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REDUCING POLLUTANTS IN
AQUACULTURE EFFLUENTS

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NUTRITIONAL STRATEGIES FOR REDUCING POLLUTANTS IN AQUACULTURE EFFLUENTS

By

Laurel J. Ramseyer

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ABSTRACT

NUTRITIONAL STRATEGIES FOR REDUCING POLLUTANTS IN AQUACULTURE EFFLUENTS

By

Laurel J. Ramseyer

A nutritional P mass balance model was constructed for coho and chinook salmon as a method of estimating P losses from the Platte River Anadromous State Fish Hatchery. For the production period of January 1993 through May 1994, the P mass balance model indicated that 37.7% of P fed was retained by fish, 21.0% was discharged in the feces, and 41.3% was discharged in dissolved form. Without any raceway solids removal, a maximum of 2.8 kg feed P·metric ton^{-1} fish produced was discharged into the hatchery's stabilization pond. This loss rate was the lowest reported for a salmonid hatchery. Efficient removal of raceway solids could have reduced hatchery P losses to 1.8 kg P·metric ton^{-1} fish produced.

Improving the digestibility and utilization of nutrients in feeds should reduce nutrients in aquaculture waste water. Dietary phytate could impair dietary protein and mineral utilization in fish by binding zinc (Zn), creating a Zn deficiency. Zn deficiency could reduce protein and mineral utilization by reducing insulin secretion, insulin sensitivity, and the activity of the digestive enzymes carboxypeptidase B and alkaline phosphatase.

Rainbow trout were fed diets containing untreated or dephytinized soybean meal and corn gluten meal with or without supplemental Zn to determine if fish dietary phytate

impairs Zn bioavailability in fish. Fish fed diets containing untreated soybean meal and corn gluten meal without supplemental Zn (basal diet) were not Zn deficient after 170 d based on growth, whole fish Zn, P and protein content, total bone Zn, and the activity of alkaline phosphatase and carboxypeptidase B. Although bone Zn concentrations were reduced in fish fed the basal diet, total bone Zn increased in all fish regardless of dietary treatment. The basal diet contained enough available Zn to offset any negative effects of phytate on Zn bioavailability. Future assessments of Zn status in fish should be based on changes in Zn-dependent metabolism or total bone Zn rather than bone Zn concentration.

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TABLE OF CONTENTS

LIST OF TABLES vi

LIST OF FIGURES ix

INTRODUCTION 1

CHAPTER 1
ESTIMATION OF PHOSPHORUS LOADING FROM THE PLATTE RIVER ANADROMOUS STATE FISH HATCHERY 5
- Introduction 5
- Literature Review 8
- Materials and Methods 18
- Results 31
- Discussion 39
- Summary and Conclusions 44

CHAPTER 2
EFFECT OF DIETARY ZINC SUPPLEMENTATION AND PHYTASE PRE-TREATMENT OF SOYBEAN MEAL OR CORN GLUTEN MEAL ON GROWTH, ZINC BIOAVAILABILITY AND PROTEIN UTILIZATION IN RAINBOW TROUT 45
- Introduction 45
- Literature Review 47
- Materials and Methods 54
- Results 66
- Discussion 73
- Summary and Conclusions 79

LIST OF REFERENCES 80

APPENDIX A
SAMPLE DIGESTION PROCEDURES 95

APPENDIX B
PHOSPHORUS DETERMINATION PROCEDURES 97

APPENDIX C
PREDICTING THE NITROGEN AND PHOSPHORUS CONTENT OF WHOLE FISH FROM FISH WEIGHT 101

APPENDIX D
ALKALINE PHOSPHATASE ASSAY PROTOCOL 114

APPENDIX E
CARBOXYPEPTIDASE B ASSAY PROTOCOL 117

LIST OF TABLES

Table 1. Starting populations of coho and chinook salmon fry at the Platte River Anadromous State Fish Hatchery beginning each January. Parental stocks originated from Lake Michigan (MI) and Lake Huron (NY). Fish stock designations (i.e., Coho '91) indicate the year eggs were fertilized 19

Table 2. Moisture and P content of feeds fed to coho and chinook salmon at the Platte River Anadromous State Fish Hatchery between January 1, 1993 and May 31, 1994 21

Table 3. Regressions of whole fish P (mg) on fish wet weight (g) for coho and chinook salmon from the Platte River Anadromous State Fish Hatchery (hatchery), the Michigan State University Fisheries Research Laboratory (MSU), or both (combined). Values ± SE 32

Table 4. Monthly P mass balance measurements and calculations from the Platte River Anadromous State Fish Hatchery 35

Table 5. Phosphorus mass balance results for the experimental period of January 1993 through May 1994 at the Platte River Anadromous State Fish Hatchery 38

Table 6. Composition of experimental diets fed to rainbow trout to determine the effects of feedstuff dephytinization and zinc supplementation on fish zinc status and zinc bioavailability. Ingredients listed on as-fed basis 57

Table 7. Mineral and phytic acid composition of experimental diets. All values are on a dry weight basis 59

Table 8. Weight gain, protein and mineral composition of whole fish and fat-free dry bone, percent Zn retention (PZR)[1], percent protein retained (PPR)[2], specific activity of alkaline phosphatase (ALP) and carboxypeptidase B (CPB) and plasma insulin concentrations of rainbow trout[3] fed experimental diets[4] containing untreated or dephytinized ingredients and/or supplemental Zn. Values ± SE. 68

Table 9. Results of nonorthogonal contrasts of treatment means for whole fish protein, Zn and P, bone Zn, P and ash, percent Zn retained (PZR), total bone Zn, percent protein retained (PPR), the specific activity of alkaline phosphatase (ALP) and carboxypeptidase B (CPB) and the concentration of plasma insulin. Significant contrasts ($P < 0.05$) were marked 'X'. . . 70

Table 10. Regression relationship between log whole fish N (g) and log wet fish weight (g) of selected fish species. Values ± SE. 104

Table 11. Sources of whole fish N and fish wet weight data, sample sizes (n) and fish wet weight ranges used in the study of whole fish N content 105

Table 12. Regression relationships between whole fish P (mg) and wet fish weight (g) for salmon and trout. Values ± SE 107

LIST OF FIGURES

Figure 1. Conceptual drawing of water and effluent flow to and from the Platte River Anadromous State Fish Hatchery 6

Figure 2. Monthly effluent P loading and fecal P production estimates for the Platte River Anadromous State Fish Hatchery for the period January 1, 1993 through May 31, 1994 36

Figure 3. Relationship between P fed and P retained by coho and chinook salmon at the Platte River Anadromous State Fish Hatchery for the period January 1, 1993 through May 31, 1994 37

Figure 4. Relationship between percent dietary Zn retained (PZR) and total Zn fed to rainbow trout receiving diets containing untreated or phytase-treated ingredients with or without supplemental Zn 72

Figure 5. Relationship between the concentration of Zn in rainbow trout bone and the dietary phytic acid:Zn molar ratio 73

Figure 6. Relationship between bone Zn concentration and the dietary phytic acid:Zn molar ratio for channel catfish and rainbow trout 78

Figure 7. Relationship between whole fish P and fish weight for trout and salmon.108

INTRODUCTION

Aquaculture facilities have had a significant impact on the quality and productivity of their receiving waters by increasing downstream nutrient concentrations (EIFAC 1982; Iwama 1991; Pillay 1992). Phosphorus (P) and nitrogen (N) from aquaculture wastes have been of special concern because they are usually the first limiting nutrients in freshwater systems, and are therefore potential eutrophicants (Schindler 1977). The addition of P and N to oligotrophic (nutrient-poor) waters may be desirable in some cases (Johnston et al. 1990). In the Great Lakes region, where many lakes and streams are mesotrophic or eutrophic (nutrient-rich), and the addition of nutrients from aquaculture would be potentially damaging. Since the passage of the Federal Water Pollution Control Act of 1972[1] and the creation of the National Pollutant Discharge Elimination System[2] (NPDES), nutrient inputs from point sources like aquaculture have been regulated.

