Fish BW (g) 316.0; Total P fed (mg/ 24h) 25.22; Total P excreted in feces (mg/ 24h) 10.35; Urine amount (%BW/ 24h) 4.40; Total P excreted in urine (mg/ 24h) 1.92; Total P retained by fish (mg/ 24h) 12.94 (%/ dry diet) 0.429.

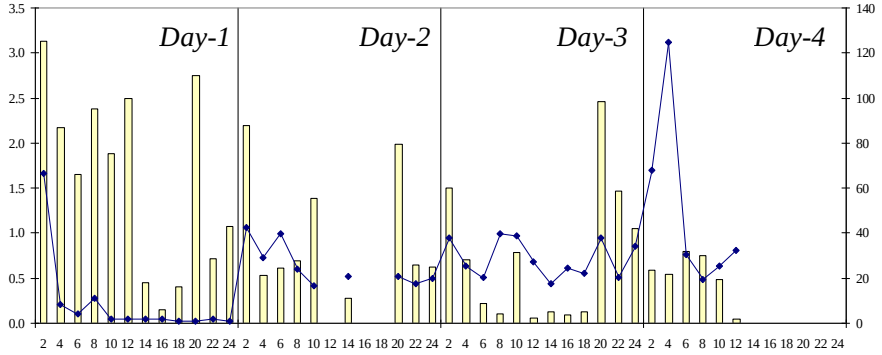


Balance Summary: Fish BW (g) 278.9; Total P fed (mg/ 24h) 22.27; Total P excreted in feces (mg/ 24h) 7.17; Urine amount (%BW/ 24h) 7.88; Total P excreted in urine (mg/ 24h) 0.21; Total P retained by fish (mg/ 24h) 14.90 (%/ dry diet) 0.560.

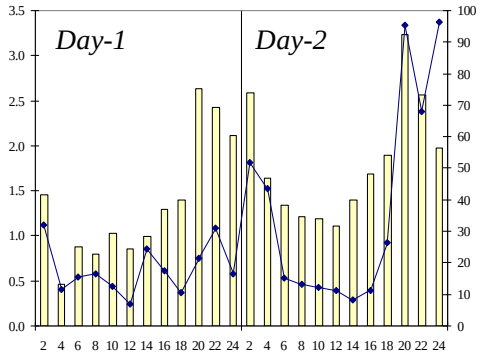


Balance Summary: Fish BW (g) 271.8; Total P fed (mg/ 24h) 21.66; Total P excreted in feces (mg/ 24h) 5.44; Urine amount (%BW/ 24h) 4.28; Total P excreted in urine (mg/ 24h) 0.10; Total P retained by fish (mg/ 24h) 16.12 (%/ dry diet) 0.623.

(Figure 47, continued)

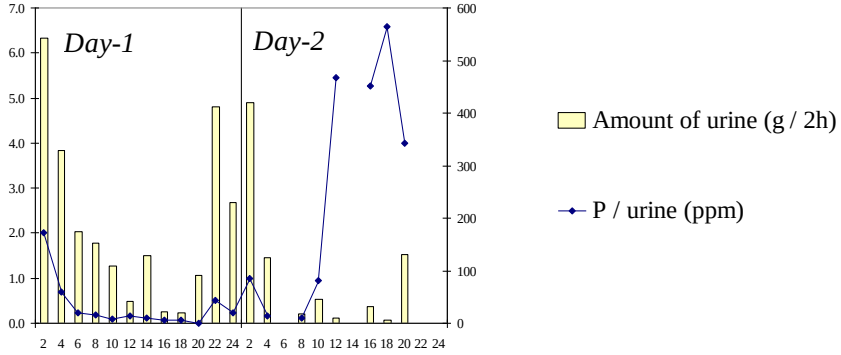


Balance Summary: Fish BW (g) 276.0; Total P fed (mg/ 24h) 22.03; Total P excreted in feces (mg/ 24h) 16.93; Urine amount (%BW/ 24h) 6.97; Total P excreted in urine (mg/ 24h) 0.28; Total P retained by fish (mg/ 24h) 4.82 (%/ dry diet) 0.183.

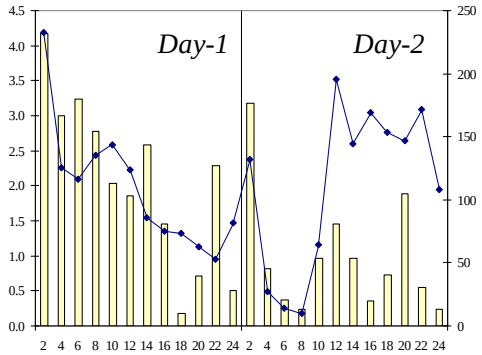


Balance Summary: Fish BW (g) 289.2; Total P fed (mg/ 24h) 26.63; Total P excreted in feces (mg/ 24h) 19.84; Urine amount (%BW/ 24h) 5.65; Total P excreted in urine (mg/ 24h) 0.33; Total P retained by fish (mg/ 24h) 6.46 (%/ dry diet) 0.203.

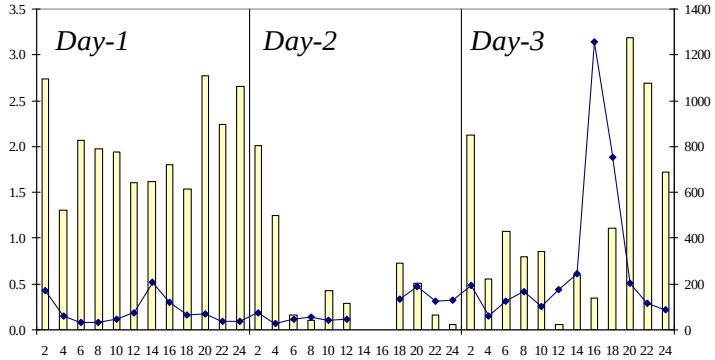
Figure 47. Urinary phosphorus excretion of rainbow trout fed diet "FB" in the metabolic chamber (Expt. 3). The diet FB contained fish bone as the source of phosphorus. Values in the X-axis show the hours after feeding experimental diets. Fish were fed every 24 h. The scales in the primary and secondary Y-axis show the amount of urine (g/2h) and the urinary phosphorus concentrations (ppm), respectively.



Balance Summary: Fish BW (g) 417.7; Total P fed (mg/ 24h) 64.49; Total P excreted in feces (mg/ 24h) 50.45; Urine amount (%BW/ 24h) 6.29; Total P excreted in urine (mg/ 24h) 1.68; Total P retained by fish (mg/ 24h) 12.35 (%/ dry diet) 0.254.

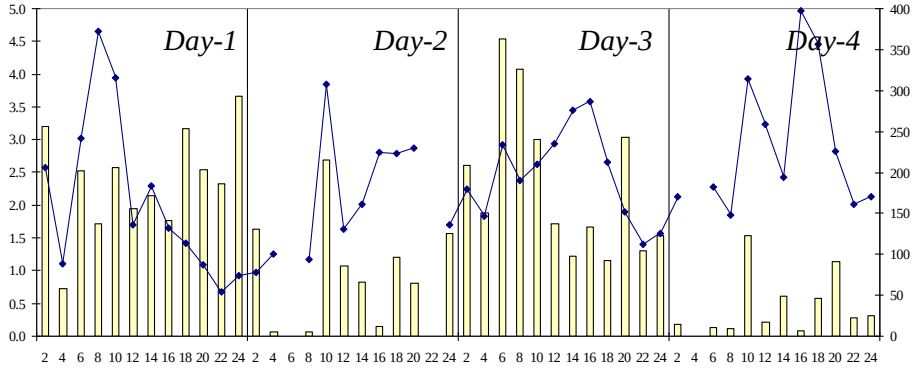


Balance Summary: Fish BW (g) 357.4; Total P fed (mg/ 24h) 55.46; Total P excreted in feces (mg/ 24h) 25.51; Urine amount (%BW/ 24h) 6.94; Total P excreted in urine (mg/ 24h) 3.17; Total P retained by fish (mg/ 24h) 26.78 (%/ dry diet) 0.639.

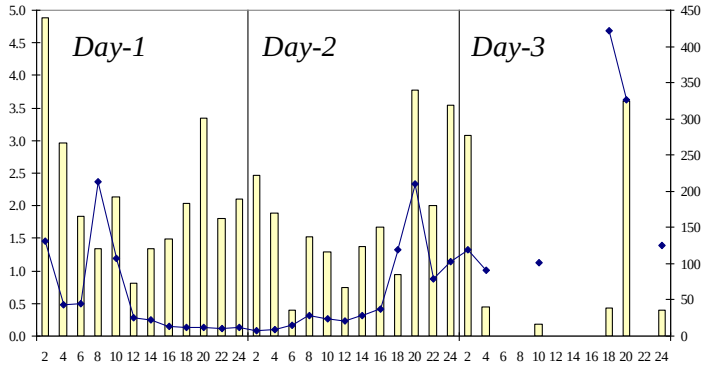


Balance Summary: Fish BW (g) 297.6; Total P fed (mg/ 24h) 45.84; Total P excreted in feces (mg/ 24h) 15.97; Urine amount (%BW/ 24h) 8.15; Total P excreted in urine (mg/ 24h) 1.91; Total P retained by fish (mg/ 24h) 27.96 (%/ dry diet) 0.808.

(Figure 48. Continued)



Balance Summary: Fish BW (g) 298.1; Total P fed (mg/ 24h) 46.08; Total P excreted in feces (mg/ 24h) 22.68; Urine amount (%BW/ 24h) 9.49; Total P excreted in urine (mg/ 24h) 4.65; Total P retained by fish (mg/ 24h) 18.74 (%/ dry diet) 0.539.



Balance Summary: Fish BW (g) 338.6; Total P fed (mg/ 24h) 46.44; Total P excreted in feces (mg/ 24h) 14.80; Urine amount (%BW/ 24h) 7.71; Total P excreted in urine (mg/ 24h) 1.55; Total P retained by fish (mg/ 24h) 30.09 (%/ dry diet) 0.858.

Figure 48. Urinary phosphorus excretion of rainbow trout fed diet "C" in the metabolic chamber (Expt. 3). The diet C was a commercial feed. Values in the X-axis show the hours after feeding experimental diets. Fish were fed every 24 h. The scales in the primary and secondary Y-axis show the amount of urine (g/2h) and the urinary phosphorus concentrations (ppm), respectively.

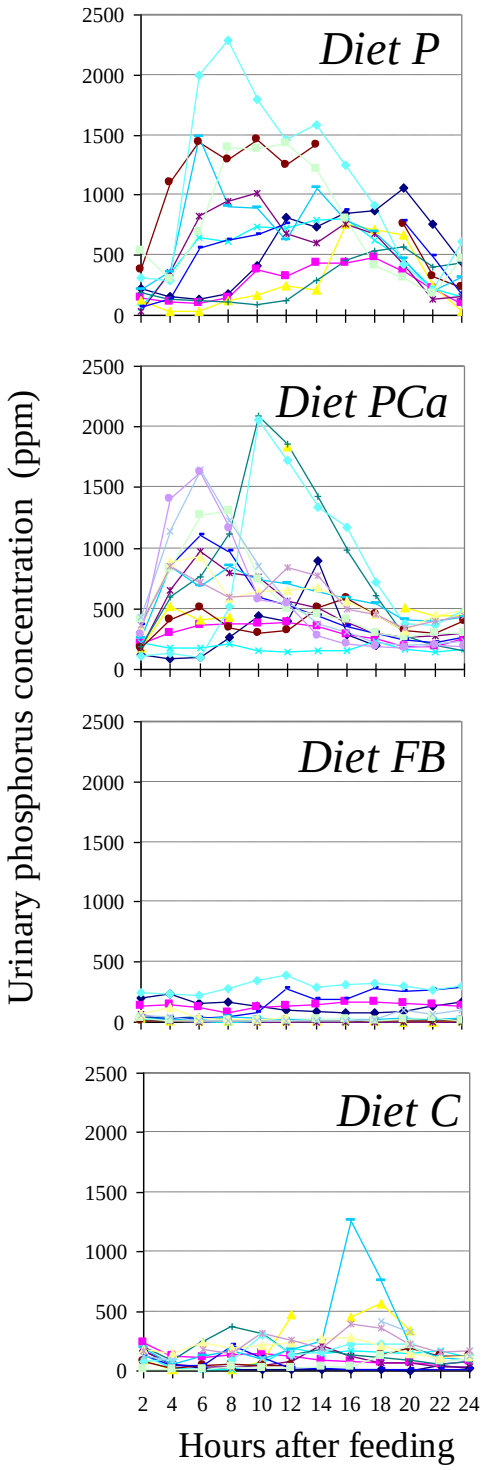
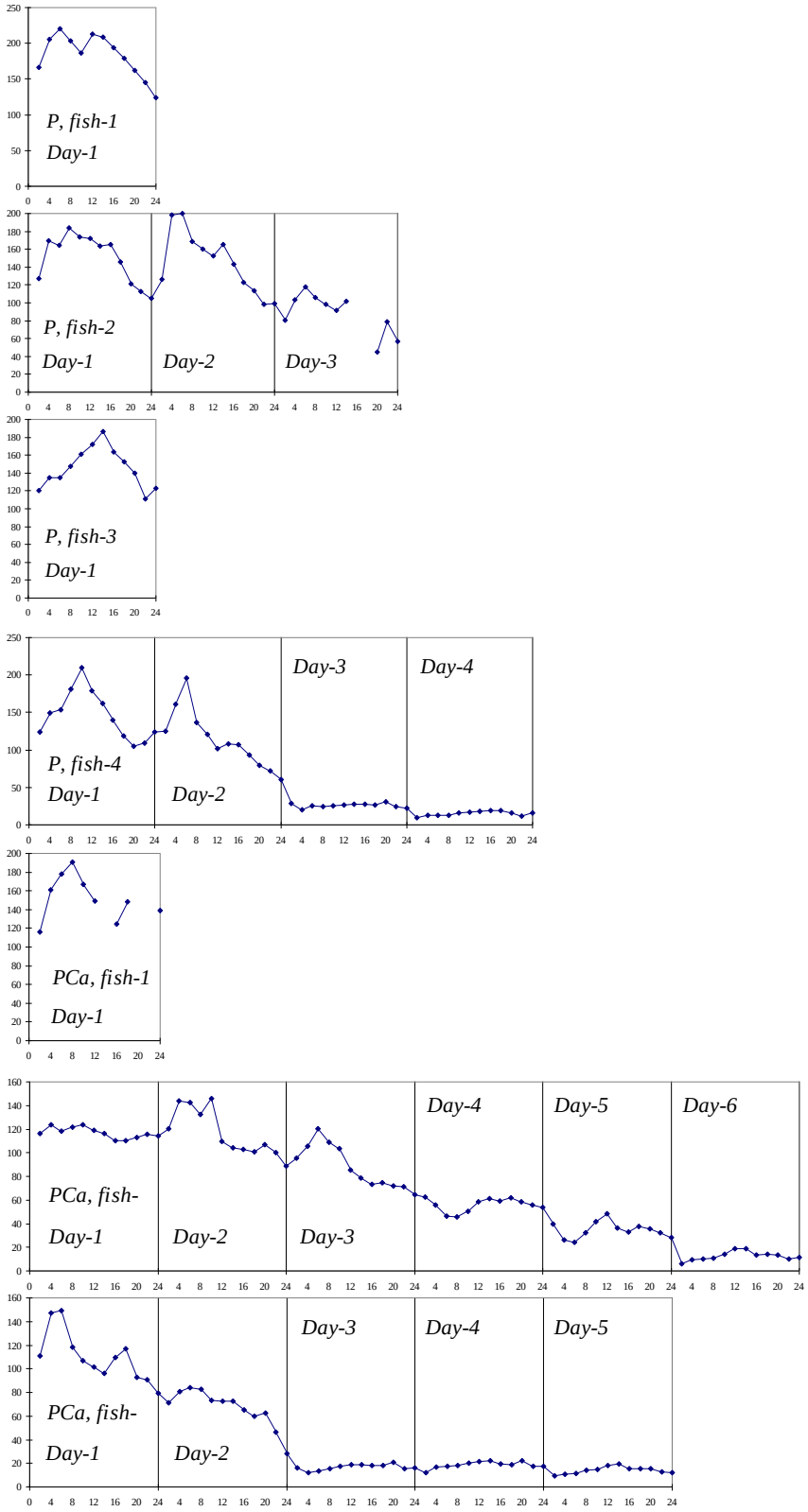


Figure 49. Overall view of urinary phosphorus concentrations after feeding (Expt. 3).



(Figure 50, continued)

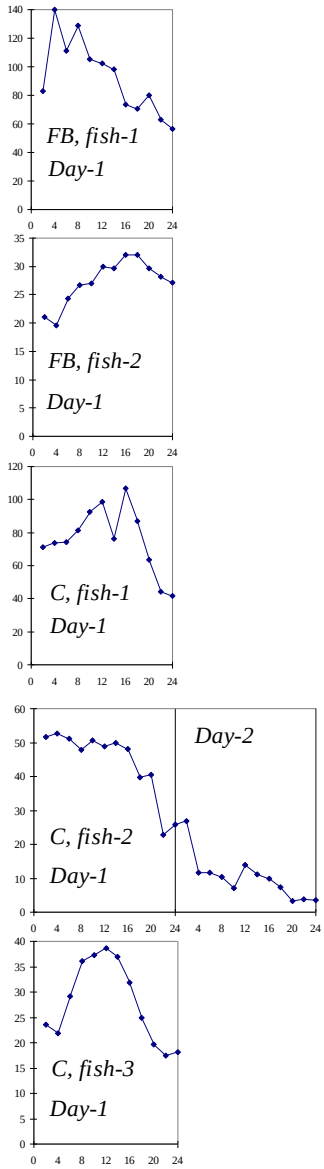


Figure 50. Urinary ascorbic acid concentrations of fish fed 4 kinds of diets (Expt. 3). P (potassium phosphate as the source of P), PCa (Diet P plus calcium carbonate), FB (fish bone as the source of P), C (commercial trout feed). Figures in rows are the samples collected from the same fish at different days. Fish were placed in the metabolism chambers at Day-1, and urine samples were collected continuously using catheter. Fish were fed at the beginning of each day. The urine collection vials were changed every 2 hours. Scales in Y-axis are ascorbic acid concentrations as ppm in urine.