In 1988, the Michigan Department of Natural Resources (MDNR) was ordered by the Ingham County Circuit Court to reduce P discharged from the Platte River Anadromous State Fish Hatchery ("Platte Hatchery", Honor, Michigan) to 421 kg P·year^{-1}, which was lower than NPDES limits. This action resulted from a lawsuit brought against the MDNR by the Platte Lake Association. The Platte Lake Association alleged that P discharged from the Platte Hatchery, located approximately 14 km upstream from Platte Lake, caused decreases in summer water clarity of Platte Lake. Due to the

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[1]33 U.S.Code §§ 1251 *et seq.*, called the Clean Water Act

[2]U.S. Public Law 92-500

costs of litigation, the MDNR is contemplating the closure of the Platte Hatchery if funds for renovating the hatchery's effluent management system are not approved by the Michigan Legislature (Kelley Smith, MDNR, personal communication).

The Platte Hatchery case and others (Axler et al. 1996) have made it clear that public perception about the environmental impacts of aquaculture may be as important as sound fish husbandry methods for the ultimate success of this 'new' industry. Finding ways to minimize the environmental impacts of aquaculture demonstrates not only good environmental stewardship, but ideally will secure the industry against future criticism.

P losses from most hatcheries have been estimated from the difference in the P concentration of water collected periodically above and below the hatchery multiplied by the water flow rate. This chemical method has not accurately estimated the amount of P released by most hatcheries evaluated (Cho et al. 1991). Many factors, such as cleaning schedules, P cycling by algae and bacteria, and changes in husbandry practices affected concentrations of effluent P on a daily or even hourly basis. Consequentially, periodic water analysis has not adequately represented the P dynamics of hatcheries.

Fish feed has been identified as the primary source of hatchery-added nutrients in effluents (Westers 1991). A nutritional mass balance model, which partitions the fate of dietary P into fish body accretion, recovered wastes, and unrecovered wastes, should produce accurate P loading estimates (Cho et al. 1991). Such a model could aid planners in locating sites for future aquaculture facilities based on projected nutrient loadings into the receiving waters. A nutritional mass balance model should also help hatchery managers plan annual fish production without compromising P discharge limits.

Maximum possible fish production can be calculated based on feed P levels, expected P retention by fish, and expected P recovered from raceway solids. Finally, the mass balance approach could be used to evaluate the efficiency of P removal from culture systems.

Nutrient loading by hatcheries has been reduced by improving the digestibility of feed ingredients (Cain and Garling 1995; Rodehutscord and Pfeffer 1995). Plant feed ingredients such as soybean meal have been important protein sources in fish feeds (NRC 1993). However, soybeans and other seeds contain phytate, a P storage molecule. Phytate is largely indigestible by fish because they do not produce phytase, the enzyme which hydrolyzes phytate. Reduced utilization of dietary P, Zn and protein have been observed in fish fed purified diets supplemented with phytic acid (Spinelli et al. 1983; Richardson et al. 1985; Gatlin and Phillips 1989), and in practical diets containing native phytate (Cain and Garling 1995; Schäfer et al. 1995). How phytate reduces protein and mineral utilization in fish has not been fully described.

The goal of this research[3] was to measure and reduce aquaculture wastes using nutritional strategies. The text is divided into two chapters with the following objectives:

Chapter 1:

1. Develop a nutritional mass balance model to estimate P retention and losses for coho and chinook salmon from first-feeding to stocking size.
2. Use the mass balance approach to evaluate the efficiency of solids P removal efforts at the Platte Hatchery.

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[3] Use of fish in the research described in this dissertation was approved by the All-University Committee on Animal Use and Care, AUF # 11/93-420-03.

Chapter 2:

1. Determine rainbow trout fed a plant-based diet high in phytate become Zn deficient based on growth, protein utilization, plasma insulin concentration, the activity of carboxypeptidase B and intestinal alkaline phosphatase, and whole fish and bone Zn, P or ash content.
2. Determine if dephytinized plant ingredients or a dietary Zn supplement is more effective in providing Zn to fish.

A collateral study was also conducted to determine if whole fish P and whole fish N could be predicted from fish weight when direct measurements are not possible. Details of the collateral study are in Appendix C.

CHAPTER 1

Estimation of Phosphorus Loading from the Platte River Anadromous State Fish Hatchery

INTRODUCTION

A nutritional P mass balance model was constructed for coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*O. tshawytscha*) at the Platte River Anadromous State Fish Hatchery ("Platte Hatchery", Honor, Michigan) to estimate the amount of P discharged from the hatchery into the stabilization pond (Figure 1). Samples of whole coho and chinook salmon, feeds, and dissected feces were collected monthly from May 1993 through April 1994 from the Platte Hatchery for P analysis. Daily fish production, water temperature, feeding, mortality, and monthly removed raceway solids records for January 1993 through May 1994 were furnished by the hatchery. The experimental period of January 1993 through May 1994 was chosen so that an entire year-class of coho salmon could be included. Five individual stocks of fish were present in the hatchery during that period, and all were sampled. Monthly P loading estimates based on water samples collected at the Platte Hatchery's stabilization pond outlet were furnished

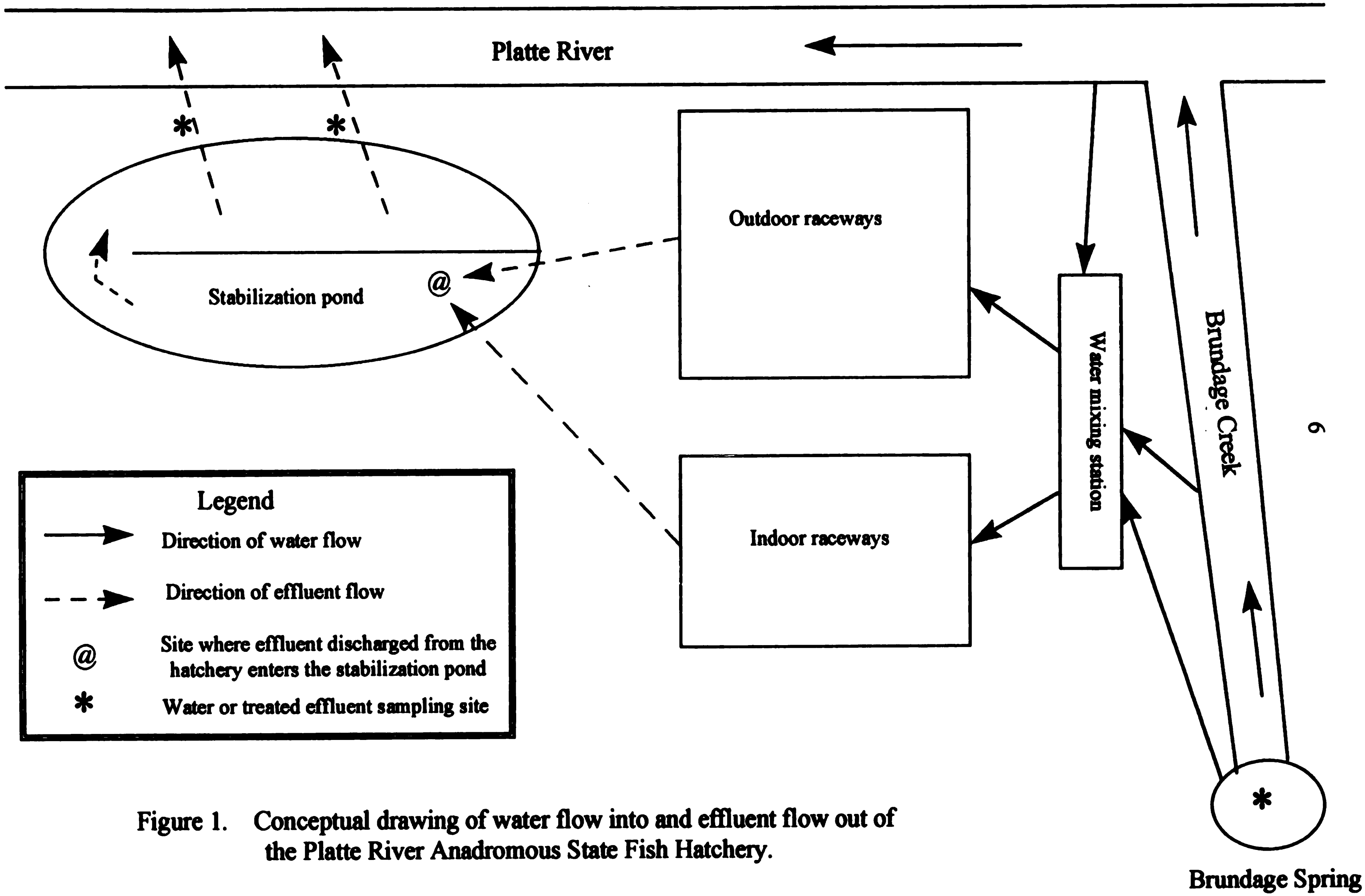

Figure 1. Conceptual drawing of water flow into and effluent flow out of the Platte River Anadromous State Fish Hatchery.

by the MDNR. Maximum and minimum possible amounts of P discharged from the Platte Hatchery into the settling basin were calculated using nutritional mass balance equations.

Live coho and chinook salmon from the Platte Hatchery were brought to the Michigan State University Fisheries Research Laboratory (MSU) each month for use in supporting experiments to 1) quantify the percentage of feed P leaving the rearing unit in dissolved form, 2) measure fecal P production, and 3) determine if fecal and whole fish P values for fish raised under controlled laboratory conditions were comparable with values from the Platte Hatchery.

Objectives

1. Develop a nutritional mass balance model to estimate P retention and losses for coho and chinook salmon from first-feeding to stocking size.
2. Use the mass balance approach to evaluate the efficiency of solids P removal efforts at the Platte Hatchery.

LITERATURE REVIEW

I. Effects of phosphorus on receiving waters

Phosphorus has been identified as the first limiting factor in primary productivity in most freshwater systems (Wetzel 1983). Additions of P into freshwater environments have caused increased productivity and eutrophication (Schindler 1977). Large P inputs have resulted in a shift of the first limiting nutrient from P to N, carbon, oxygen or light. Phosphorus loading which decreases the N:P ratio below 10 has precipitated shifts in the dominant phytoplankton species, favoring nitrogen-fixing bloom-forming bluegreen algae (Schindler 1977). Blue green algae, or cyanobacteria, have adversely affected freshwater productivity by covering the water surface and severely reducing light penetration. The decay of large algal blooms have in turn reduced dissolved oxygen to concentrations that are suboptimal or lethal to fish and other aquatic organisms.

Freshwater primary productivity has been positively correlated with total P concentration. In a review of experimental lake enrichment studies, Elser et al. (1990) found that P and P+N enrichment of North American lakes enhanced algal growth. Because primary productivity has been positively correlated with fish production (Oglesby 1977; Jones and Hoyer 1982), the P enrichment of a lake or river has had a profound effect on fish biomass by expanding the food base. Yurk and Ney (1989) found that the P concentration in 22 southern Appalachian reservoirs could predict 75% of the variation (*P* < 0.001) in fish standing stock. In one Virginian reservoir which had been subject to a P reduction program, total P concentration and fish biomass decreased together over an 11-year period, and total P concentration explained 66% of the variation in fish standing stock. Downing et al. (1990), in a review of lake studies worldwide, found that fish production was closely correlated with annual phytoplankton production ($r^2 = 0.79$, $P < 0.001$), mean total P concentration ($r^2 = 0.67$, $P < 0.002$) and annual average fish standing stock ($r^2 = 0.67$, $P < 0.001$).

Numerous studies have indicated that fish hatchery effluents may significantly alter the nutrient status and productivity of receiving waters (Hinshaw 1973; EIFAC 1982; Bergheim et al. 1984; Merican and Phillips 1985; Wiesmann et al. 1988; Kendra 1991). Szluha (1974) found periphyton production below the Jordan Valley National Fish Hatchery, Michigan, to be 5 to 7 times greater than above the hatchery. Eloranta and Palomaeki (1986) found significant increases in lake phytoplankton biomass, chlorophyll *a* concentrations, rate of primary productivity, and changes in phytoplankton community structure as a result of nutrient loading from a Finnish fish farm.

The effects of P loading have been site-specific and depend on the concentration and consistency of P inputs, the chemical form of P released, the presence of other nutrients in the effluent, and the prior nutrient status of the receiving water. Consequently, P loading may not be detrimental in certain locations. Non-productive (nutrient-poor) rivers have been deliberately fertilized with N and/or P to increase fish production (Johnston et al. 1990). Oberdorff and Porcher (1994) found an increase in the density and biomass of most fish species downstream from trout farms in France. However, sensitive species were often replaced by pollution-tolerant and exotic species.