Appendix II. *Data Figures for Chapter II*

Table 72. Correlation summary[1] in the digestibility study-1.

	Digestibility or Availability											
	DM				CP				Ash			
	Animal		Plant		Animal		Plant		Animal		Plant	
	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout
TDN	1.00	1.00	1.00	1.00	0.42	0.55	0.12	0.32	-0.02	0.19	0.39	-0.12
CP	0.56	0.60	1.00**	1.00**	-0.37	-0.30	0.12	0.33	-0.21	-0.28	0.40	-0.08
Ash	0.12	-0.23	-0.22	-0.27	-0.02	-0.10	-0.40	-0.93*	-0.71	-0.70	-0.52	-0.32
Ca	0.08	-0.27	0.02	-0.05	-0.09	-0.18	-0.10	-0.77	-0.85*	-0.83*	-0.21	-0.09
Cu	-0.39	-0.27	0.29	0.26	-0.84*	-0.79	-0.72	-0.82	0.33	0.27	-0.64	-0.76
Fe	-0.55	-0.74	0.23	0.18	-0.83*	-0.87*	-0.60	-0.87	-0.48	-0.51	-0.60	-0.67
K	0.41	0.21	-0.21	-0.27	0.38	0.37	-0.36	-0.93*	0.14	0.08	-0.50	-0.31
Mg	0.12	-0.02	-0.59	-0.61	0.49	0.44	-0.44	-0.77	-0.43	-0.30	-0.57	-0.22
Mn	-0.84*	-0.86*	-0.58	-0.57	-0.74	-0.78	-0.26	-0.41	-0.16	-0.15	-0.31	0.02
Na	0.25	0.16	0.24	0.16	-0.02	0.00	0.26	-0.37	-0.24	-0.18	0.12	0.11
P	0.27	-0.13	-0.50	-0.49	0.08	-0.01	-0.72	-0.74	-0.71	-0.74	-0.76	-0.51
Sr	0.21	0.37	-0.02	-0.09	-0.01	0.03	-0.23	-0.85	-0.71	-0.58	-0.34	-0.22
Zn	-0.44	-0.45	-0.26	-0.27	-0.94**	-0.93**	-0.23	-0.44	-0.02	-0.11	-0.20	0.00

(Table 72, continued)

	Digestibility or Availability											
	Ca				Cu				Fe			
	Animal		Plant		Animal		Plant		Animal		Plant	
	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout
TDN	-0.37	-0.07	0.32	0.17	0.20	0.29	0.01	0.41	-0.24	-0.23	0.14	0.05
CP	-0.28	-0.28	0.32	0.19	-0.48	-0.23	-0.07	0.40	-0.07	-0.24	0.16	0.08
Ash	-0.69	-0.42	-0.63	-0.64	0.52	-0.59	0.41	-0.87	-0.79	-0.77	-0.26	-0.41
Ca	-0.80*	-0.56	-0.31	-0.37	0.42	-0.70	0.63	-0.63	-0.82*	-0.80*	0.02	-0.13
Cu	0.58	0.38	-0.71	-0.83	-0.92**	0.22	0.10	-0.73	0.78	0.75	-0.59	-0.75
Fe	-0.14	-0.15	-0.60	-0.76	-0.36	-0.68	0.35	-0.69	-0.06	0.01	-0.54	-0.65
K	-0.06	0.12	-0.57	-0.61	0.64	0.08	0.50	-0.83	-0.32	-0.38	-0.25	-0.39
Mg	-0.54	-0.16	-0.77	-0.62	0.81*	-0.13	-0.02	-0.93*	-0.75	-0.65	-0.22	-0.40
Mn	0.22	0.13	-0.62	-0.37	-0.44	-0.41	-0.35	-0.72	0.26	0.40	0.02	-0.18
Na	-0.30	0.04	0.27	0.07	0.24	0.05	0.96*	-0.02	-0.38	-0.38	0.14	0.18
P	-0.76	-0.54	-0.94*	-0.81	0.56	-0.54	-0.33	-0.95*	-0.81*	-0.85*	-0.49	-0.67
Sr	-0.73	-0.46	-0.43	-0.49	0.01	-0.72	0.58	-0.71	-0.68	-0.62	-0.12	-0.27
Zn	0.29	0.04	-0.59	-0.37	-0.89**	-0.20	-0.28	-0.73	0.53	0.50	0.11	-0.17

(Table 72, continued)

	Digestibility or Availability											
	K				Mg				Mn			
	Animal		Plant		Animal		Plant		Animal		Plant	
	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout
TDN	0.70	0.66	0.86	0.29	-0.03	0.29	0.54	0.26	-0.53	-0.29	0.54	0.35
CP	0.23	0.21	0.84	0.33	-0.19	-0.12	0.53	0.27	-0.66	-0.73	0.53	0.38
Ash	0.30	0.16	0.29	0.57	-0.93**	-0.96**	-0.62	-0.60	-0.68	-0.59	-0.32	-0.49
Ca	0.12	-0.03	0.52	0.80	-0.99**	-0.99**	-0.30	-0.29	-0.75	-0.63	0.05	-0.16
Cu	-0.62	-0.36	0.59	0.34	0.31	0.36	-0.50	-0.75	0.28	0.20	-0.40	-0.64
Fe	-0.60	-0.66	0.58	0.41	-0.55	-0.49	-0.39	-0.66	-0.27	-0.27	-0.31	-0.57
K	0.86*	0.74	0.30	0.57	-0.19	-0.32	-0.55	-0.55	-0.17	-0.24	-0.28	-0.46
Mg	0.47	0.45	-0.16	0.27	-0.61	-0.64	-0.89*	-0.67	-0.29	-0.12	-0.57	-0.58
Mn	-0.82*	-0.82*	-0.29	0.18	-0.14	-0.07	-0.83	-0.47	0.21	0.20	-0.46	-0.36
Na	0.45	0.62	0.52	0.62	-0.53	-0.55	0.36	0.20	-0.41	-0.31	0.44	0.22
P	0.36	0.20	-0.19	0.01	-0.90**	-0.96**	-0.99**	-0.87	-0.74	-0.65	-0.79	-0.79
Sr	0.11	-0.07	0.48	0.72	-0.53	-0.35	-0.39	-0.41	-0.71	-0.59	-0.08	-0.29
Zn	-0.78	-0.67	0.08	0.46	0.03	0.09	-0.74	-0.43	0.02	-0.05	-0.27	-0.26

(Table 72, continued)

Digestibility or Availability												
	Na				P				Sr			
	Animal		Plant		Animal		Plant		Animal		Plant	
	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout	Coho	Trout
TDN	0.63	0.71	0.25	0.16	-0.21	0.13	0.19	-0.02	-0.29	0.12	0.23	0.05
CP	0.00	0.03	0.28	0.19	-0.18	-0.23	0.20	0.01	0.29	0.08	0.22	0.08
Ash	0.58	0.02	-0.62	-0.48	-0.94**	-0.86*	-0.64	-0.56	-0.60	-0.66	-0.34	-0.31
Ca	0.45	-0.03	-0.35	-0.21	-0.98**	-0.94**	-0.35	-0.35	-0.64	-0.70	-0.01	-0.02
Cu	-0.63	-0.65	-0.77	-0.74	0.46	0.44	-0.81	-0.86	0.78	0.54	-0.61	-0.68
Fe	-0.45	-0.79	-0.74	-0.69	-0.37	-0.39	-0.71	-0.83	0.18	-0.06	-0.49	-0.58
K	0.74	0.27	-0.61	-0.47	-0.30	-0.18	-0.60	-0.56	-0.18	-0.25	-0.30	-0.28
Mg	0.78	0.53	-0.56	-0.42	-0.70	-0.54	-0.68	-0.38	-0.78	-0.54	-0.48	-0.32
Mn	-0.73	-0.82*	-0.26	-0.13	0.06	0.02	-0.48	-0.06	0.36	0.22	-0.35	-0.13
Na	0.69	0.31	-0.02	0.04	-0.58	-0.40	0.16	-0.12	-0.28	-0.26	0.39	0.25
P	0.67	0.08	-0.73	-0.64	-0.96**	-0.90**	-0.87	-0.58	-0.69	-0.77	-0.76	-0.61
Sr	-0.08	0.35	-0.47	-0.34	-0.49	-0.52	-0.47	-0.47	-0.22	-0.06	-0.14	-0.16
Zn	-0.74	-0.84*	-0.22	-0.09	0.20	0.11	-0.48	-0.10	0.62	0.31	-0.27	-0.08

(Table 72, continued)

Digestibility or Availability				
	Zn			
	Animal		Plant	
	Coho	Trout	Coho	Trout
TDN	0.41	0.02	0.33	0.14
CP	-0.27	-0.54	0.35	0.16
Ash	-0.37	-0.64	-0.67	-0.69
Ca	-0.49	-0.75	-0.39	-0.43
Cu	-0.46	0.18	-0.76	-0.86
Fe	-0.96**	-0.53	-0.72	-0.78
K	0.34	0.00	-0.65	-0.65
Mg	0.21	-0.13	-0.67	-0.67
Mn	-0.78	-0.12	-0.40	-0.43
Na	0.00	-0.16	0.03	0.06
P	-0.29	-0.66	-0.82	-0.84
Sr	-0.30	-0.68	-0.51	-0.54
Zn	-0.76	-0.20	-0.35	-0.46

[1] The correlation coefficients between nutrient contents in diets (left column) and digestibility or availability of nutrients in diets (rows). n=7 for animal sources (herring meal, anchovy meal, menhaden meal, deboned whitefish meal, poultry byproduct meal, feather meal and casein diet); n=5 for plant sources (soybean meal, wheat gluten meal, corn gluten meal, wheat middling, wheat flour). Values with asterisks are statistically significant * ($P< 0.05$), ** ($P< 0.01$).

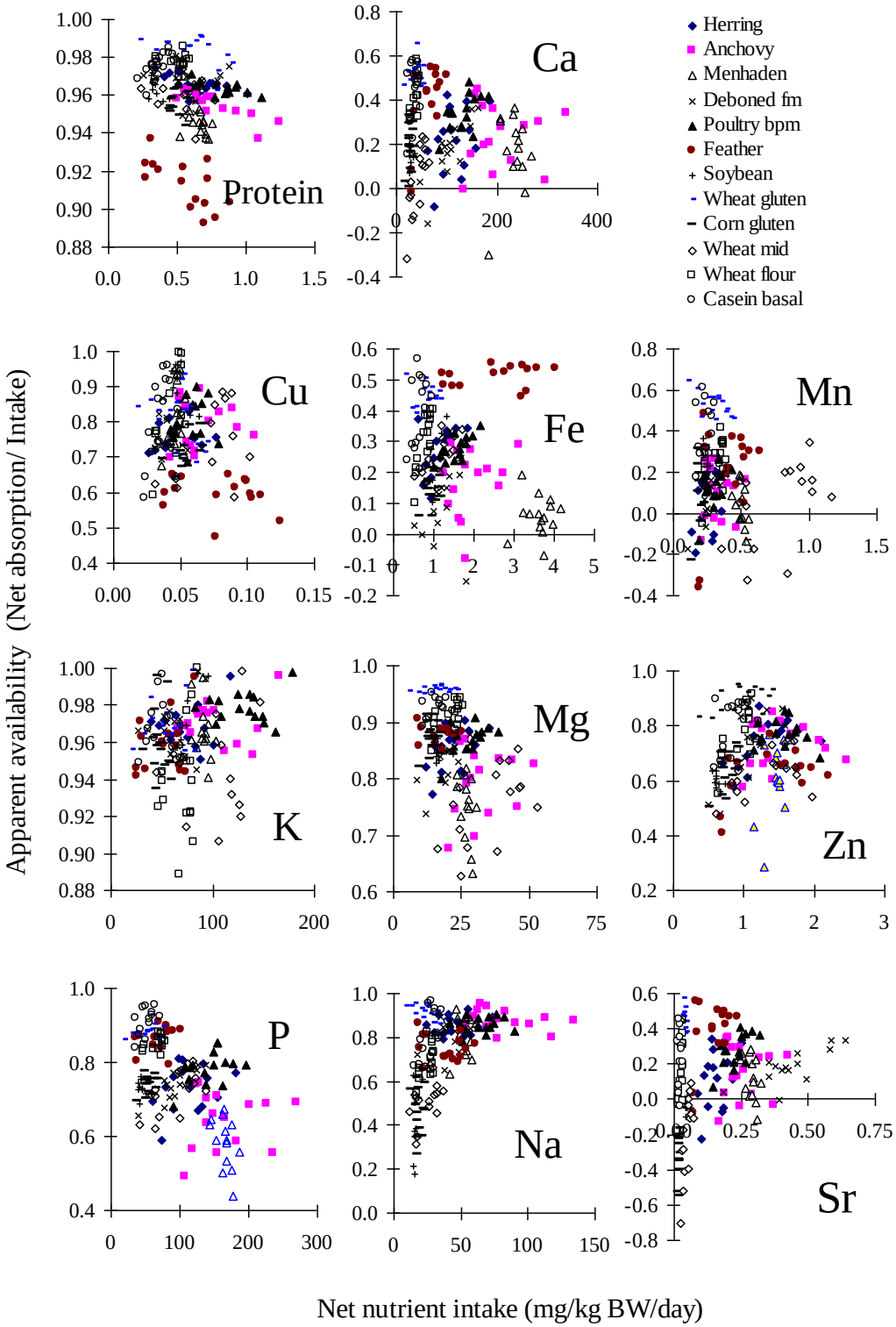


Figure 51. Apparent digestibility of protein and availability of minerals at various levels of intake (data from Study-1 with coho salmon only).

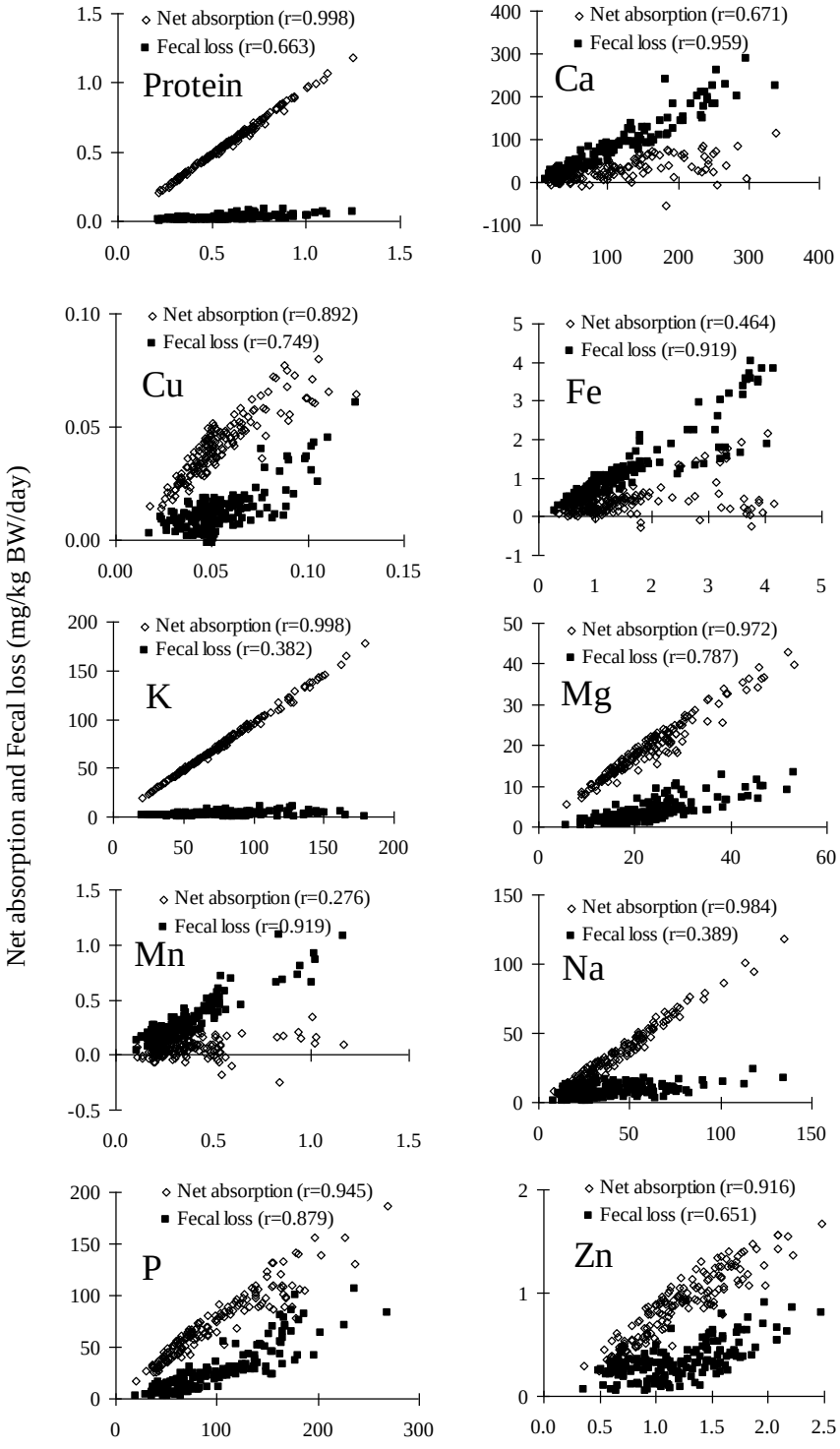


Figure 52. Net absorption and fecal loss of nutrients at various levels of intake (data from Study-1 with coho salmon only).