II. Strategies for decreasing phosphorus in hatchery effluents

A. Dietary formulation

Westers (1991) identified feed in the form of feces and uneaten feed as the primary source of P in hatchery effluents (Westers 1991). Reductions in effluent P have been achieved by feeding experimental diets providing dietary P at near-requirement levels and in highly digestible forms (Ketola 1985; Ketola et al.1991; Ketola and Richmond 1994; Cain and Garling 1995). However, minimizing effluent P through feed formulation has been difficult in practice. Substantial quantities of native P occur in key feed ingredients like soybean meal, corn gluten meal and fish meal (NRC 1993). Much of this P is present in forms that are poorly digested by fish (Lovell 1978; Satoh et al. 1987). Riche and Brown (1996) found that P in fish meal, primarily from bone, was 21-55% digestible by rainbow trout. Ogino et al. (1979) observed fish meal P was even less digestible in fish such as the common carp which lack acid digestion. Although most fish have been shown to require 5-8 g $P \cdot kg^{-1}$ dietary dry matter (NRC 1993), commercial fish feeds often contain 10.5-14 g $P \cdot kg^{-1}$ dietary dry matter to compensate for poor P digestibility. Low P commercial feeds (approximately 7 g $P \cdot kg^{-1}$) based on deboned fish meal are currently available (i.e., Bioproducts, Warrenton, Oregon). However, the expense of the deboning process has increased feed costs substantially.

Commercial fish feeds have traditionally contained fish meal as the primary protein source (250-500 g kg^{-1}). However, the high cost of fish meal has prompted an increased use of plant protein sources such as soybean meal and corn gluten meal. These feed ingredients contain phytate, a P storage molecule which has been shown to be poorly digested by fish (Cain and Garling 1995; Richie and Brown 1996). Fifty to 70% of soybean meal P was bound in phytate (Lolas et al. 1976; Lott 1984). The stability of phytate was not effected by feedstuff processing techniques such as defatting and heating (Davies and Reid 1979; Clydesdale and Camire 1983).

Phytate can be dephosphorylated by the enzyme phytase (*myo*-inositol hexaphosphate phosphohydrolase, EC 3.1.3.8). However, monogastric fish like rainbow trout do not produce intestinal phytase. Additions of P supplements have been required in fish feeds which contained untreated soybean meal or other plant seed products as major protein sources (Ketola 1975; Lovell 1978; Cain and Garling 1995). The bioavailability of phytate-P in plant feedstuffs has been greatly improved for poultry and swine by pre-treating the feedstuff with fungal phytase (Nelson et al. 1971; Lei et al. 1993b; Qian et al. 1997). Cain and Garling (1995) found that dietary P supplements could be eliminated from plant-based rainbow trout feeds when soybean meal was pre-treated with phytase.

The addition of microbial phytase or phytase-containing transgenic seeds directly to the feed has also improved the bioavailability of dietary P for swine (Lei et al. 1993b,c; Young et al. 1993) and poultry (Nelson et al. 1971; Pen et al. 1993; Qian et al. 1997). Little or no inorganic P supplementation was required in the phytase-added diets. Phosphorus availability has also been improved for common carp and rainbow trout fed diets supplemented with phytase (Rodehutscord and Pfeffer 1995; Schäfer et al. 1995; Riche and Brown 1996). In the experiments with fish, most dephosphorylation of phytate by phytase probably occurred during diet preparation. However, Rodehutscord and Pfeffer (1995) found that P utilization (P deposition·P intake^{-1} x 100) in phytase-added diets was influenced by fish rearing temperature. Since phytase activity has been shown to be temperature dependent (Han et al. 1987), Rodehutscord and Pfeffer (1995) concluded that some phytase activity may have occurred in the fish gut.

Adding phytase directly to fish feeds may be beneficial if a stable form of low cost phytase becomes available. However, pre-treating feedstuffs with phytase or preparing feeds in a way that promoted phytase activity during feed processing would avoid inconsistent results due to loss of enzyme activity during feed storage or as a result of low fish rearing temperatures (10-15°C).

B. Solids management

Since P is rapidly leached from feed and feces left in water for periods as brief as 5-60 min (Windell et al. 1978; Smith et al. 1980; Brown et al. 1989; Phillips et al. 1993), Westers (1991) recommended careful handling of settleable solids to reduce P in hatchery effluents. Clark et al. (1985) observed that solids were fragile and easily broken into smaller particles by husbandry practices and fish movement. Nutrient leaching should be higher in smaller particles because they have greater surface-to-volume ratios (Westers 1991). Boerson and Westers (1986) designed a baffle system to continually move solids down the raceway to a screened-off settling area where fish movement would not resuspend or break the settling waste. Merican and Phillips (1985) and Åsgård et al. (1991) emphasized that the most efficient method of reducing solids in raceways was to minimized feed inputs.

Pellet binders could improve the efficiency of solids removal by increasing the stability of feces in water (Storebakken 1985; Storebakken and Austreng 1987; Hardy 1989). Westers (1991) reported that intact fish feces had a rapid settling velocity and could be efficiently removed from raceways, whereas fragmented fecal matter had a reduced settling velocity and was difficult to collect.

C. <u>Recovery of dissolved P from effluents</u>

Even well balanced feeds fed appropriately will result in the loss of some P in dissolved form. Efforts to recapture dissolved P from hatchery effluents have included the production of economically important species such as the agar-producing algae *Gracilaria* spp. in raceway effluents (Buschmann et al. 1994). Mussel and oyster culture conducted in conjunction with finfish culture have also benefited from the dissolved nutrients (Folk and Kautsky 1989; Shpigel et al. 1993). The production and removal of polyphosphate-accumulating bacteria in effluents has been effective in removing dissolved P, although such a method may be cost-prohibitive (Kortstee et al. 1994).

In Michigan, some effluent P could be reclaimed by diverting hatchery effluents to baitfish ponds or hydroponics facilities. However, the perturbation of pond sediments by baitfish could resuspend particulate P and actually increase P losses. And, the N and P concentrations in hatchery effluents were too dilute for vegetable production (Rakocy and Hargreaves 1993). Formal research on the value of secondary production for waste reduction has been lacking for the northern temperate region.

Constructed wetlands have been used as 'biological sponges' for the treatment of industrial, agricultural and municipal waste (Bavor and Mitchell 1994). Recent research has indicated that constructed wetlands may be useful in treating effluents released periodically from aquaculture ponds (Schwartz and Boyd 1995). Before constructed wetlands can be recommended as a treatment for effluents from flow-through raceway systems, the issues of large solids loads, high flow rates, large land requirements and wetland capacity to reduce P must be addressed.

III. Estimating phosphorus loading

Cho et al. (1991) suggested that a dietary mass balance model could be used to calculate P lost from fish hatcheries. Mass balance studies have been useful for back-calculating dietary nutrient concentrations of deer (Howery and Pfister 1990), moose (Leslie et al. 1989), and domestic livestock (Belonje and Van den Berg 1980; Holechek et al. 1982, 1985). Mass balance models have been used to estimate P and N cycling by fish in lakes (Kraft 1992), P turnover from sediments beneath salmon rearing pens (Holby and Hall 1991) and changes in effluent P resulting from the dephytinization of dietary soybean meal (Cain and Garling 1995).

The quantity of P in hatchery effluents can be described by:

$$P_{ef} = P_{in} + P_{ho} - P_{abs} \quad (1)$$

where:

P_{ef} = P in hatchery effluent

P_{in} = P in incoming water

P_{ho} = P of hatchery origin (from dissolved excreta, leachate from feces and uneaten feed)

P_{abs} = P absorbed from water by fish

Since absorption of P from water was negligible by fish at the low P concentrations of 10-20 $\mu g \cdot L^{-1}$ generally present in incoming water (Solomatina and Arsan 1980; Lall 1991), P_{abs} may be omitted from the equations under ordinary situations. Therefore P_{ef} should only change as a result of P_{ho}.

Two models used to estimate P_{ho} have been the chemical model and the nutritional model (Cho et al. 1991). In the chemical model, P concentrations were measured in water samples, and P_{ho} was estimated as:

$$CP_{ho} = (P_{out} - P_{in}) \times W \tag{2}$$

where:

CP_{ho} = P in effluent of hatchery origin estimated using the chemical model

P_{out} = P in hatchery outflow

P_{in} = P in hatchery incoming water

W = Water flow rate

The P content of fish, feed and fecal matter were used to estimate P_{ho} in the nutritional model:

$$NP_{ho} = P_{fed} - [P_f + (P_{tm} - P_{ti}) + P_m] \tag{3}$$

where:

NP_{ho} = P in effluent of hatchery origin estimated using the nutritional model

P_{fed} = P in feed fed

P_f = P in feces and uneaten feed (solids)

P_{tm} = P in fish at the end of the growth period

P_i = P in fish at the beginning of the growth period

P_m = P in dead fish

Equation 3 assumes that fish do not consume fecal matter or any non-feed item. Based on personal observations, this assumption is reasonable for trout or salmon reared in cement raceways.

Although the chemical method has been the accepted method for determining hatchery compliance with NPDES regulations in Michigan, it may result in erroneous estimates of hatchery P losses. Krom and Neori (1989) and Cho et al. (1991) found the chemical method to overestimate P loading by 1.1 and 1.8 times, respectively, as compared to the nutritional method. Krom and Neori (1989) sampled water twice daily for the duration of their month-long experiment, and Cho et al. (1991) combined hourly samples over a weeks time for weekly average P concentrations. These sampling frequencies were inadequate since many factors like cleaning schedules, uptake by periphyton in the raceway, or changes in feeds or rearing practices may have affected day-to-day P concentrations in effluent water (Bergheim et al. 1991; Foy and Rosell 1991a). Since feces left in water have been shown to lose a significant proportion of their P content within 5 min of excretion, even hourly samples could have missed P loading peaks (Phillips et al. 1993).

In contrast to the conclusions of Cho et al. (1991) and Krom and Neori (1989) that the chemical model did not accurately measure P losses, Foy and Rosell (1991a) found close agreement between the two models in a year-long survey of effluents at a Northern Ireland hatchery. The nutritional model they used was:

$$P_{ho} = (FCR \times P_{fc}) - P_{tm} \tag{4}$$

where:

FCR = Feed conversion ratio (kg food fed$\cdot$kg^{-1} fish produced)

P_{fc} = Feed P concentration

P_{tm} = P content of fish (fish growth × P concentration in 500 g fish)

To estimate effluent P using the chemical model (Equation 2), they sampled inflow and outflow hourly for 24 h about every 5th day.

When calculating P_{ho} according to Equation 4, Foy and Rosell (1991a) used the FCR as an estimate of digestibility, thereby avoiding the need for feces collection. However, their FCR of 1.8 indicated overfeeding and feed wastage, and they noted that a FCR of 1.5 or less would have been closer to expectations. They also assumed that the percent whole body P was the same for 3-500 g trout. Based on data from Shearer (1984), that assumption was not valid. This error could have resulted in an overestimate of P_{ho} by underestimating the P content of fish at the beginning of the experiment.

MATERIALS AND METHODS

Fish

Adult coho and chinook salmon were captured between September and December each year as they returned to Michigan rivers to spawn. Eggs were also collected from spawners returning to New York rivers because New York populations were thought to be free of bacterial kidney disease which had devastated Michigan populations. Egg collection data and the condition of return spawners may be found in Pecor (1992). Eggs were stripped, artificially fertilized then incubated in Heath trays at the Platte Hatchery (Westers 1979). Newly hatched coho and chinook salmon (fry) were introduced into indoor 38.2 m^3 cement raceways from late December through early February (Table 1). Chinook salmon remained in the indoor raceways until release the following May or June. About 9 months after hatching, coho salmon were transferred to outdoor 56.6 m^3 cement raceways where they remained until release in their second spring (May or June), approximately 17 months after hatching.

Coho and chinook salmon were never placed in the same raceways. Michigan and New York fish were kept in separate raceways for 2-12 months, then mixed. Until fish were mixed, separate growth curves were developed for Michigan and New York fish.

Average fish weight was calculated monthly by hatchery personnel using a fish growth model based on water temperature history. The model was designed specifically for Platte Hatchery coho and chinook salmon (Roger Hubble, MDNR, personal communication).

Table 1. Starting populations of coho and chinook salmon fry at the Platte River Anadromous State Fish Hatchery beginning each January. Parental stocks originated from Lake Michigan (MI) and Lake Huron (NY). Fish stock designations (i.e., Coho '91) indicate the year eggs were fertilized.

		Number of fry introduced to raceways		
Fish stock	year introduced	MI	NY	Total
Coho '91	1992	3,053,268	588,000	3,641,268
Coho '92	1993	1,936,761	327,600	2,264,361
Coho '93	1994	2,751,533	351,600	3,103,133
Chinook '92	1993	3,811,961	1,702,484	5,514,445
Chinook '93	1994	5,087,976	692,640	5,780,616

Platte Hatchery rearing conditions

Fish were reared without supplemental lighting. Natural light intensity was reduced in the hatchery building (where indoor raceways were located) by green fiberglass windows. Outdoor raceways were subject to full sun.

Three water sources were used by the Platte Hatchery: Brundage Spring, Brundage Creek, and the Platte River. Eggs were incubated in water from Brundage Spring. Mixtures of water from all three sources were used to rear fish. The proportion of the total flow coming from each water source was determined by volume, temperature and dissolved oxygen requirements and water clarity. Water flow rates provided 1-4 exchanges·h^{-1} depending on fish density and size. Water temperature fluctuated seasonally between 6 and 13° C. Standard rearing techniques were employed (Westers 1979).

Platte Hatchery feeds and feeding

All feeds used during the 17 month experimental period were obtained from Bioproducts, Inc. (Warrenton, Oregon) except for smaller quantities of feed from Zeigler Bros., Inc. (Gardners, Pennsylvania) used January through March, 1993. Bioproducts, Inc. feeds were formulated from Kodiak white fish meal[4], feather meal, cereal tailings (cooked oats, wheat, barley or rice), spray dried blood meal, herring meal, processed deboned fish, vitamin and mineral supplements and preservatives. The P content of the feeds varied from 6.7-14.8 g P·kg^{-1} (as fed basis). Most feeds were "low P", containing 10 g P·kg^{-1} or less. During periods when Kodiak white fish meal was unavailable to the manufacturer (usually December through March), feeds contained 10 g P·kg^{-1} or more (Dennis Rolie, Bioproducts, Inc., personal communication).