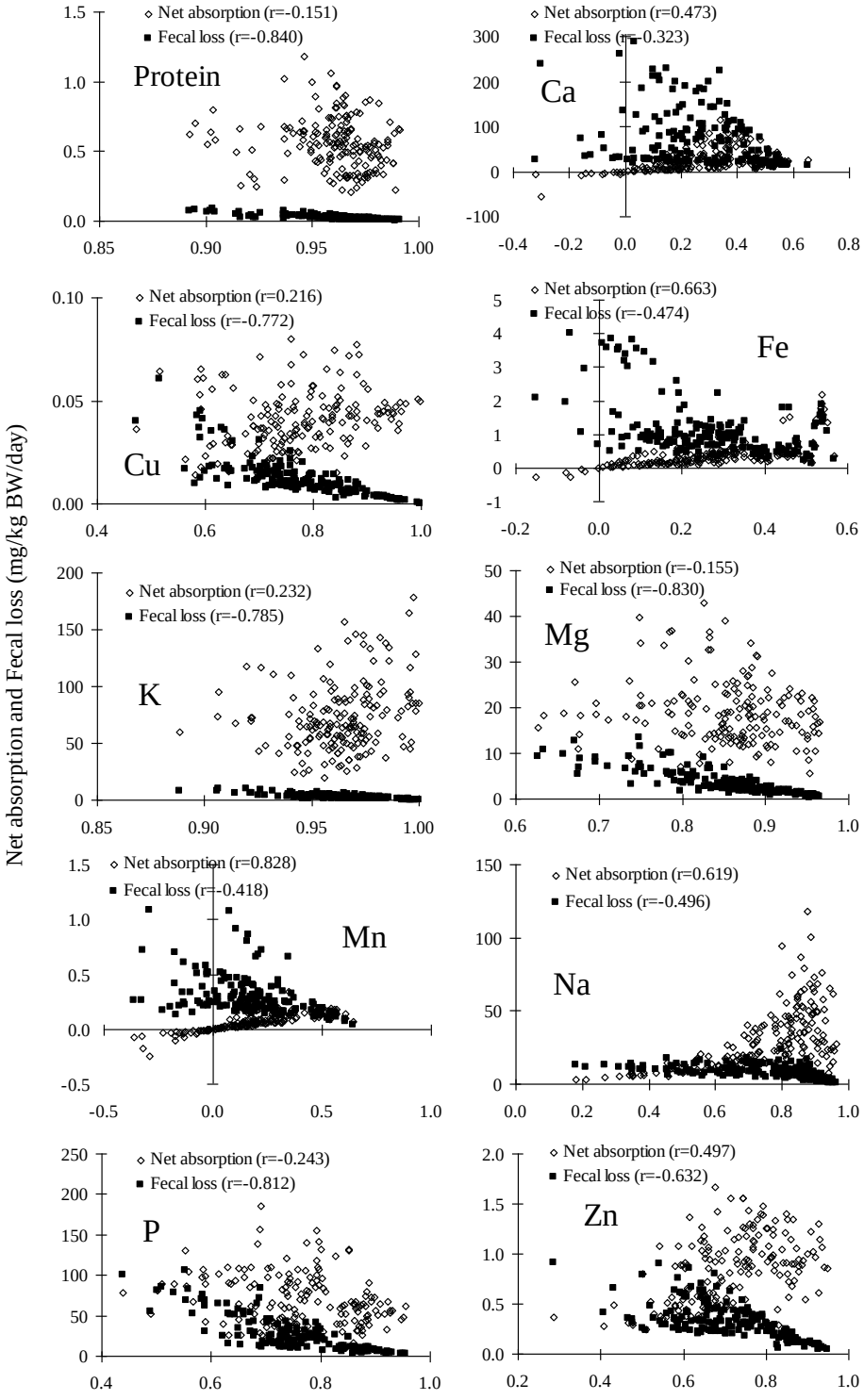
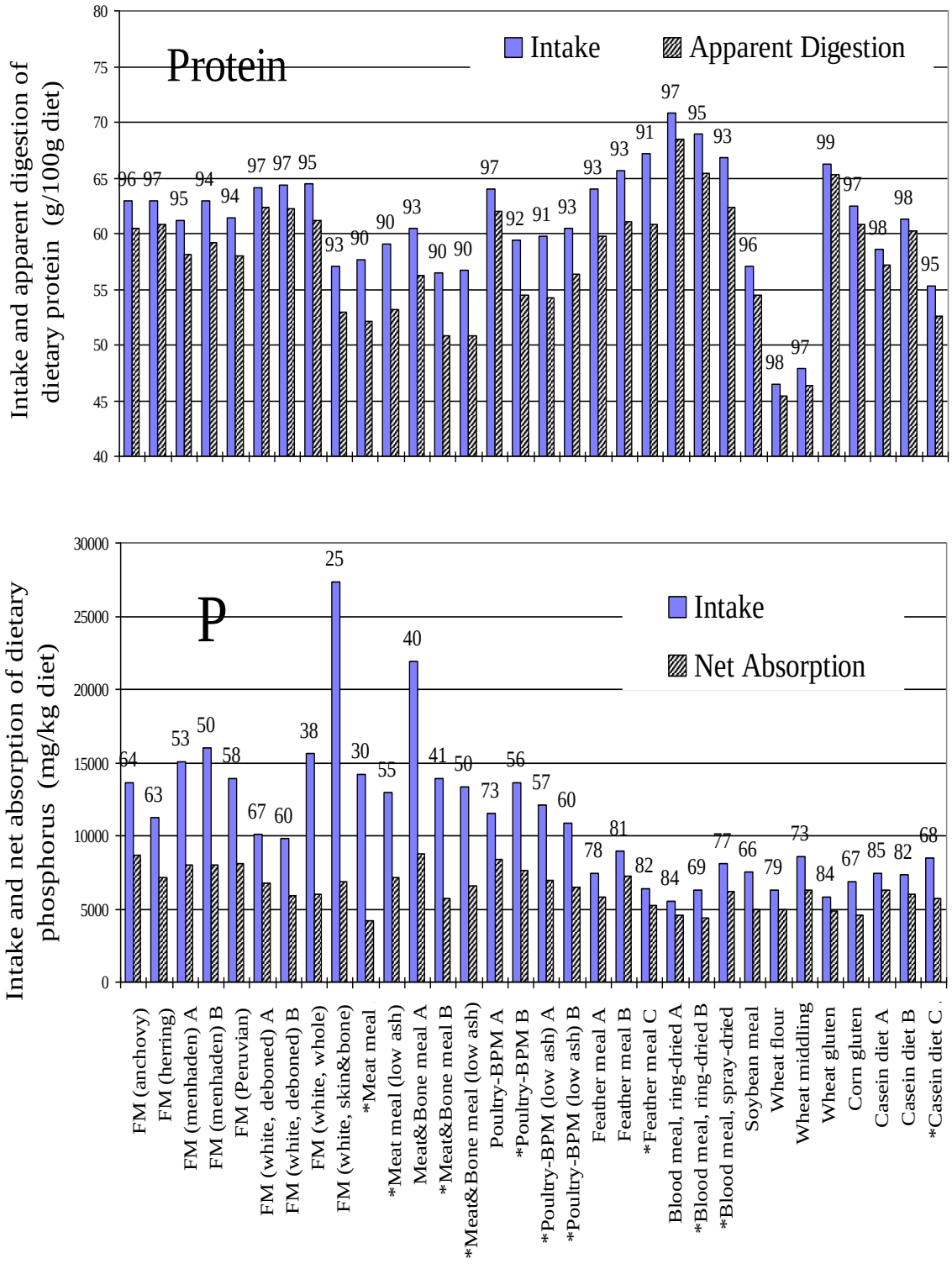
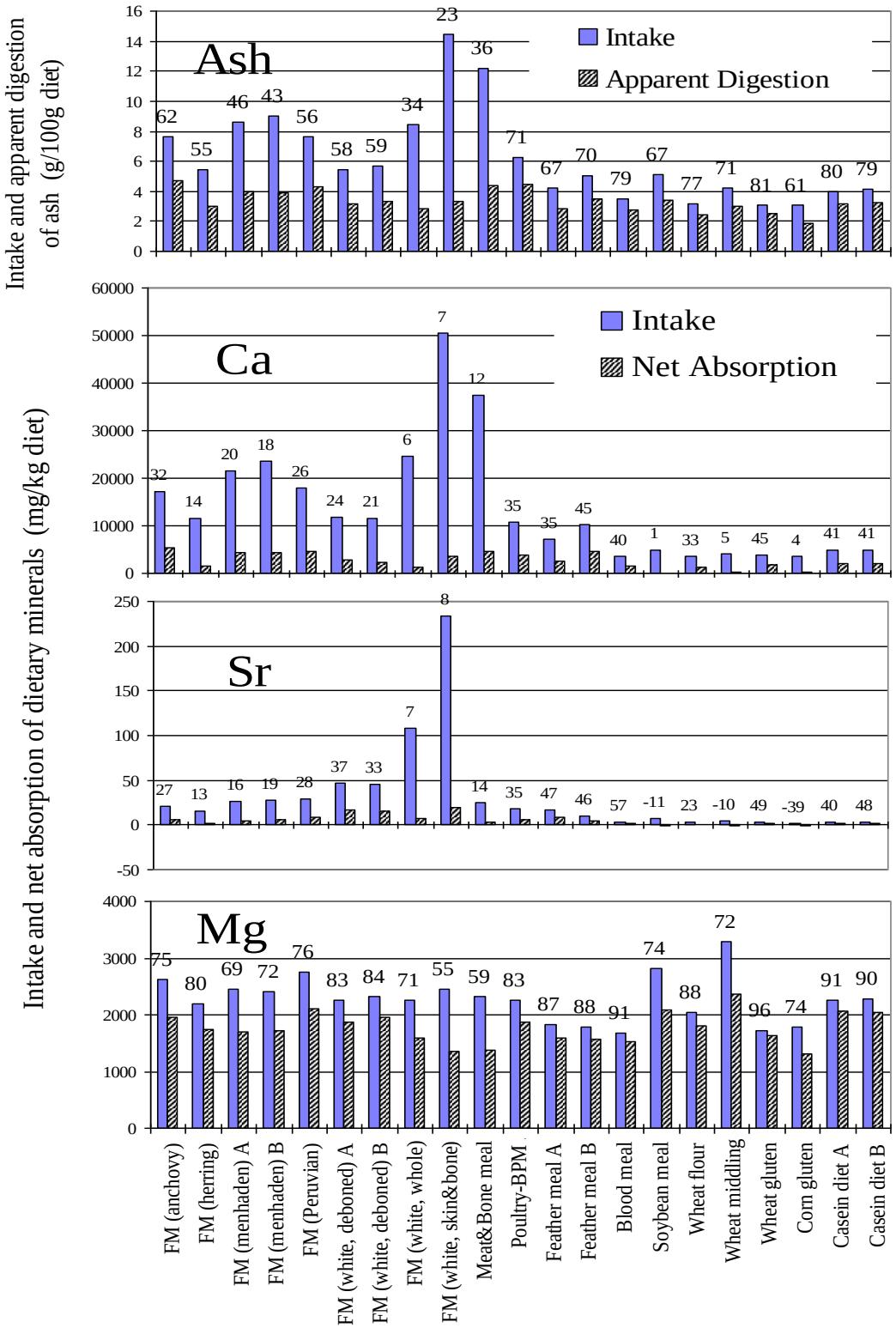


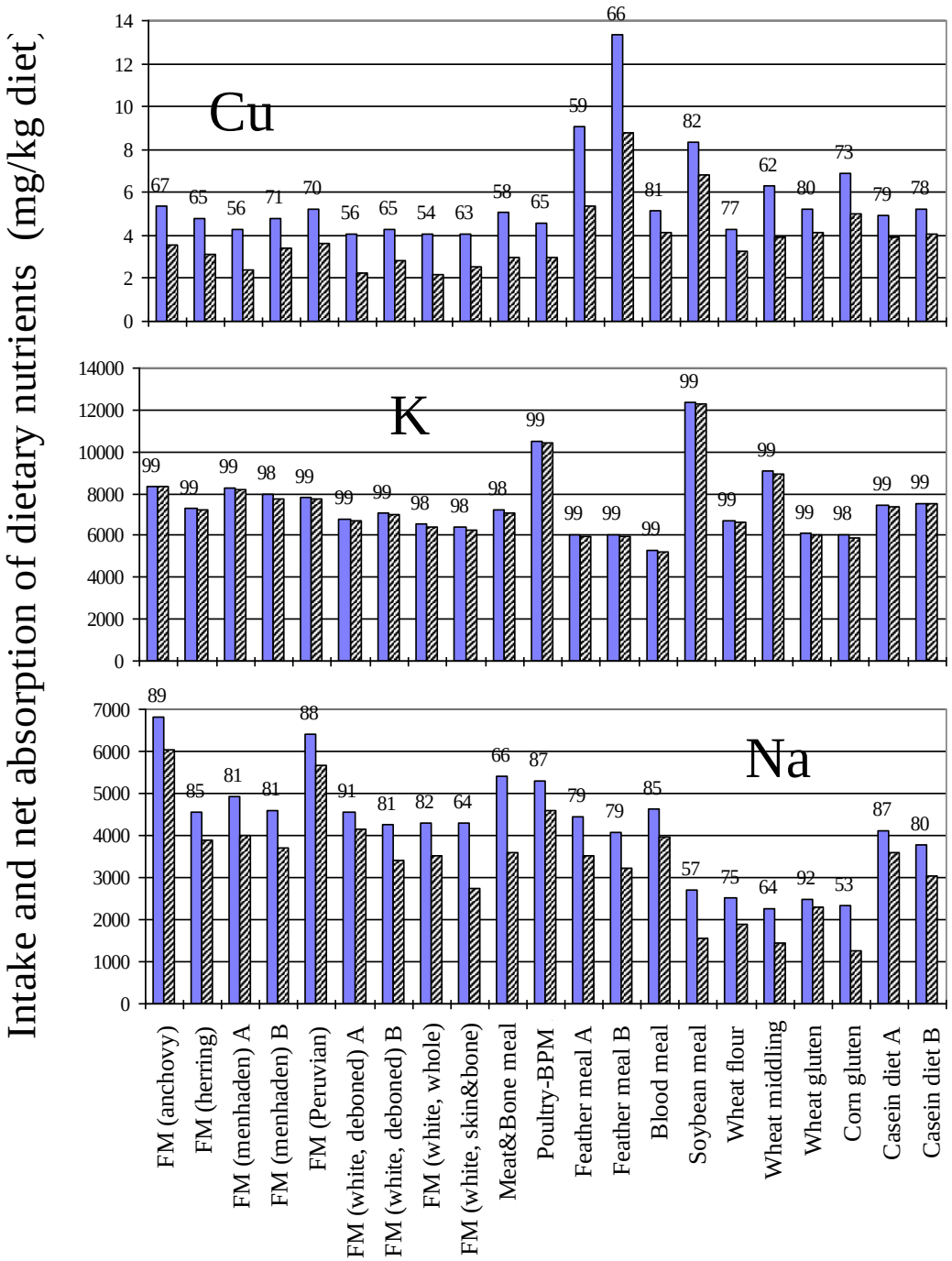
Figure 53. Relationship between digestibility of dietary nutrients and the net absorption or fecal loss of nutrients (data from Study-1 with coho salmon only).