Most feed lots received by the Platte Hatchery were analyzed for total P by Lancaster Laboratories, Inc., Lancaster, Pennsylvania (Table 2). However, few of the medicated feeds were sent to Lancaster Laboratories, Inc for analysis. Feed samples were digested using nitric and perchloric acids (AOAC 1984) and P was determined using the

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Kodiak white fish meal is a low P fish protein concentrate made by pressing ground raw fish (often pollack) through a screen to remove bone, skin and scales (Dennis Rolie, Bioproducts, Inc., personal communication).

Table 2. Moisture and P content of feeds[1] fed to coho and chinook salmon at the Platte River Anadromous State Fish Hatchery between January 1, 1993 and May 31, 1994

Feed	Designation	Moisture (% as fed)[2]	P ($g \cdot kg^{-1}$ diet as fed)
Bio Diet Starter	# 3	20.5	12.0-13.0
Bio Diet Grower	1 mm	20.5	11.0
Bio Dry	1 mm	11.0	11.0
Bio Dry	1.3 mm	11.0	8.0-10.0
Bio Dry	1.5 mm	11.0	8.4-10.0
Bio Dry	2 mm	11.0	8.5-9.2
Bio Dry	3 mm	11.0	7.6-8.7
Bio Dry 1000 LP	1 mm	11.0	8.6-9.2
Bio Moist	1.3 mm	26.0	9.5
Bio Moist	3 mm	26.0	6.7-6.9
Bio Moist + Gallimycin	1.3 mm	26.0	14.6
Bio Moist + Gallimycin	1.5 mm	26.0	7.5
Bio Moist + Gallimycin	3 mm	26.0	14.8
Bio Moist + Terrimycin	1.5 mm	26.0	07.5
NY	3/32	unknown	12.0
NY	1/8	unknown	12.0

1 All feeds manufactured by Bioproducts, Inc., Warrenton, Oregon except "NY" diets, which were manufactured by Zeigler Bros., Gardners, Pennsylvania.

2 Based on information provided by the manufacturer.

ascorbic acid-molybdenum method (APHA et al. 1985). Feed P concentrations were confirmed for several feeds at MSU using the method of Gomori (1942) after digestion in nitric and perchloric acids (AOAC 1984). Up to eight different feeds were in use during any given month at the hatchery. The use of multiple feeds was not communicated to MSU until after the experiment was concluded. Therefore, the P concentration of several diets was not confirmed at MSU, but was estimated based on information provided by the manufacturer (Dennis Rolie, Bioproducts, Inc., personal communication). Sample digestion and P determination protocols used at MSU are detailed in Appendices A and B, respectively.

Feeds were stored at approximately 7°C and were generally fed within six months of receipt. However, feeds for fry and medicated feeds were occasionally fed one year after receipt. Individual lots of feed were fed in the order in which they were received.

Feed was offered at 2% wet body weight$\cdot d^{-1}$ (as fed basis) to all size classes of salmon. A portion of the daily ration was distributed every 30 min during the 8 h workday by an automated pneumatic system. Feed was distributed over about 80% of the raceway surface. Feed was withheld on days when heavy rain and silt reduced feed visibility to the fish. In winter, when low water temperatures resulted in decreased feeding activity, feeds were offered every second or third day. Daily feed distribution records were maintained by Platte Hatchery personnel.

Platte Hatchery solids management

Indoor raceways were outfitted with a screen and baffle system which allowed solids to accumulate at the end of each raceway (Boerson and Westers 1986). A new vacuum system removed water and solids from the end of indoor raceways every 30 min. The vacuumed slurry was filtered with a 40 μm screen and the resulting sludge removed from the hatchery system. Filtrate was discharged into the hatchery stabilization pond. Platte Hatchery personnel collected random samples of sludge for P analysis. Raceways were scrubbed and siphoned once daily when the vacuum system was not in operation and in raceways not equipped with the system. Siphoned water and solids were discharged directly into the stabilization pond. Outdoor raceways were scrubbed and siphoned about every 3 d.

Platte Hatchery fish and feces sampling procedures

Monthly, 10 coho salmon and 10 chinook salmon were randomly collected from raceways with a net. Tricaine methane sulfonate (MS-222) was used at 15-30 mg·L^{-1} for sedation prior to weighing, and at 100-200 mg·L^{-1} (lethal dose) prior to dissection. Feces were collected by dissection (Brown 1993) for P_f calculations. An incision was made along the fish's left side with a scissors. The intestine was ligated with forceps at the transition point between the anterior and posterior intestine to prevent movement of material between the intestinal segments. The posterior intestine was cut away from the anus, and fecal matter was gently squeezed from the posterior intestine into a pre-weighed aluminum dish. The emptied intestinal segment was returned to the fish, and the carcass was frozen until processed. The weight of fresh fecal samples was determined by subtracting the initial weight of the pan from the weight of the pan containing the sample. Fecal samples were dried at 105°C for 24 h in a forced air oven. After cooling in a desiccator, total fecal dry matter was determined by subtracting the initial weight of the pan from the weight of the pan containing the dry fecal matter. Dried samples were stored in a desiccator until digestion in nitric and perchloric acids (AOAC 1984). The P concentration was determined according to APHA et al. (1985).

Frozen whole fish were sliced into 1-cm sections and lyophilized 72 h, dried at 60°C for 12 h in a vacuum oven and cooled in a desiccator. Whole fish dry matter was determine by the same method as fecal matter. Dried fish were ground in liquid nitrogen using a stainless steel Waring blender (Waring, New Hartford, Connecticut) , then frozen in plastic bags until digestion in nitric and perchloric acids (AOAC 1984).

Platte Hatchery water sampling procedures

Water which passed through the hatchery enters a stabilization pond before being discharged into the Platte River (Figure 1). Platte Hatchery personnel collected composite 24 h water samples beginning every Monday and Thursday morning at the Brundage Creek water intake site (representing P in water entering the hatchery) and at the stabilization pond outlets (representing P leaving the stabilization pond). The sample collection device was a 10 L polypropylene bottle with an air release outlet and water intake hole in the lid. The rate of water intake was regulated by a 30 gauge needle attached to the end of the water intake hole.

A 250 mL subsample of each 24 h water sample was refrigerated in a Teflon topped glass bottle containing 3 mL sulfuric acid. Water samples were analyzed for P weekly by Great Lakes Environmental Laboratories (Traverse City, Michigan). Unfiltered 8 mL samples from the glass bottles were digested using the sulfuric acid-ammonium persulfate method (APHA et al. 1985). Total P was quantified with a Technicon autoanalyzer using the ascorbic acid-molybdenum method (APHA et al. 1985). The minimum detection limit was 3 $\mu g \cdot L^{-1}$. A standard curve was made by diluting an external Environmental Protection Agency standard to 5-60 $\mu g \cdot L^{-1}$. Total P values were used by the MDNR to calculate monthly discharges of P from the stabilization pond.