(Figure 54, continued)



(Figure 54, continued)



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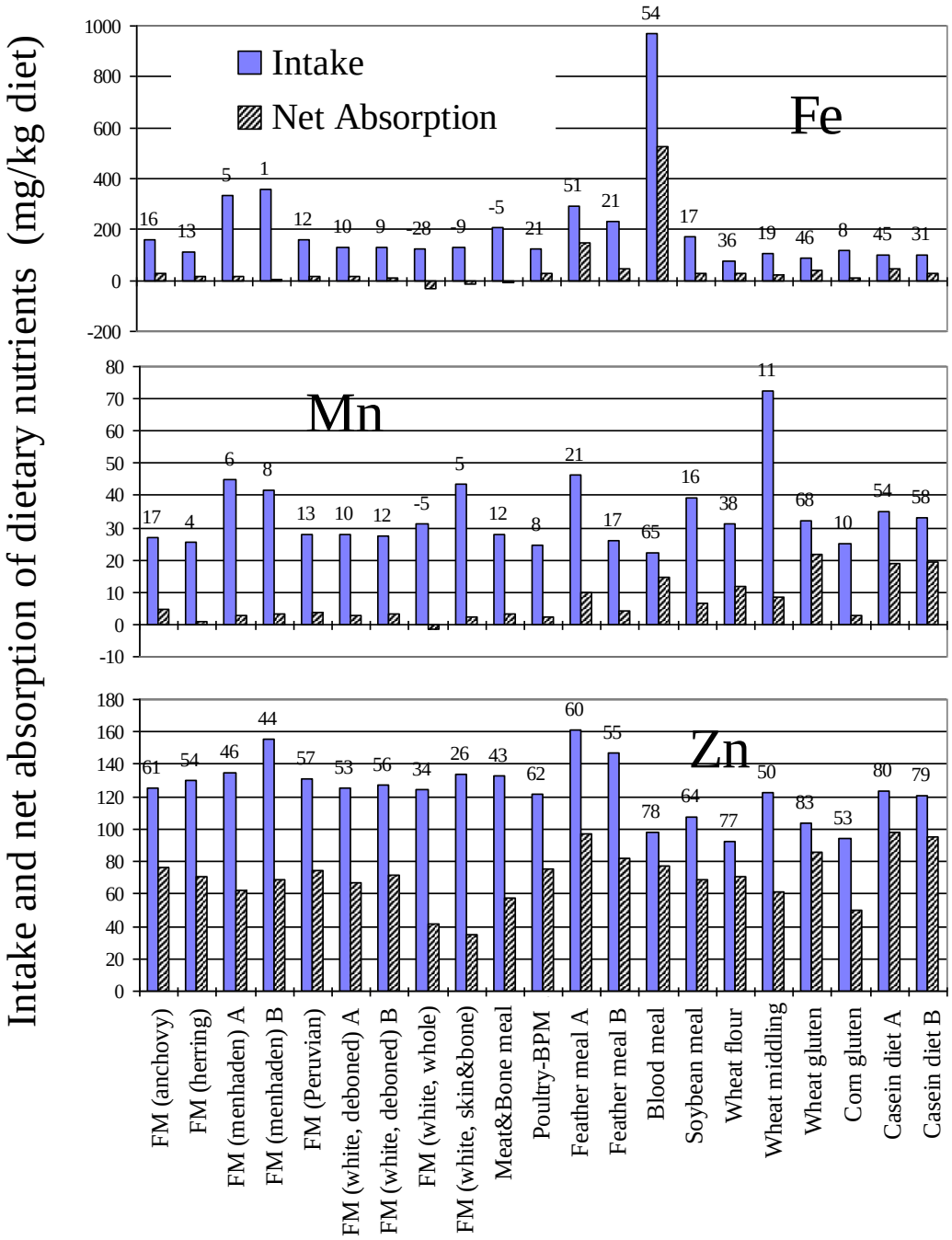


Figure 54. Intake and apparent digestion (net absorption) of dietary protein and minerals in test diets (whole diet) by rainbow trout (data from Study-1 and 2). All diets contained the casein basal diets at 70% and one of the test ingredients at 30% (as-is basis). The number in each column indicates the apparent digestibility (availability) of specified nutrients in the diets expressed as the percentage of dietary intake. For protein and phosphorus, feces were collected either by settling (no asterisk) or by stripping (with an asterisk). For other minerals, feces were collected by settling. The test ingredients of the same name but with different letters (A, B or C) were obtained from different suppliers; the letter does not indicate the quality or grade of the product.

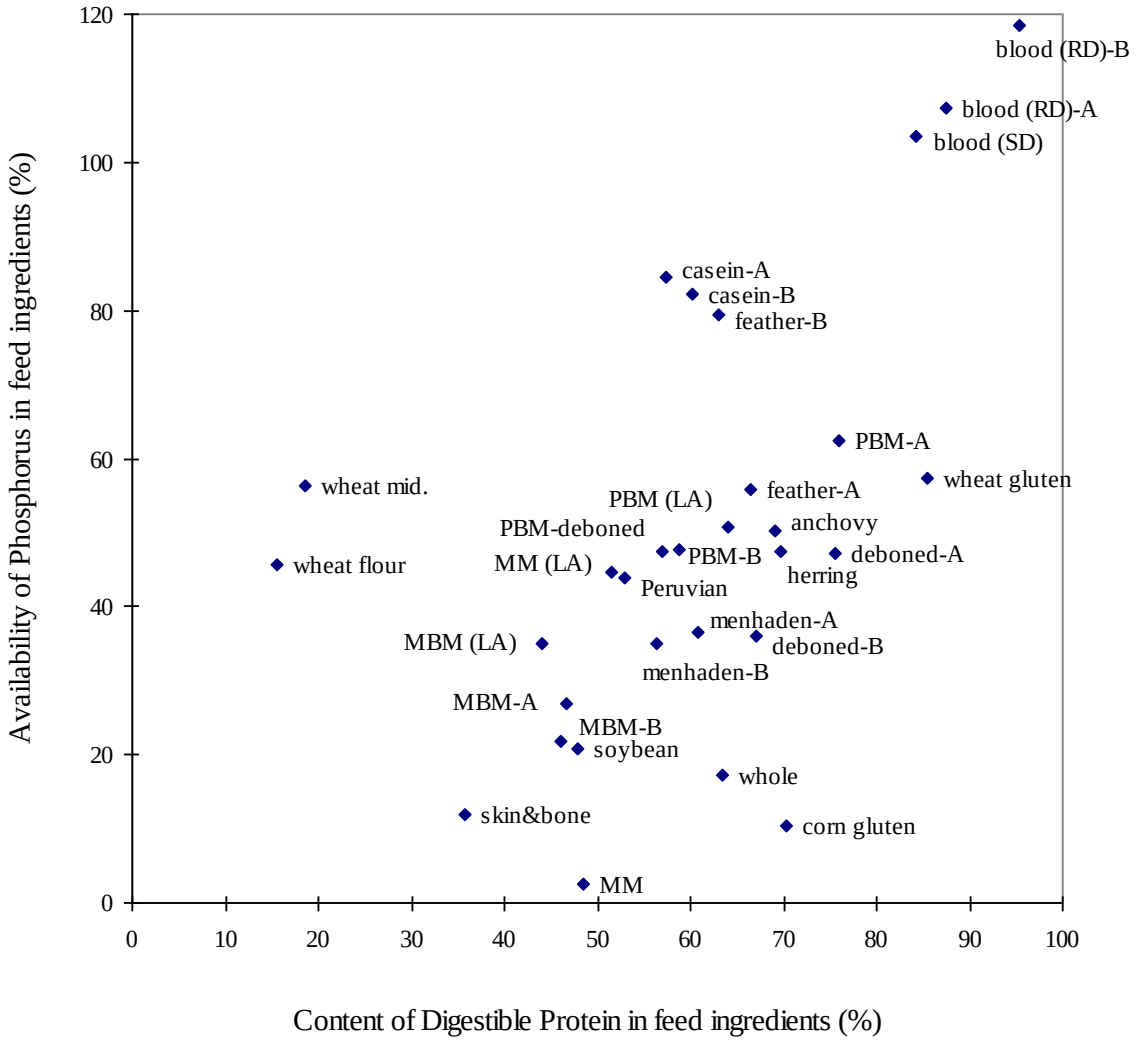


Figure 55. Schematic diagram showing the preferred ingredients for low-pollution feeds (data from Study-1 and 2 with rainbow trout). Note: the availability of blood meals for phosphorus, which is over 100%, is primarily due to its extremely low phosphorus content and its dilution effect of total phosphorus levels in diets. Abbreviations used (given in alphabetical order): anchovy= anchovy meal; blood (SD)= blood meal-spray dried; blood (RD)= blood meal-ring-dried; casein= casein basal diet; deboned= deboned whitefish meal; feather= feather meal; herring= herring meal; menhaden= menhaden meal; MBM= meat&bone meal; MBM (LA)= meat&bone meal-low ash; MM= meat meal; MM (LA)= meat meal-low ash; Peruvian= Peruvian fish meal; PBM= poultry byproduct meal; PBM (LA)= poultry byproduct meal-low ash; skin&bone= skin&bone whitefish meal; wheat mid.= wheat middling; whole= whole whitefish meal. Ingredients of the same trade name but obtained from different suppliers were identified with the letter “A” or “B”. The letter does not indicate the grade or quality of the product.

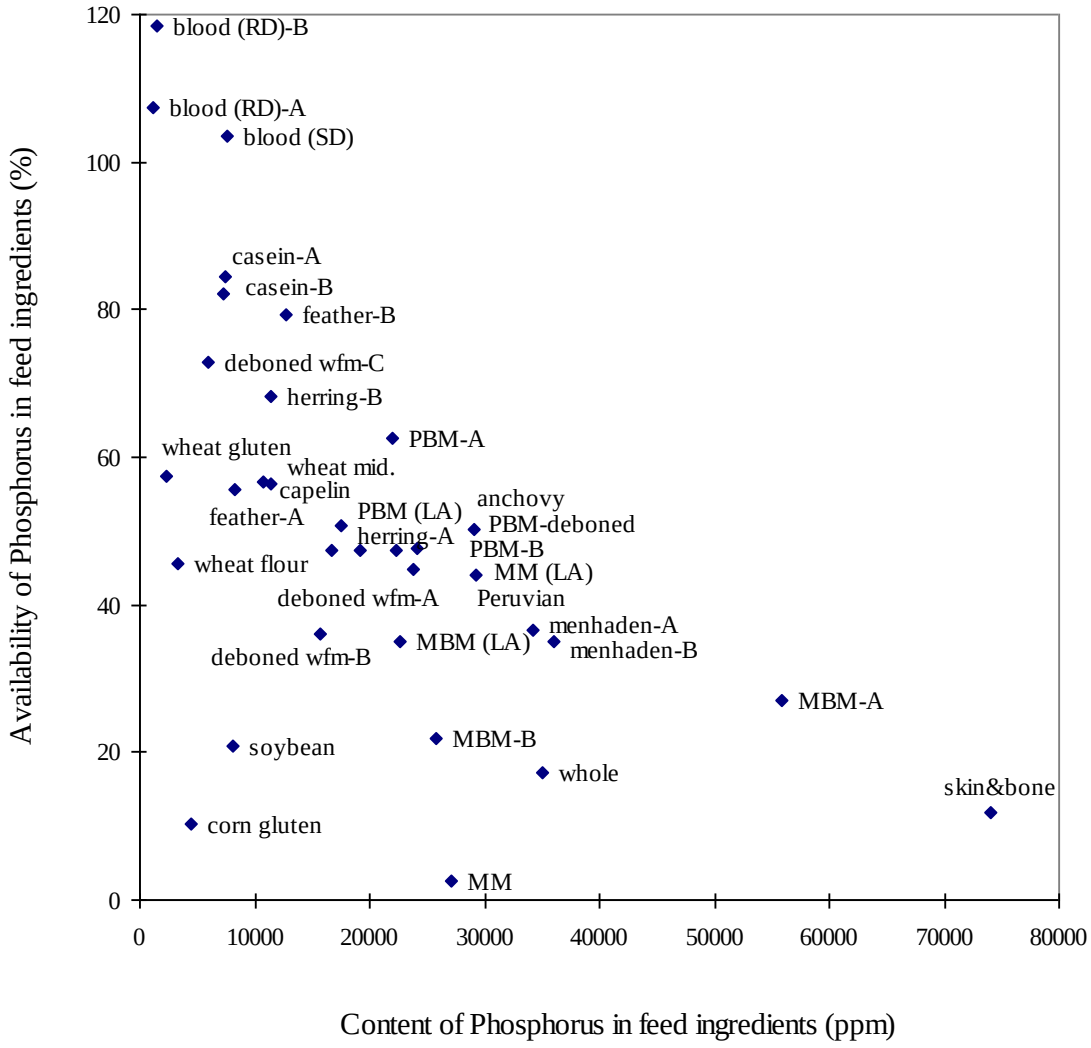


Figure 56. Content of phosphorus in feed ingredients and their apparent availabilities (ingredient basis) (data from Study-1 and 2 with rainbow trout). Abbreviations used (in alphabetical order): anchovy= anchovy meal; blood (SD)= blood meal-spray dried; blood (RD)= blood meal-ring-dried; capelin= capelin meal; casein= casein basal diet; deboned= deboned whitefish meal; feather= feather meal; herring= herring meal; menhaden= menhaden meal; MBM= meat&bone meal; MBM (LA)= meat&bone meal-low ash; MM= meat meal; MM (LA)= meat meal-low ash; Peruvian= Peruvian fish meal; PBM= poultry byproduct meal; PBM (LA)= poultry byproduct meal-low ash; skin&bone= skin&bone whitefish meal; wheat mid.= wheat middling; whole= whole whitefish meal. Ingredients of the same trade name but obtained from different suppliers were identified with the letter "A, B or C". The letter does not indicate the grade or quality of the product.

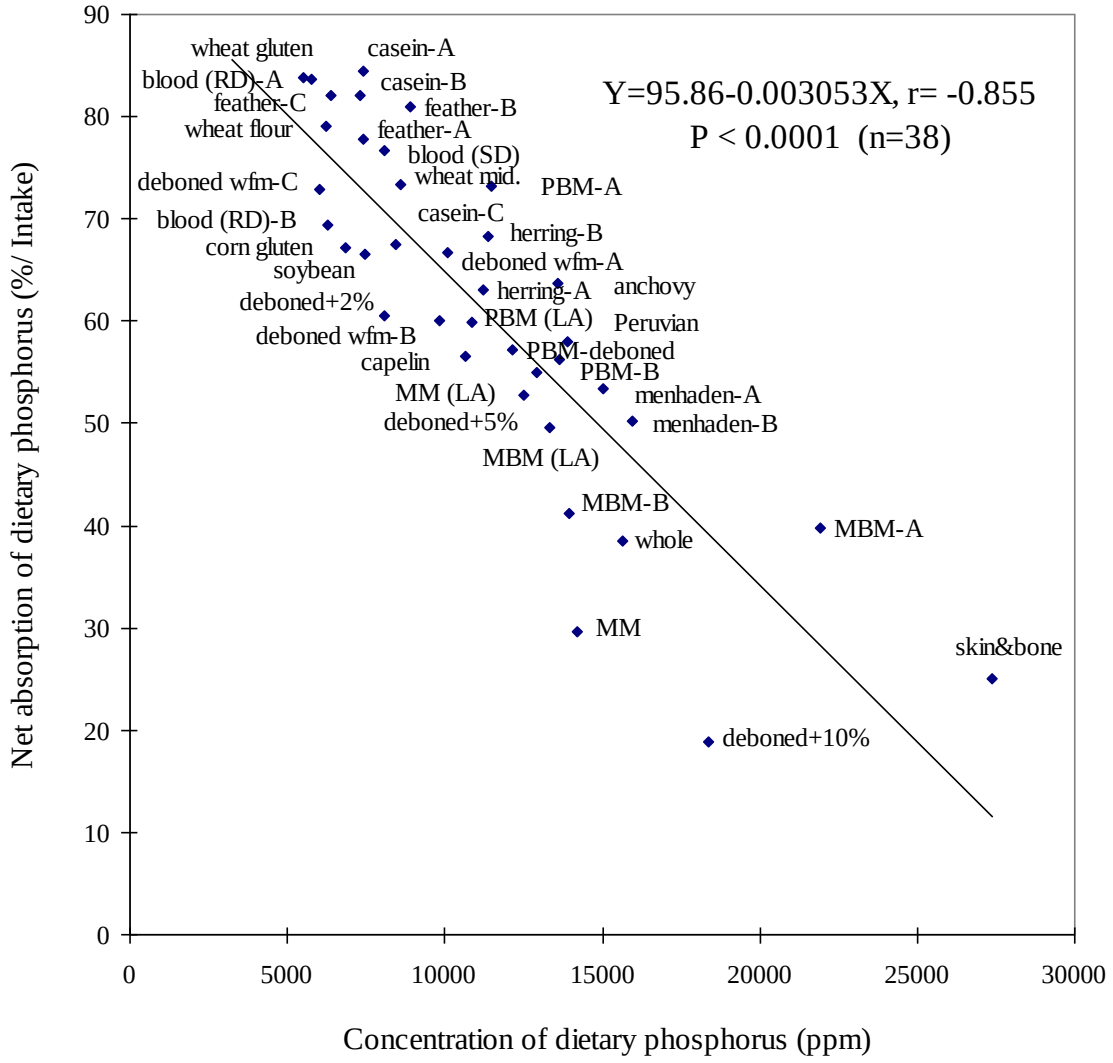


Figure 57. Net absorption (apparent availability) of phosphorus in diets containing test ingredients at 30% and the casein basal diet at 70% levels (dry basis) (data from Study-1, 2 and 3 with rainbow trout). Note: since dietary phosphorus concentrations change proportionally with calcium, ash or other bone minerals, the x-axis could be any of these minerals without considerable difference from the given figure. Abbreviations used (given in alphabetical order): anchovy= anchovy meal; blood (SD)= blood meal-spray dried; blood (RD)= blood meal-ring-dried; casein= casein basal diet; deboned= deboned whitefish meal; deboned+2, 5 or 10%= deboned whitefish meal + fish bone at 2, 5 or 10% per dry diet; feather= feather meal; herring= herring meal; menhaden= menhaden meal; MBM= meat&bone meal; MBM (LA)= meat&bone meal-low ash; MM= meat meal; MM (LA)= meat meal-low ash; Peruvian= Peruvian fish meal; PBM= poultry byproduct meal; PBM (LA)= poultry byproduct meal-low ash; skin&bone= skin&bone whitefish meal; wheat mid.= wheat middling; whole= whole whitefish meal. Ingredients of the same trade name but obtained from different suppliers were identified with the letter "A, B or C". The letter does not indicate the grade or quality of the product.

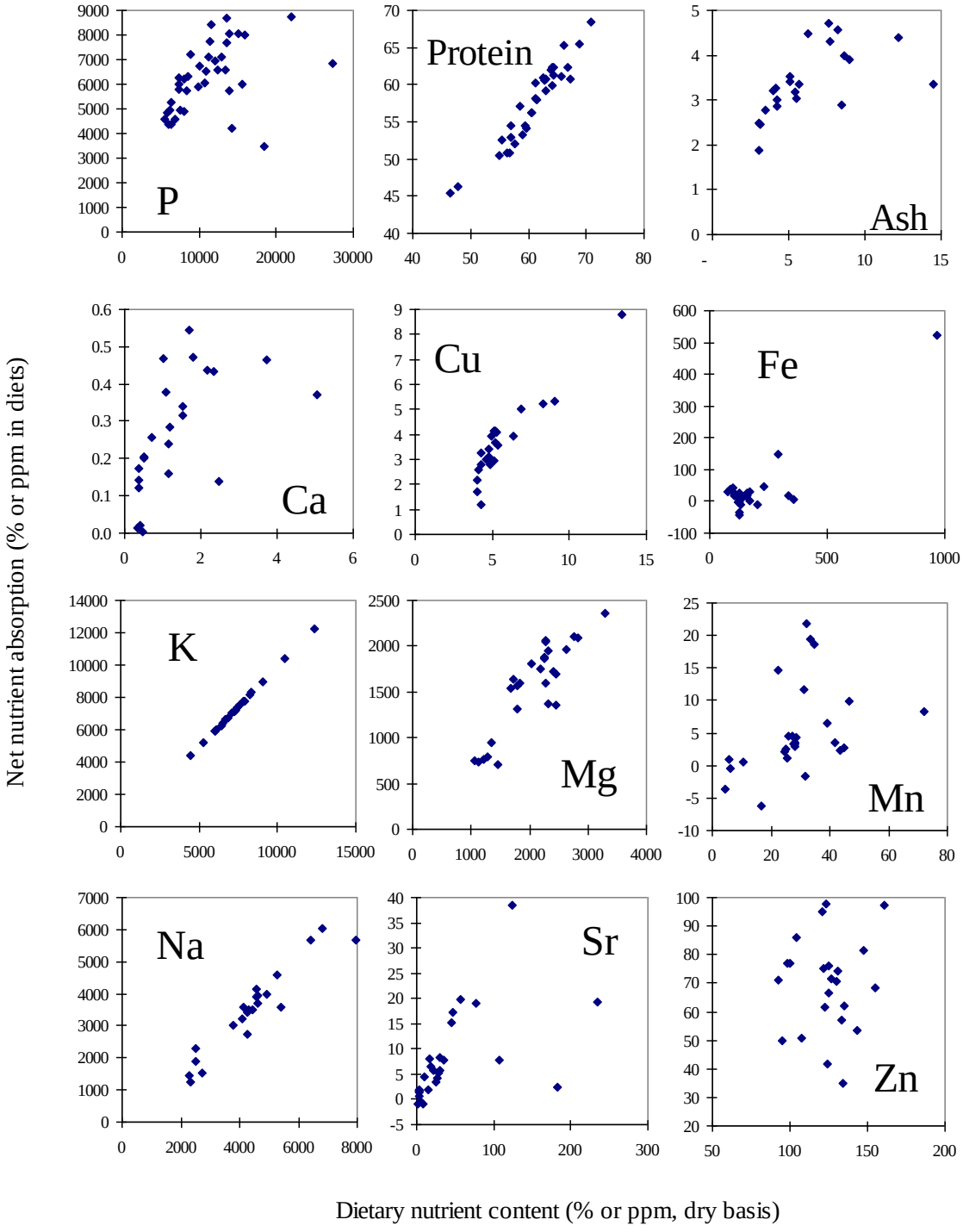


Figure 58. Net absorption of nutrients from diets containing animal byproduct meals (30%) and the casein basal diet (70%) (Study-2).

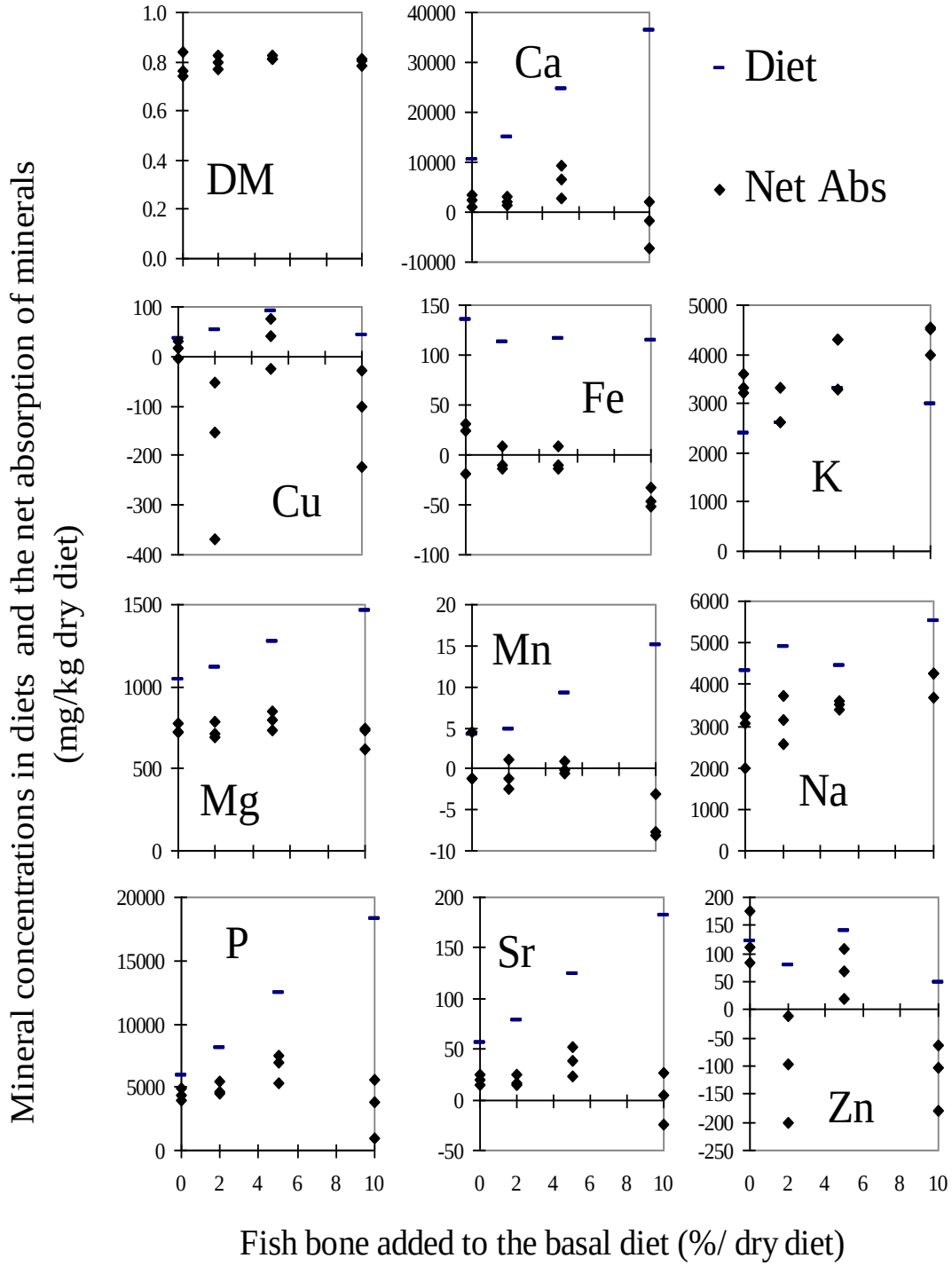


Figure 59. Net absorption of minerals from diets containing varied levels of fish bone (Study-3).

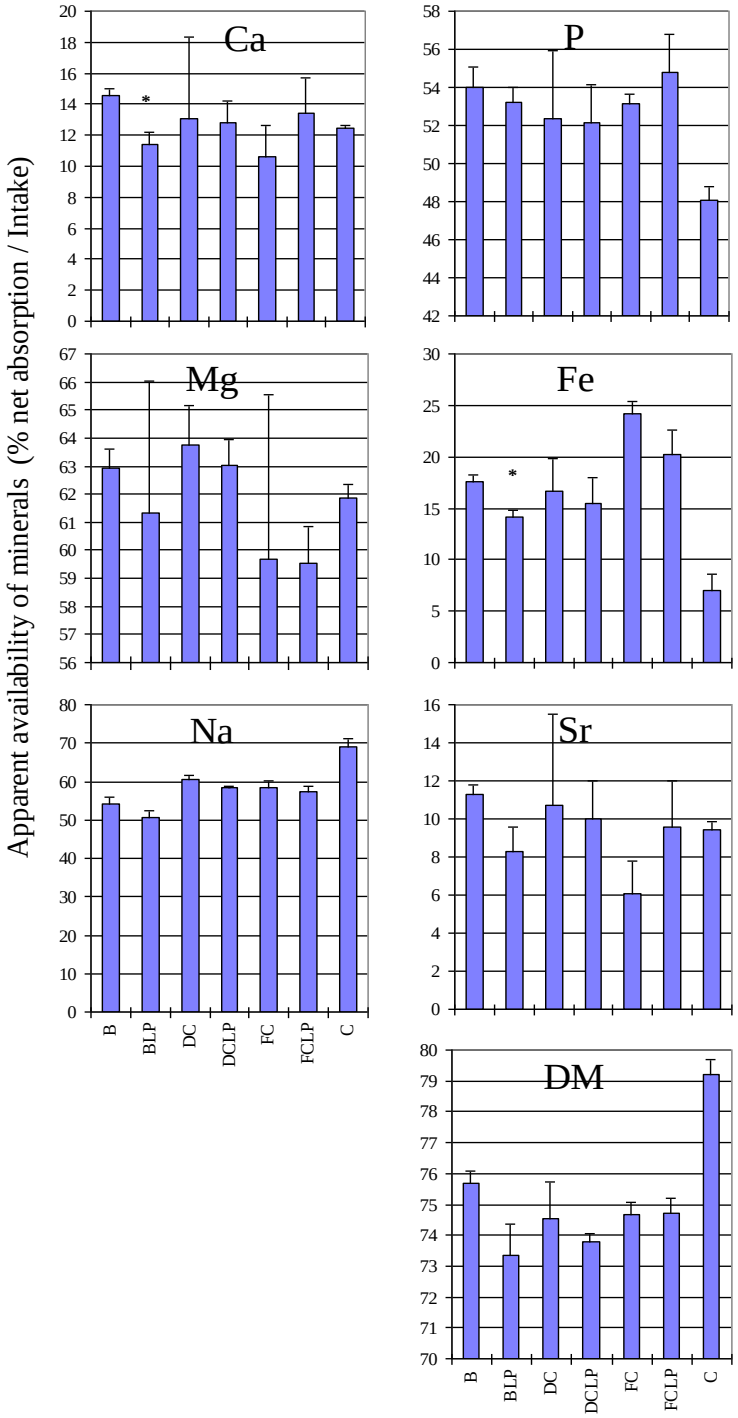


Figure 60. Apparent availability of minerals in fish meal diets containing ordinary or low-phytate grains (Study-5). Abbreviations: B (barley), BLP (barley of low-phytate), DC (dent corn), DCLP (dent corn of low-phytate), FC (flint corn), FCLP (flint corn of low-phytate), C (control fishmeal diet containing no grains). Each column represent the mean of 3 tanks (with sem indicated as the error bar). Columns with asterisks are significantly different from its ordinary counterpart (* P< 0.05; ** P< 0.01).

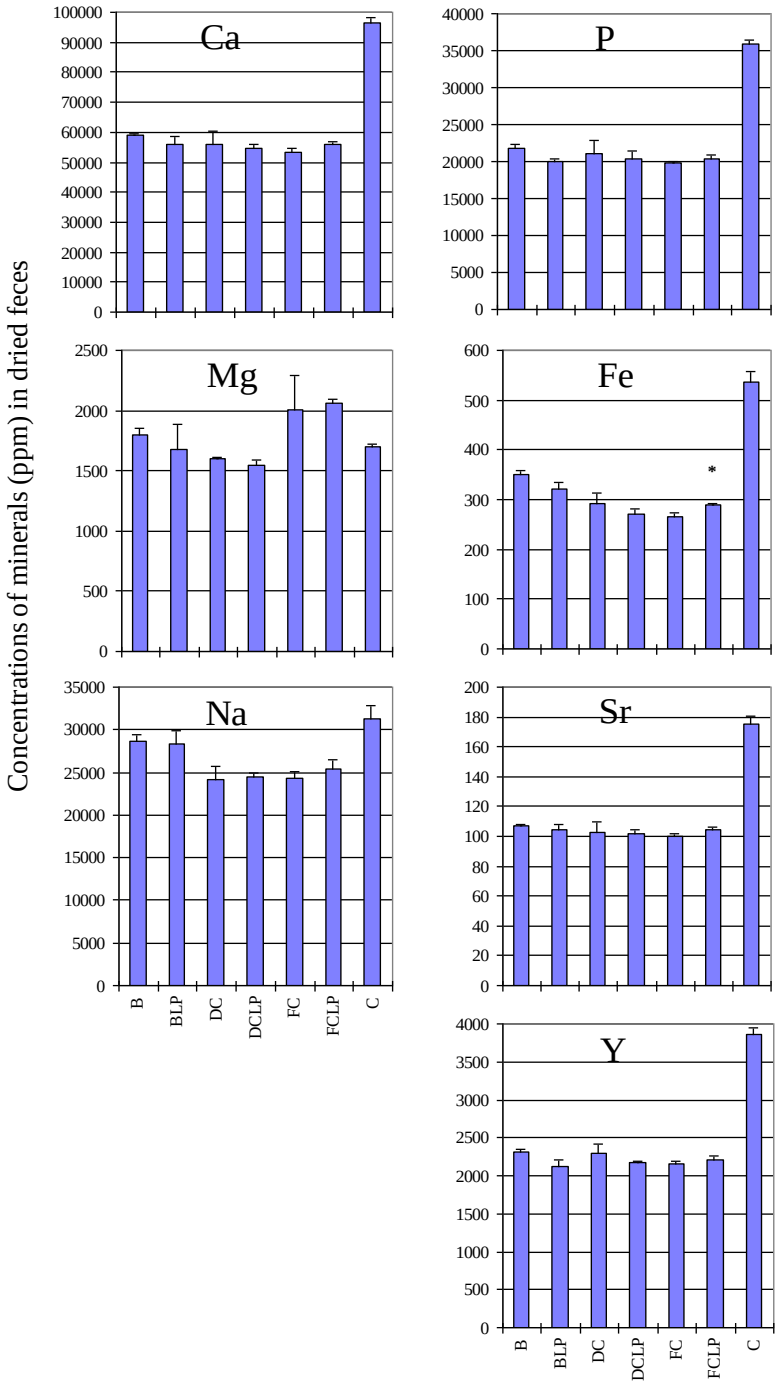


Figure 61. Content of minerals in the feces of fish fed fish meal diets containing ordinary or low-phytate grains (Study-5). Abbreviations: B (barley), BLP (barley of low-phytate), DC (dent corn), DCLP (dent corn of low-phytate), FC (flint corn), FCLP (flint corn of low-phytate), C (control fishmeal diet containing no grains). Each column represent the mean of 3 tanks (with sem indicated as the error bar). Columns with asterisks are significantly different from its ordinary counterpart (* $P< 0.05$; ** $P< 0.01$).

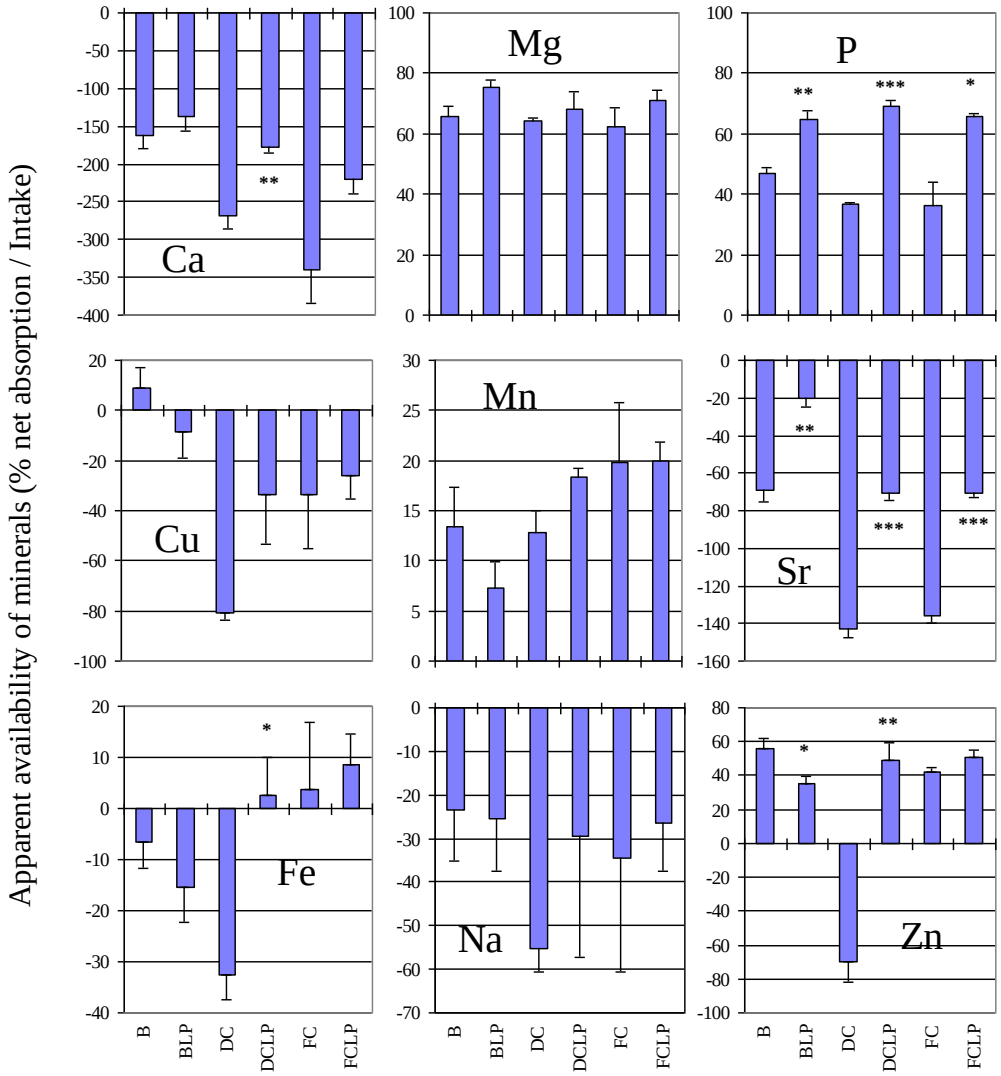


Figure 62. Apparent availability of minerals in ordinary and low-phytate grains (Study-5). Abbreviations: B (barley), BLP (barley of low-phytate), DC (dent corn), DCLP (dent corn of low-phytate), FC (flint corn), FCLP (flint corn of low-phytate). Each column represent the mean of 3 tanks (with sem indicated as the error bar). Columns with asterisks are significantly different from its ordinary counterpart (* P< 0.05; ** P< 0.01; *** P< 0.001).