Rearing conditions at MSU

Triplicate groups of coho salmon and/or chinook salmon were reared in 41 L or 125 L rectangular plastic tanks. Tanks were supplied with aerated well water at 12 ± 1 °C. Supplemental light was provided by overhead fluorescent lights on automatic timers following the natural light:dark photoperiod. Light intensity was lowered by covering the light fixtures with 1 mil brown plastic. New fish were obtained from the Platte Hatchery every 4-8 weeks. After an initial 7-10 d acclimation period, fish were randomly distributed to tanks and the weight of fish in each tank was determined. Initial fish loading rates were 0.5-2 $kg \cdot (L \text{ per min})^{-1}$ (Westers 1979). Each batch of fish was reared for 4 weeks. Weekly removal of fish for fecal collection maintained loading and density at approximately initial levels.

Fish were fed 2% wet body weight·d^{-1} on a dry matter basis. Feed dry matter was determined by the same method as fecal dry matter. The daily ration was divided into two feedings, at least 4 h apart. Feed was fed by hand at a rate of 1-2 pellets·s^{-1}. At this feeding rate, all feed should have been consumed (Westers 1987). Fish and fecal samples were collected weekly using the Platte Hatchery procedures.

Effluent P study at MSU

To measure the proportion of feed P leaving the rearing unit in dissolved form, effluent water samples were collected from three 41 L tanks containing 17 coho salmon each. Average fish weight (± SD) was 6.0 ± 0.4 g and total tank weight was 102 ± 5 g. Water flow rate was 2.5 L·min^{-1}. Fish were fed 1.3 mm Bio Dry 1000 which contained 9 g P·kg^{-1} on a dry matter basis (Bioproducts, Inc.). The effluent experiment began 48 h before the end of a 28 d growth period. Sampling of effluent water began 1 h before the first feeding and continued at 30 min intervals for 24 h. Each 50 mL water sample was stored at 4°C in a polypropylene bottle containing 1 mL concentrated HCl. Water samples were digested within 24 h of collection using the sulfuric acid-ammonium persulfate method (APHA et al. 1985). P concentrations were determined according to APHA et al. (1985). The minimum detection limit was 10 μg P·L^{-1}

Feces production and P content

Acid insoluble ash was measured as an internal marker to calculate feed P digestibility and fecal P production. Acid insoluble ash was isolated according to Atkinson et al. (1984). Feed and feces were dried for 24 h at 105°C, then ashed for 16 h at 600°C. Samples were cooled in a desiccator, then weighed to determine percent ash. Ash was boiled in 100 mL 2M HCl for 5 min, filtered hot through Whatmann No. 42 ashless filter paper, and the cake washed with boiling deionized distilled water. Filter papers were then dried and ashed for 16 h at 600°C. The residue was considered acid insoluble ash.

A literature search was conducted to find estimates of dietary dry matter digestibility for juvenile salmonids fed feeds similar to those used at the Platte Hatchery. Dietary dry matter digestibilities reported by Kim and Kaushik (1992), Shearer et al. (1992), Gomes et al. (1993) and Oliva-Teles et al. (1994) were averaged, then subtracted from 100 to give an estimate of the percent of dietary dry matter not retained by the fish. Monthly P_f values were then calculated as:

(kg dry matter fed) × (100-dry matter digestibility)·$(100)^{-1}$ × (g P·kg^{-1} dry dissected feces).

Mass balance models

The Platte Hatchery estimated the amount of P released from the stabilization pond into the Platte River as:

$$CP_{sp} = (P_{out} - P_{in}) \times W \quad (5)$$

where:

CP_{sp} = P exiting the stabilization pond

P_{out} = P in stabilization pond outflow

P_{in} = P in Brundage Spring water entering the hatchery

W = Water flow rate

Since fecal P rapidly leaches into water upon excretion, measuring P_f in feces taken directly from the posterior intestine (i.e., before any leaching occurs) will result in a "minimum possible" P_{ho} ($MINP_{ho}$). Minimum possible P_{ho} ($MINP_{ho}$) was calculated as:

$$MINP_{ho} = P_{fed} - [P_f + (P_{tm} - P_{ti}) + P_m] \qquad (6)$$

where:

$MINP_{ho}$ = minimum possible P of hatchery origin in effluents entering the stabilization pond

P_{fed} = P in feed

P_f = P in dissected feces

P_{tm} = P in fish at the end of the month

P_{ti} = P in fish at the beginning of the month

P_m = P in dead fish

Equation 6 was modified by omitting P_f to estimate the maximum possible P_{ho} ($MAXP_{ho}$):

$$MAXP_{ho} = P_{fed} - [(P_{tm} - P_{ti}) + P_m] \qquad (7)$$

where:

$MAXP_{ho}$ = maximum possible P of hatchery origin entering the stabilization pond

A P_f represented by P concentrations found in collected raceway solids (P_s) should provide an "actual" P_{ho} (AP_{ho}). Actual P_{ho} (AP_{ho}) was calculated as:

$$AP_{ho} = P_{fed} - [P_s + (P_{tm} - P_{ti}) + P_m] \qquad (8)$$

where:

AP_{ho} = actual amount of P released from the hatchery into the stabilization pond

P_s = P concentration in collected raceway solids

The efficiency of P removal from raceways through solids removal was calculated as:

$$(MINP_{ho} / AP_{ho}) \times 100 \qquad (9)$$

P_{ho} was calculated at monthly intervals for each fish stock or substock (e.g., New York coho salmon spawned in 1993). P_{fed} was calculated as (g P·kg^{-1} feed × kg feed fed·month^{-1}). P_f was calculated as (mg P·kg^{-1} dry feces × kg dry feces produced·month^{-1}). P_{tm} and P_{ti} were calculated as (mg P·fish^{-1} × number of fish at the end and beginning of each month, respectively). Monthly estimates of average fish weight within each substock was regressed against day of the year to estimate the weight of dead fish on any given day. P_m was calculated daily and summed for each month. Estimates of whole fish P for both living and dead fish were made using whole fish P vs. fish wet weight regressions describing fish sampled at the Platte Hatchery and/or MSU. The percent of feed P retained in fish was calculated as $(P_{tm} - P_{ti} + P_m) / P_{fed}$. Separate monthly P_{ho} values for each fish stock or substock were summed to make monthly hatchery totals. The ratio of P lost in dissolved form vs. P lost in feces (d:f ratio) was calculated as (% P fed lost in dissolved form : % P fed lost in feces) using 17 month totals.

Statistical analysis

Linear regression relationships were calculated for whole fish P vs. fish weight, fecal percent dry matter vs. fish weight, and fecal P concentration vs. fish weight using the SAS software General Linear Model procedure (SAS Institute 1997). Data for fish reared at MSU and the Platte Hatchery were combined if they regressed homogeneously. Regression relationships were considered significant if $P \leq 0.05$.