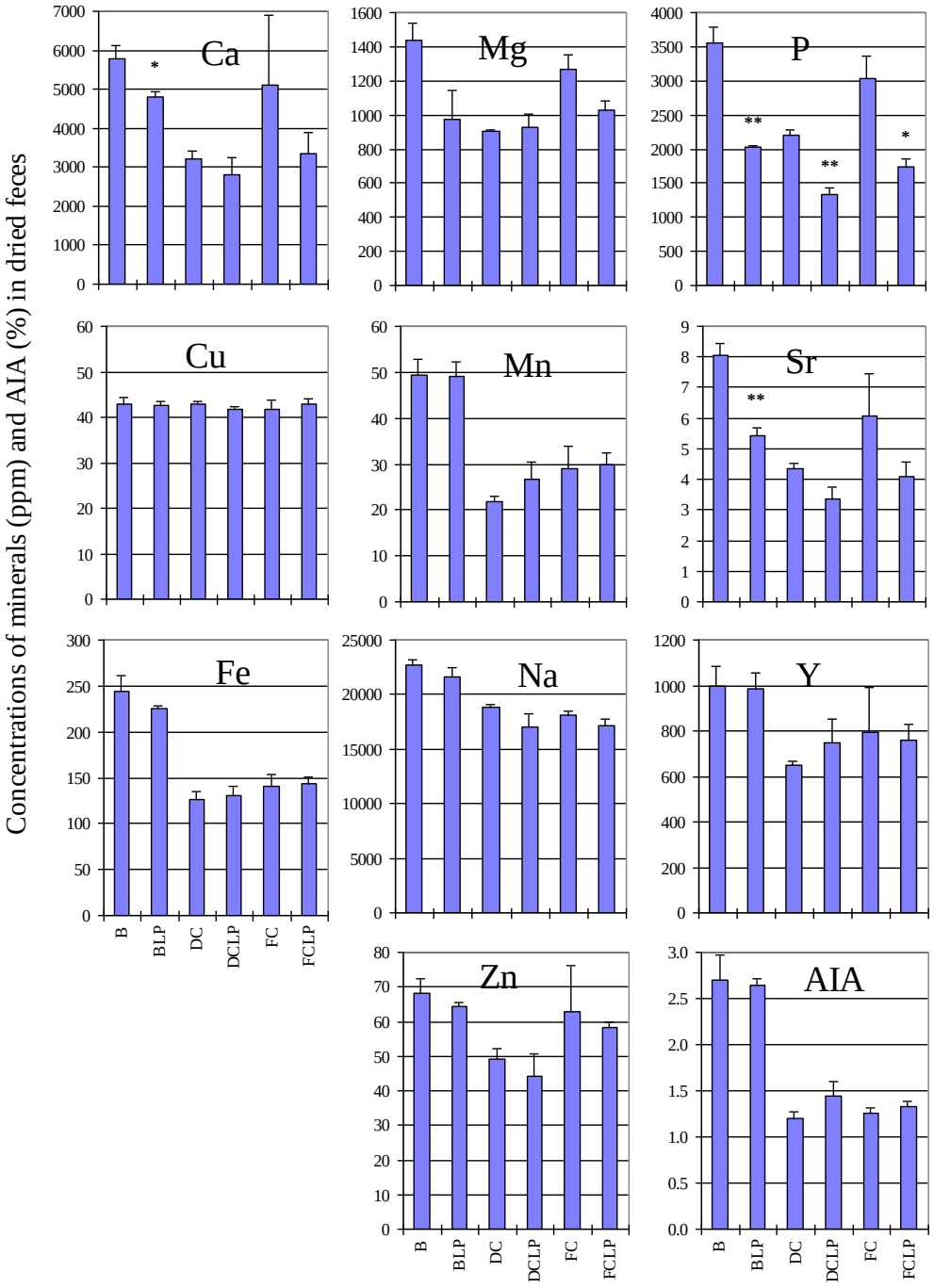


Figure 63. Content of minerals and acid-insoluble ash (AIA) in the feces of fish fed ordinary and low-phytate grains (Study-5). Abbreviations: B (barley), BLP (barley of low-phytate), DC (dent corn), DCLP (dent corn of low-phytate), FC (flint corn), FCLP (flint corn of low-phytate). Each column represent the mean of 3 tanks (with sem indicated as the error bar). Columns with asterisks are significantly different from its ordinary counterpart (* P< 0.05; ** P< 0.01).

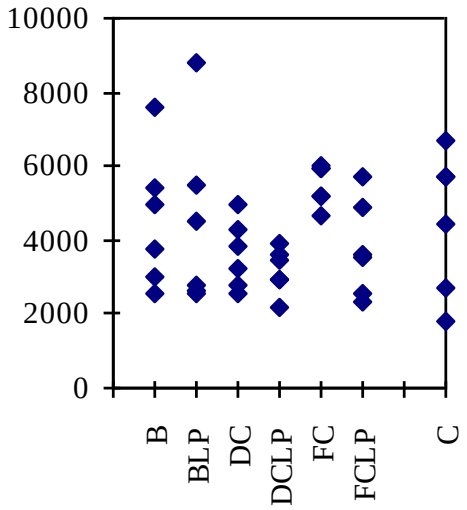
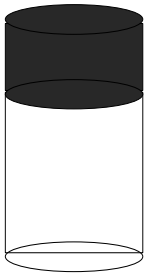


Figure 64. Fecal content of P (in ppm; Y axis) in fish fed casein basal diet containing ordinary or low-phytate grains (30%/ diet) (Study 5). Diet abbreviations (in X-axis): B (barley); BLP (barley of low-phytate); DC (dent corn); DCLP (dent corn of low-phytate); FC (flint corn); FCLP (flint corn of low-phytate); C (casein basal diet containing no grains). Each point represents one fish.

$$ADC_{Ingredient} = \frac{(ADC_{TD} - 0.7 \times ADC_{BD})}{0.3}$$

$$\frac{(Nutr_{TD} \times ADC_{TD} - 0.7 \times Nutr_{BD} \times ADC_{BD})}{0.3 \times Nutr_{Ing}}$$

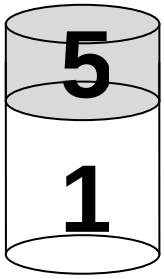


30% Test Ingredient

70% Basal diet

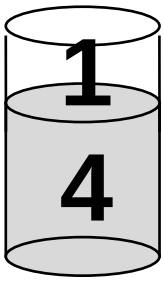
Practical examples

1. P concentration in menhaden meal was 5 times higher than that in the basal diet.



□ Using "simple formula,"
ADC of P in menhaden meal is –23%
□ Using "nutrient contribution formula,"
ADC of P in menhaden meal is 40%

2. Protein concentration of the basal diet was 4 times higher than that in wheat flour.
<u>Suppose</u> ADC of protein in the test diet (wheat flour + basal diet) is 81%, then



□ Using "simple formula,"
ADC of protein in wheat flour is 61%
□ Using "nutrient contribution formula,"
ADC of protein in wheat flour is 0%

Figure 65. Calculation of the apparent digestibility (availability) coefficients: ADC by the conventional formula (NRC, 1993) and the nutrient contribution formula. Slide (top) and transparency (below) presented at the VII International Symposium on Nutrition and Feeding of Fish. College Station, Texas, Aug. 11-15, 1996.

Appendix III. Data Figures for Chapter III

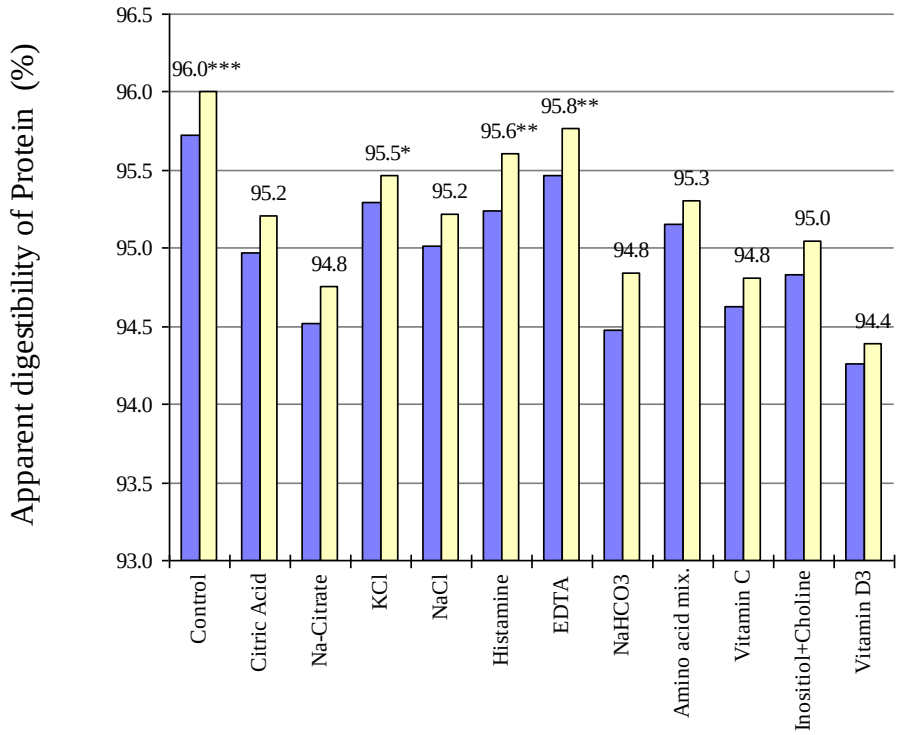


Figure 66. Effects of various dietary supplements on the apparent digestibility of protein in a fish meal-based diet (Study-1). Solid bars (left) indicate the apparent digestibility coefficient determined based on chromium; hollow bars (right) indicate those determined based on yttrium as non-absorbable dietary markers. Values above hollow columns indicate the ADC value (on yttrium). Values with asterisks are significantly higher than the median value of all groups (* P< 0.05; ** P< 0.01; *** P< 0.001).

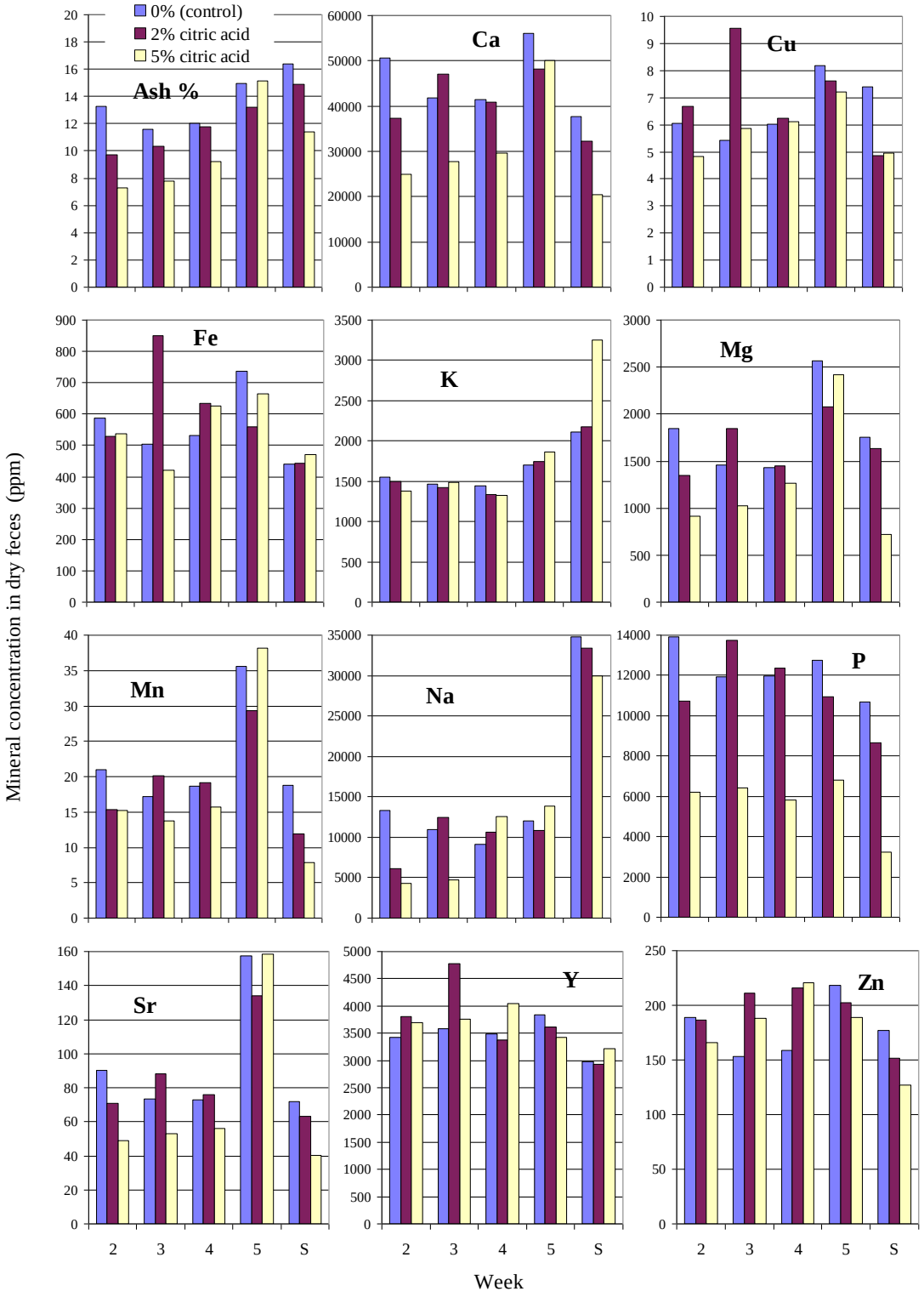


Figure 67. Mineral content in the feces of rainbow trout fed 0,2 or 5% citric acid in diets (Study-2). Feces were collected by settling using digestibility tanks (wk 2,3,4,5) or by stripping at the end of the experiment (S). Water hardness was increased during wk 5.

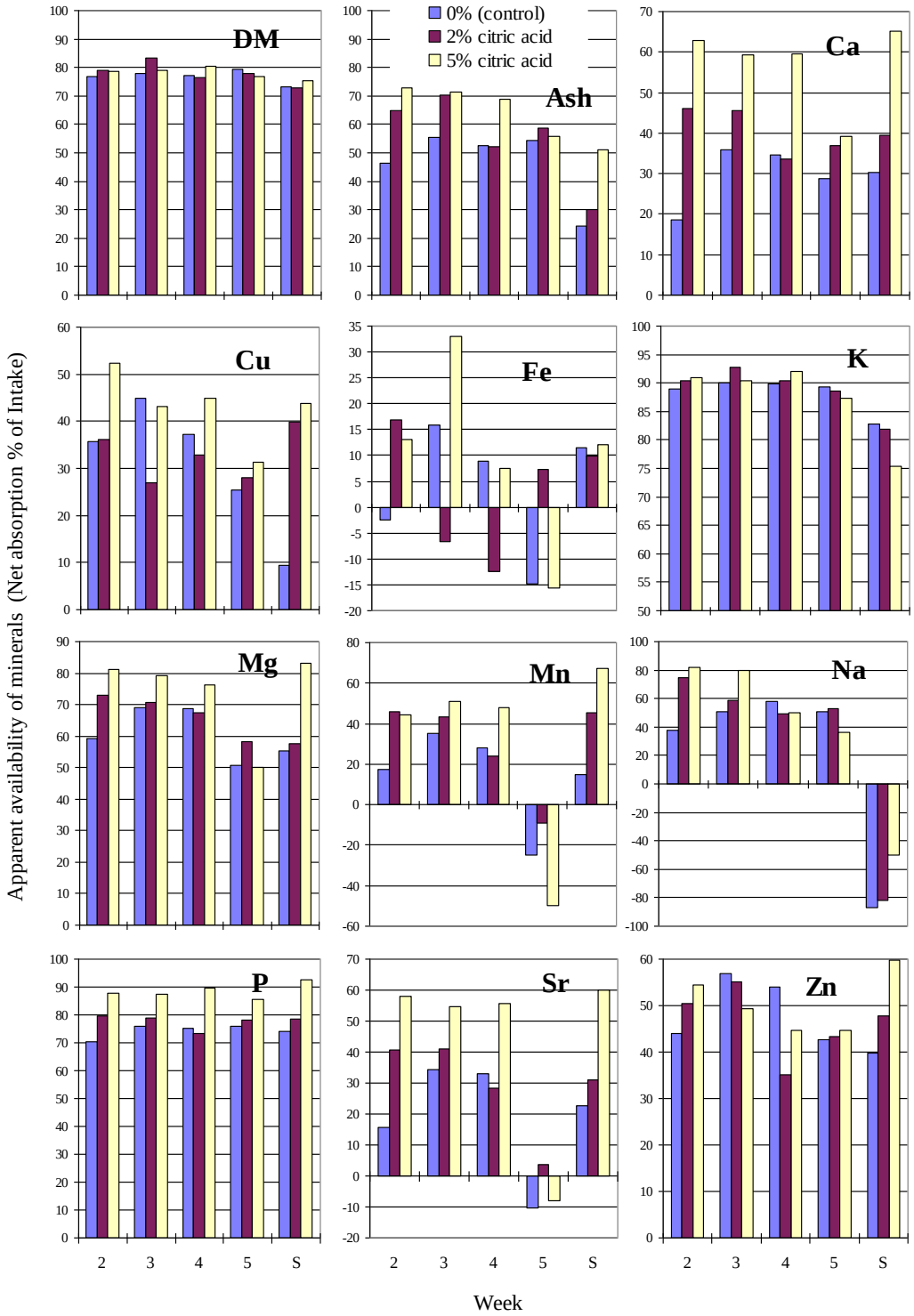


Figure 68. Apparent availability of minerals in diets supplemented with citric acid at 0,2 or 5% per diet (dry basis) (Study-2). Feces were collected by settling (wk 2,3,4 and 5) or by stripping at the end of the experiment (indicated as "S"). During wk-5, water hardness was increased by dissolving $CaSO_4$ in the recirculating water.

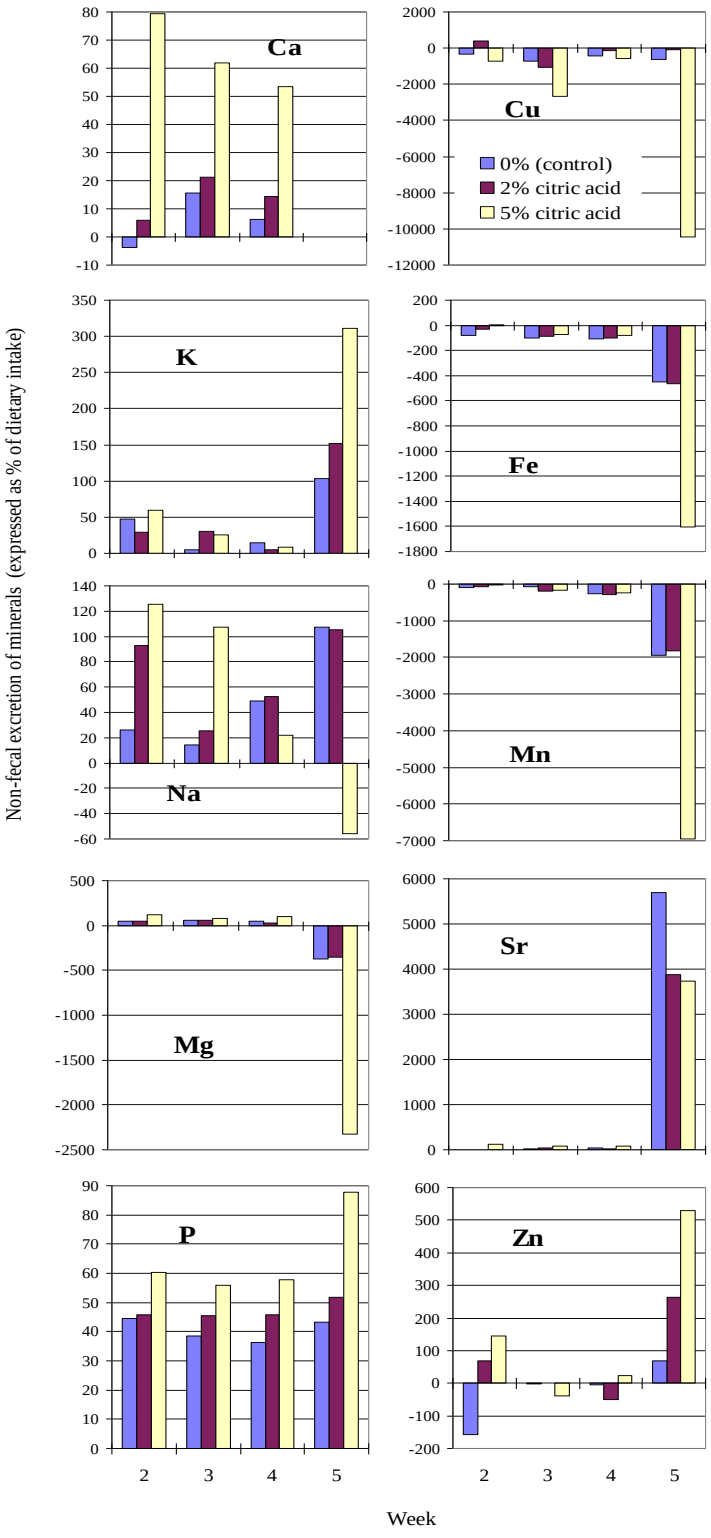


Figure 69. Non-fecal excretion of minerals in rainbow trout fed citric acid 0,2 or 5% in diets for 5 weeks (Study-2). During wk-5, water hardness was increased ca. 10 fold by dissolving $CaSO_4$ in the recirculating water.

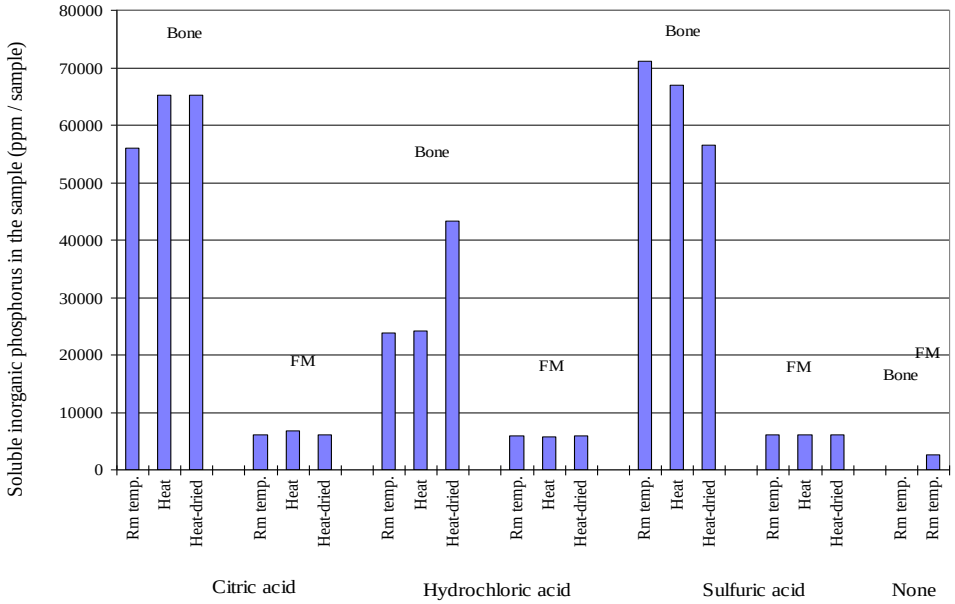


Figure 70. Soluble phosphorus content in fish bone and fish meal treated with citric, hydrochloric or sulfuric acid. Procedures: 0.125g bone or 2.0g fish meal + 3.5g distilled water containing either citric acid mono-hydrate 0.105g, HCl conc. 0.152g, or H_2SO_4 conc. 0.075g. The mixture was treated either room temp. (1 h. at room temp. with occasional stirring), 70°C- 1h. (1 h. in a water bath at 70°C; vials were capped), or 70°C- dried (dried in 1 h. in a water bath). After 1 h of the treatment, net weight of the contents were adjusted to 10.13g (for bone soln.) or 12.0g (for fish meal soln.) by adding distilled water. Then, pH of the soln. was measured using pH electrode. The soln. was then back-titrated with 1N-NaOH to pH 7 to determine remaining or unreacted acid in the soln (data given in the following table). This neutralized soln. containing solid matter was diluted to 1 L with tap water and shaken for ca. 1 min. The supernatant was determined for P concentration (Taussky and Shorr, 1953).

Table 73. Soluble phosphorus content in fish bone[1] and fish meal[2] treated with citric, hydrochloric or sulfuric acid.

Sample	Acid used	treatment	pH	1N-NaOH (ml)	Soluble-P (ppm/sample)
Bone (fish meal)	Citric acid	Rm temp.	3.23 (4.34)	1.23 (1.41)	55982 (6155)
Bone (fish meal)	Citric acid	Heat	3.38 (4.36)	1.17 (1.33)	65219 (6824)
Bone (fish meal)	Citric acid	Heat-dried	3.39 (4.36)	1.18 (1.35)	65219 (6097)
None	Citric acid	Rm temp.	2.06	1.61	
Bone (fish meal)	HCl	Rm temp.	1.67 (3.93)	1.29 (1.47)	23834 (5866)
Bone (fish meal)	HCl	Heat	1.61 (3.84)	1.29 (1.45)	24203 (5693)
Bone (fish meal)	HCl	Heat-dried	1.89 (3.86)	1.12 (1.40)	43233 (5831)
None	HCl	Rm temp.	0.84	1.58	
Bone (fish meal)	H_2SO_4	Rm temp.	1.88 (4.20)	0.95 (1.41)	71132 (6109)
Bone (fish meal)	H_2SO_4	Heat	1.93 (4.18)	0.92 (1.29)	66882 (6155)
Bone (fish meal)	H_2SO_4	Heat-dried	1.88 (4.17)	0.95 (1.22)	56536 (6155)
None	H_2SO_4	Rm temp.	1.05	1.55	
Bone (fish meal)	water	Rm temp.	7.41 (5.76)	------- (0.24)	0 (2575)

[1] Values for fish bone were indicated without parenthesis.
[2] Values for fish meal were indicated in parenthesis.

Inorganic Acid Feeding Trial

Table 74. Analytical composition of the experimental diets[1].

		Control	HCl	H_2SO_4
Crude Protein	(%/diet, dry basis)	42.2	41.7	41.2
Ash	(%/diet, dry basis)	6.32	6.15	6.64
Acid Insoluble Ash	(%/diet, dry basis)	1.11	1.09	1.05
P	(%/diet, dry basis)	0.552	0.515	0.520
Ca	(%/diet, dry basis)	0.751	0.746	0.759
Moisture	(%/diet, as-is basis)	30.5	27.7	27.5
pH[2]		6.12	1.97	2.44

[1] Ingredient composition is the same as that of the performance trial (Table 51, diet FM). The diet abbreviations: Control (no acidification); HCl (hydrochloric acid added at 76 g/kg dry diet; equivalent to 5 % citric acid per diet in H^+ strength); H_2SO_4 (sulfuric acid added at 37.5 g/kg dry diet; equivalent to 5 % citric acid per diet in H^+ strength).

[2] Feeds (frozen-stored & thawed) were homogenized with distilled water using a flat-bottom test tube, and the pH of the supernatant was measured.

Table 75. Analytical composition of feces collected by stripping and settling[1].

		Control	HCl	H_2SO_4
Crude Protein	(%/dry matter)	10.20 (9.03)	9.62 (9.68)	9.94 (10.57)
P	(%/dry matter)	0.793 (0.721)	0.322 (0.352)	0.126 (0.130)
Ca	(%/dry matter)	2.56	2.08	1.79
ADC[2]-Dry matter	(%/ intake)	80.1 (81.0)	80.5 (81.6)	78.5 (80.4)
ADC-Crude Protein	(%/ intake)	95.2 (95.9)	95.5 (95.7)	94.8 (95.0)
ADC-P	(%/ intake)	71.4 (75.2)	87.8 (87.4)	94.8 (95.1)
ADC-Ca	(%/ intake)	32.1	45.4	49.2
pH		8.44	8.39	8.03

[1] Values show the data of feces collected by stripping (no parenthesis) or by settling (in parentheses).

[2] ADC: Apparent Digestibility (Availability) Coefficient.

Table 76. Effects of inorganic acids on the partition of dietary protein and phosphorus[1].

			Control	HCl	H_2SO_4
Excretion[2]	CP	%/ Intake	4.07 (72.4)	4.28 (71.7)	5.01 (64.9)
	CP	%/feed	1.72 (30.5)	1.78 (29.9)	2.07 (26.8)
	P	%/ Intake	24.8 (2.6)	12.6 (12.4)	4.9 (11.7)
	P	%/feed	0.14 (0.01)	0.06 (0.06)	0.03 (0.06)
	P	%/N-retention	8.9 (0.9)	4.1 (4.0)	1.3 (3.1)
Retention[3]	CP	%/ Intake	22.8	23.8	30.0
	CP	%/feed	9.60	9.92	12.35
	P	%/ Intake	68.8	75.4	83.1
	P	%/feed	0.38	0.39	0.43
	P	%/N-retention	24.7	24.5	21.9

[1] Fish were fed to satiation for 23 days. The data were collected at 23rd day using metabolic tanks. The daily feed intake of fish (at satiation feeding, %/BW/day, averages of 23 days, dry basis) were 1.55 (Control); 1.15 (HCl) and 1.31 (H_2SO_4).

[2] Excretion in feces (without parenthesis) and in water (with parenthesis).

[3] Retention by fish (estimated: retention = amount fed - amount excreted in feces and water).

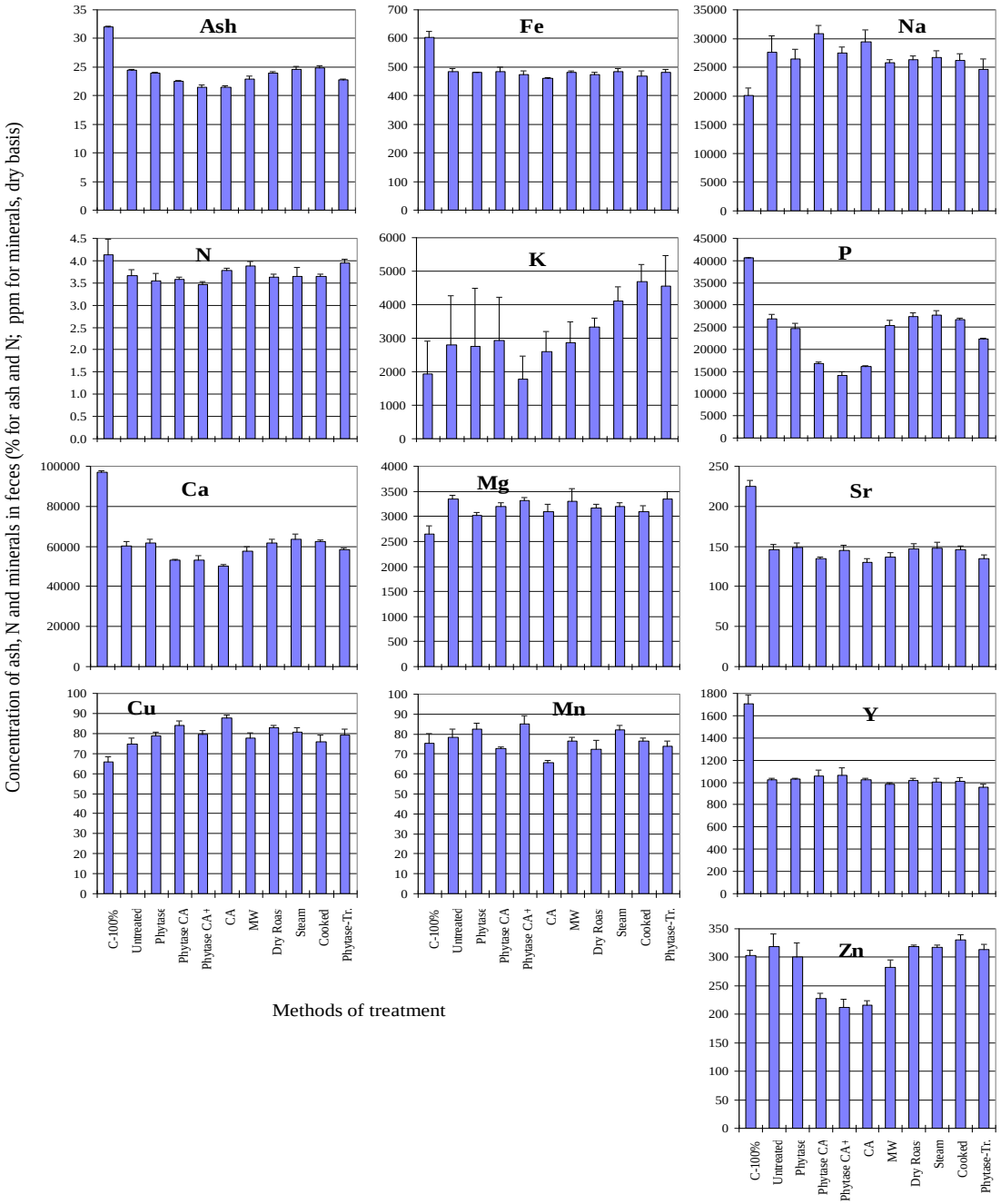


Figure 71. Mineral content in the feces of fish fed fish meal-soybean meal complex diets (Study-5). The control diet (C-100%) contained no soybean meal; the other diets contained ca. 30% soybean meal (dry basis) treated with various methods. Exact ratios of soybean meal to the control diet were determined from the concentration of yttrium in each test diet and that in the control diet. Diet abbreviations (from the left): C-100% (control diet), Untreated (untreated-soybean meal), Phytase (phytase-added to the untreated-soybean meal diet), Phytase CA (phytase and citric acid added), Phytase CA+ (Phytase CA diet was incubated 24 h at 50°C before drying), CA (citric acid added), MW (microwaved soybean meal), Dry Roast (dry roasted soybean meal), Steam (steam-heated soybean meal), Cooked (cooked soybean meal), Phytase-Tr. (phytase treated soybean meal).

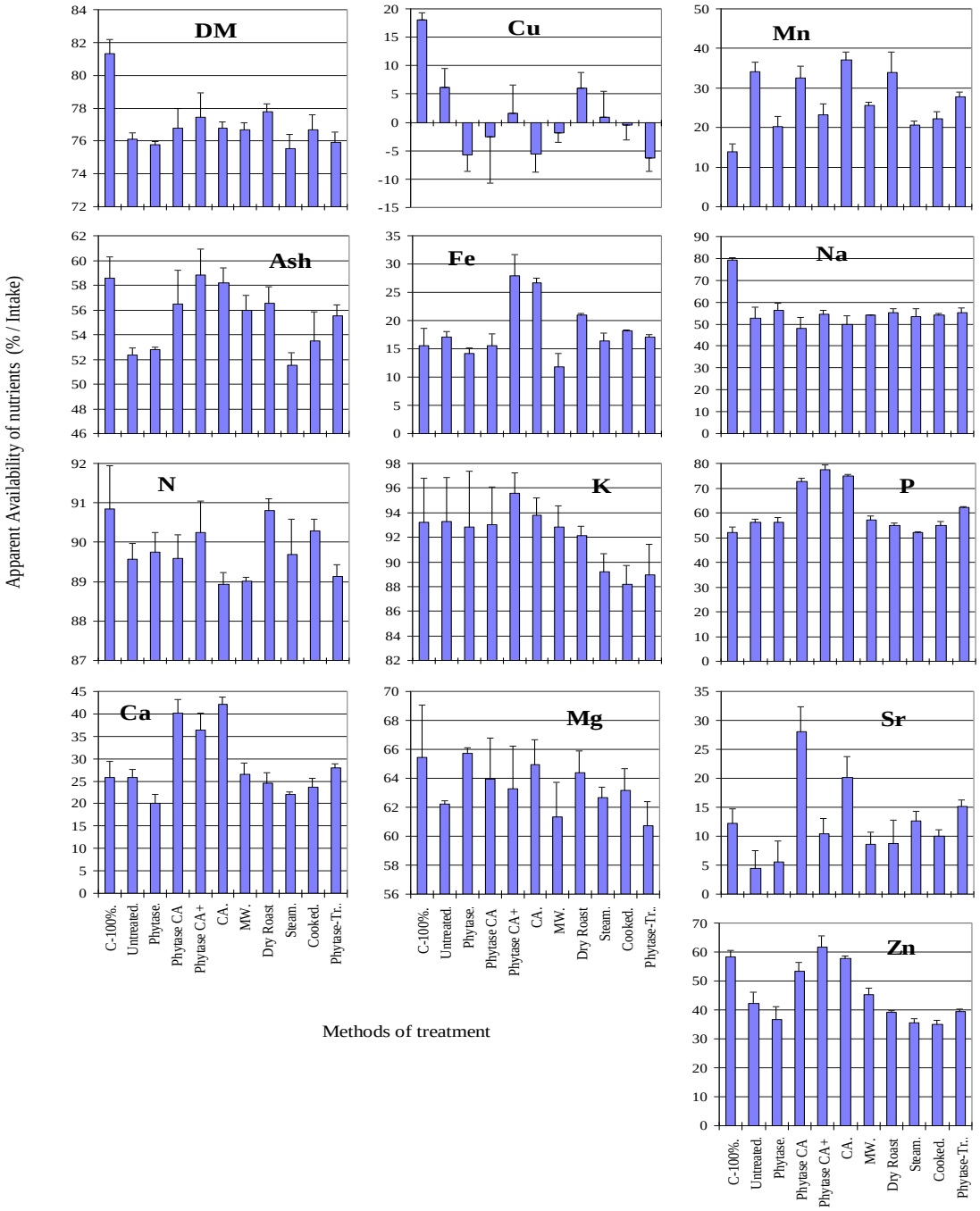


Figure 72. Apparent availability of dietary nutrients in fish meal-soybean meal complex diets (Study-5). The control diet (C-100%) contained no soybean meal; the other diets contained ca. 30% soybean meal treated with various methods. Diet abbreviations (from the left): C-100% (fish meal-based control diet), Untreated (untreated-soybean meal), Phytase (phytase-added to the untreated-soybean meal diet), Phytase CA (phytase and citric acid added), Phytase CA+ (Phytase CA diet was incubated 24h at 50°C before drying), CA (citric acid added), MW (microwaved soybean meal), DryRoast (dry roasted soybean meal), Steam (steam-heated soybean meal), Cooked (cooked soybean meal), Phytase-Tr. (phytase treated soybean meal).

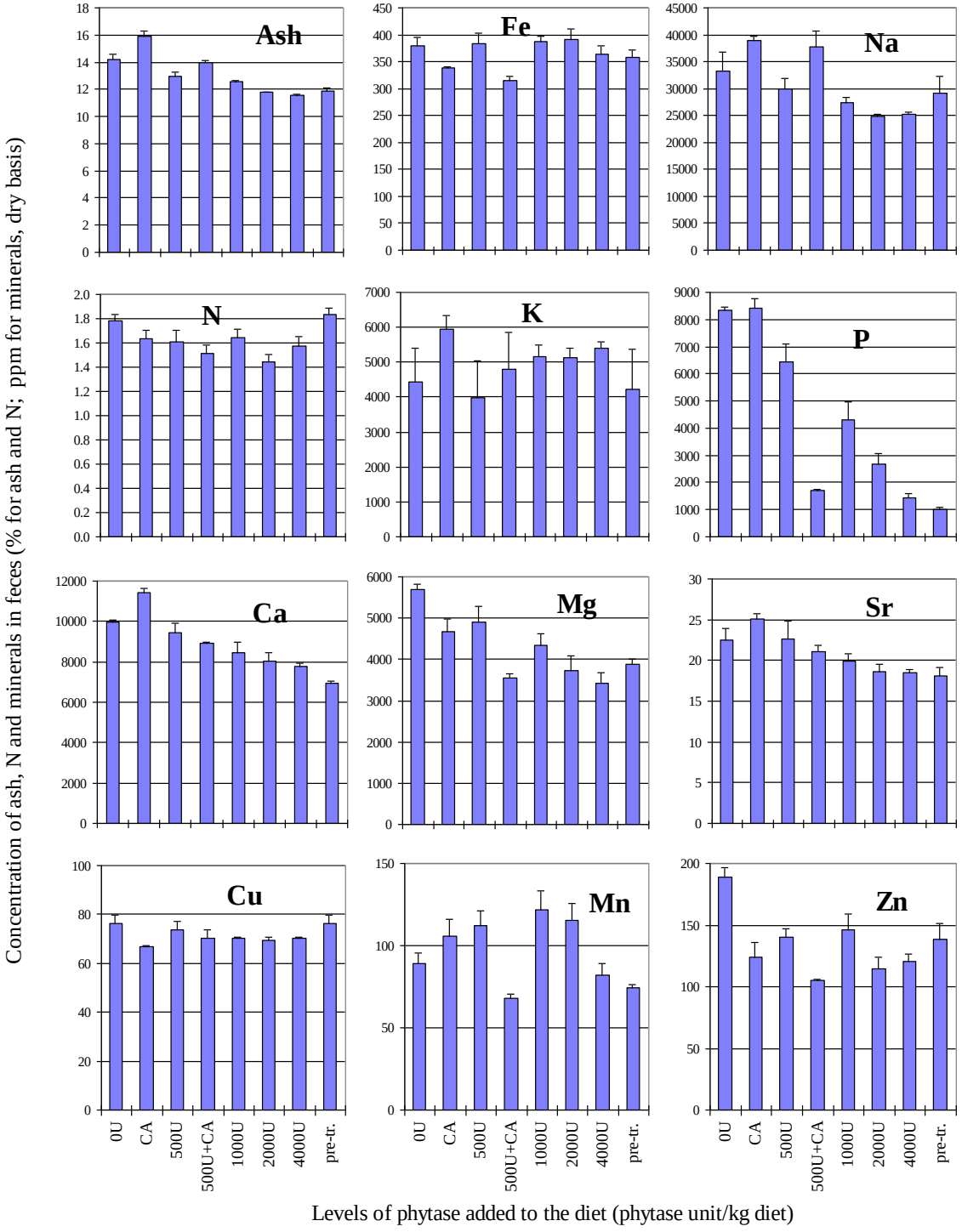


Figure 73. Mineral content in feces of fish fed low-ash soybean meal diets (Study-5). Each column represents the average of 3 tanks (with sem shown as the error bar). Abbreviations; CA (citric acid), pre-tr. (pretreated soybean meal for 24h at 50°C with 200U phytase/kg soybean meal, equivalent to 100U phytase/kg diet).

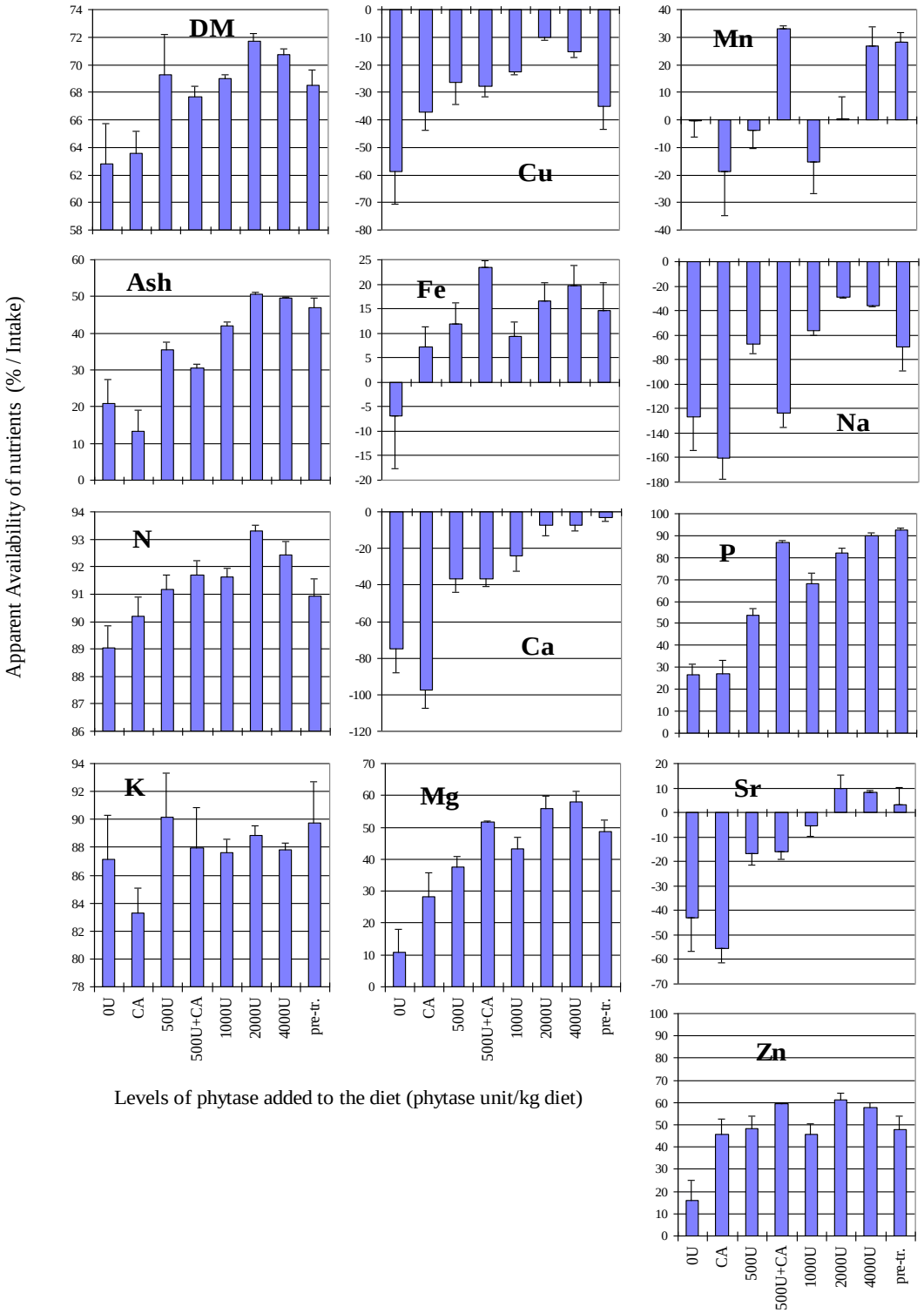


Figure 74. Apparent availability of dietary nutrients in low-ash soybean meal diets (Study-5). Each column represents the average of 3 tanks (with sem shown as the error bar). Abbreviations; CA (citric acid), pre-tr. (pretreated soybean meal for 24h at 50°C with 200U phytase/kg soybean meal, equivalent to 100U phytase/kg diet).

Table 77. Mineral content[1] in feces of fish fed low-ash soybean meal diets (Study-5)[2].

	Ash	N	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Y	Zn
Phytase[3]	-------- % -----		---------- ppm ----------										
0U	14.2	1.78	9951	76.2	380	4445	5700	89.0	33244	8324	22.5	1005	189
CA[3]	15.9*	1.64	11436**	67.0	338*	5931	4671*	106.0	38887	8400	25.1	1018	124**
500U	13.0	1.61	9465	73.8	384	3981	4896	112.1	29951	6423*	22.7	1225	141**
500U+CA[4]	14.0	1.51*	8931**	70.4	315*	4798	3558***	67.9*	37785	1703***	21.1	1144	105***
1000U	12.5*	1.64	8461	70.3	387	5168	4353**	121.6	27453	4320**	20.0	1191	146*
2000U	11.8**	1.45*	8030*	69.3	392	5116	3722**	115.5	24875	2689***	18.7	1308*	114**
4000U	11.6**	1.57	7757***	70.1	363	5394	3415**	81.8	25262	1419***	18.4	1262*	121**
pre-tr.[5]	11.8**	1.83	6921***	76.3	359	4214	3880***	74.6	29242	999***	18.0	1177	138*

[1] Values are dry basis. Asterisks show the difference from the control (0U) group; P values * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). Each value is the mean of 3 tanks.

[2] See Tables 57, 58 (Ch. 3) for the composition of the diet.

[3] Phytase unit added to the diet (unit/kg dry diet).

[4] Citric acid.

[5] pre-treated soybean meal for 24h at 50°C with 200U phytase/kg soybean meal (equivalent to 100U phytase/kg diet).

Table 78. Mineral content[1] in the fish meal-soybean meal complex diets (Study-5).

	SBM[3]	Ash	N	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Y	Zn
Diet[2]	--------- % ---------			---------- ppm ----------										
C-100%	0	14.4	8.48	24416	15.0	133	5478	1441	16.3	17980	15835	47.9	318	136
Untreated	23.1	12.2	8.40	19389	19.0	139	9912	2117	28.4	13954	14693	36.5	245	132
Phytase	21.5	12.3	8.39	18664	18.0	136	9231	2130	25.0	14725	13630	38.2	250	115
PhytaseCA	23.2	12.0	8.01	20654	19.1	133	9388	2065	25.0	13797	14352	43.5	244	113
PhytaseCA+	25.2	11.7	8.04	18833	18.2	148	9341	2041	24.9	13625	14017	36.4	238	124
CA	25.4	11.9	7.92	20147	19.3	146	9685	2049	24.2	13652	14996	37.9	237	119
MW	28.2	12.2	8.22	18228	17.7	127	9388	1986	24.0	13064	13720	34.8	228	120
DryRoast	29.0	12.3	8.80	18132	19.6	133	9445	1977	24.4	13035	13519	35.8	226	116
Steam	23.2	12.4	8.70	19854	20.0	141	9394	2089	25.3	14104	14108	41.2	244	120
Cook	26.3	12.5	8.77	19059	17.6	133	9337	1961	22.9	13312	13852	37.7	234	118
Phytase-tr.	27.5	12.3	8.74	19440	17.9	140	9976	2046	24.6	13249	14152	38.1	230	125

[1] Values are dry basis.

[2] The control diet (C-100%) contained no soybean meal; the other diets contained ca. 30% soybean meal treated with various methods. Diet abbreviations (from the left): C-100% (fish meal-based control diet), Untreated (untreated-soybean meal), Phytase (phytase-added to the untreated-soybean meal diet), Phytase CA (phytase and citric acid added), Phytase CA+ (Phytase CA diet was incubated 24h at 50°C before drying), CA (citric acid added), MW (microwaved soybean meal), DryRoast (dry roasted soybean meal), Steam (steam-heated soybean meal), Cooked (cooked soybean meal), Phytase-Tr. (phytase treated soybean meal).

[3] Percentage of soybean meal (SBM) was determined from the concentration of yttrium in the control diet and the test diets.

Table 79. Mineral content[1] in feces of fish fed fish meal-soybean meal complex diets (Study-5).

	Ash	N	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Y	Zn
Diet[2]	-------- % -------		---------- ppm ----------										
C-100%	31.9***	4.14	96846***	66.0	603**	1928	2648*	75.5	20058	40563***	225**	1708***	303
Untreated	24.4	3.67	60291	74.8	483	2791	3349	78.5	27673	26910	146	1024	319
Phytase	24.0	3.55	61602	78.7	481	2755	3016*	82.3	26465	24698	149	1031	301
PhytaseCA	22.4**	3.59	53241*	84.1	485	2929	3193	72.7	30799	16851***	135	1057	227*
PhytaseCA+	21.4**	3.47	53218	79.4	474	1787	3314	85.1	27513	14034***	145	1063	212*
CA	21.4***	3.78	50197**	87.6*	461	2604	3093	65.6*	29455	16130***	130	1022	216*
MW	22.9*	3.88	57474	77.7	482	2857	3305	76.6	25792	25280	137	981	282
DryRoast	24.0	3.63	61556	83.0	473	3342	3165	72.5	26335	27450	147	1016	319
Steam	24.6	3.65	63401	80.8	484	4115	3191	82.0	26734	27632	147	1001	317
Cook	24.9	3.65	62418	75.9	469	4699	3098	76.5	26199	26756	146	1007	330
Phytase-tr.	22.8**	3.94	58284	79.3	482	4550	3342	74.0	24689	22262*	134	958	314

[1] Values are dry basis. Asterisks show the difference from the "Untreated" group; P values * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). Each value is the mean of 3 tanks.

[2] See footnote 2 of Table 78.