RESULTS

Body composition

Regression relationships between whole fish P and fish weight are summarized for coho and chinook salmon in Table 3. All regression relationships were significant and highly correlated ($r^2 > 0.98$). Coho salmon reared at the Platte Hatchery and MSU did not differ in the whole fish P vs. fish wet weight relationship. Hatchery and MSU data sets for coho salmon were merged, and the combined regression equation was used to estimate coho salmon P content in the mass balance study. Regressions of whole fish P on fish wet weight differed significantly for chinook salmon reared at the Platte Hatchery and MSU. The phosphorus content of chinook salmon in the mass balance experiment was estimated using the hatchery equation (Table 3).

Feces characteristics and production

Rearing location had no effect on fecal percent dry matter or mg $P \cdot g^{-1}$ dry feces in coho or chinook salmon. No relationship was found between fecal percent dry matter or mg $P \cdot g^{-1}$ dry feces and the weight of chinook salmon. Chinook salmon feces averaged (± SE) 16.0 ± 5.6 % dry matter and 5.23 ± 4.7 mg $P \cdot g^{-1}$ dry feces. Fecal percent dry matter and fecal P content were significantly effected by fish weight for coho salmon:

$$\text{coho salmon fecal dry matter (\%)} = -0.0521 \cdot [\text{fish wet weight (g)}] + 15.3. \qquad (10)$$

Table 3. Regressions of whole fish P (mg) on fish wet weight (g) for coho and chinook salmon from the Platte River Anadromous State Fish Hatchery (hatchery), the Michigan State University Fisheries Research Laboratory (MSU), or both (combined). Values ± SE.

Species	location	slope	intercept	r^2	n	fish weight range (g)
coho salmon	hatchery	4.35 ± 0.08	-1.11 ± 1.27	0.98	56	0.267-41.2
coho salmon	MSU	4.18 ± 0.06	-1.73 ± 1.30	0.98	82	0.267-46.8
coho salmon	combined†	4.21 ± 0.05	-1.19 ± 0.93	0.98	138	0.267-46.8
chinook salmon	hatchery†	4.13 ± 0.06	-0.57 ± 0.08	0.99	48	0.278-3.15
chinook salmon	MSU	3.78 ± 0.08	-0.53 ± 0.15	0.99	34	0.400-4.07

† Equation used to estimate whole chinook salmon P content for mass balance calculations.

$$\text{mg P}\cdot\text{g}^{-1} \text{ dry coho salmon feces} = 0.30[\text{fish wet weight (g)}] + 6.14. \quad (11)$$

Coho salmon feces averaged 14.4 ± 3.0% dry matter and 8.76 ± 5.4 mg P·g^{-1} dry feces.

Measurable amounts of acid insoluble ash were recovered from feeds (0.2-0.7% dry matter), but not from the pooled dissected feces of thirty-five 15 g coho salmon (300 mg dry fecal matter). A literature review and consultation with a Bioproducts, Inc. nutritionist (Dennis Rolie, personal communication) revealed no data on the dry matter digestibility of Bioproducts feeds for juvenile salmonids. However, the average dry matter digestibility of feeds similar to the Bioproducts, Inc. feeds used at the Platte Hatchery was 78.0 ± 0.9% SD for 14-158 g rainbow trout and Atlantic salmon. Digestibility estimates should have been more or less the same for juvenile coho and chinook salmon, rainbow trout and Atlantic salmon, and should not have been affected by fish weight or age in juvenile fish (Karl Shearer, National Marine Fisheries Service, personal communication). Based on an assumed retention of 78.0% feed dry matter, 19,016 kg dry feces were produced over the 17 month experimental period. Total feces were estimated to contain 146.8 kg P.

Since the feeds fed at the Platte Hatchery contained less than 1% fines, and fish were reported to consume all feed offered (Roger Hubble, MDNR, personal communication), feces should have comprised 98-99% of raceway solids.

Hatchery personnel were unable to provide information on the amount or P content of raceway sludge (P_s) collected by the vacuum system during the experimental period. The inability to provide this data was not communicated until well after the experiment ended. Consequently, data on the P content of removed solids (P_s) for the experimental period was not available. In addition, the needles which regulated the flow of water into the Platte Hatchery's water sampling devices often either clogged or admitted too much water into the containers. When operating properly, containers should have been 50-75% full after 24 h (Roger Hubble, MDNR, personal communication). Approximately 75% of the time, containers were full after 24 h.

Phosphorus loading

Monthly values for P mass balance parameters are summarized in Table 4 and Figures 2 and 3. Without P_s values, AP_{ho} could not be calculated and conclusions could not be made on the efficiency of the solids P collection system used by the hatchery. Monthly values for $MAXP_{ho}$ varied from -0.24 kg in June 1993 to 103 kg in April 1993 (Table 4). Monthly values for $MAXP_{ho}$, $MINP_{ho}$, CP_{sp}, P retained by fish, and P fed values followed similar patterns except during March-May 1993 (Figures 2 and 3). CP_{sp} measurements approached or exceeded $MAXP_{ho}$ estimates from October through December 1993 (Figure 2).

The percent of feed P retained by fish (including mortalities) was 37.7% (Table 5). $MAXP_{ho}$, which represented feed P lost in feces and dissolved wastes, was 62.3% of P fed. Feces contained 21.0% of P fed based on dietary dry matter digestibility estimates and the P concentration in dry feces. Dissolved P entering the stabilization pond was not measured at the Platte Hatchery. Dissolved P concentrations in effluent water collected from the effluent study completed at MSU were below the detection limit. Consequently dissolved waste, or the percentage of feed P not accounted for in fish and feces, was

Table 4. Monthly P mass balance measurements and calculations from the Platte River Anadromous State Fish Hatchery.

	kg P				metric tons		% feed P	kg			fecal P as %
Date	fed	growth	dead fish	feces	feed fed	fish on hand	retained	CP_{sp}	$MAXP_{ho}$	$MINP_{ho}$	of $MAXP_{ho}$
1/93	24.86	8.822	0.008	5.328	2.772	66.09	35.5	6.328	16.03	10.71	33.2
2/93	35.56	9.374	0.665	6.862	3.764	68.64	28.3	10.669	25.52	18.66	26.9
3/93	76.64	22.71	0.154	13.182	7.394	74.27	29.8	15.468	53.78	40.60	24.5
4/93	119.0	16.16	0.083	19.097	10.933	45.68	13.7	14.561	102.75	83.65	18.6
5/93	66.75	21.66	0.180	23.117	7.779	7.985	32.7	22.920	44.91	21.79	51.5
6/93	12.04	12.26	0.014	9.787	1.793	4.064	101.9	5.579	-0.238	-10.02	
7/93	39.74	16.35	0.013	7.718	3.999	7.675	41.2	4.078	23.38	15.66	33.0
8/93	50.71	22.76	0.091	12.02	6.340	13.22	45.1	8.138	27.86	15.84	43.1
9/93	32.93	0.000	0.080	9.243	4.790	13.20	0.242	6.396	32.85	23.61	28.1
10/93	20.67	10.29	0.054	5.388	2.792	15.46	50.0	12.655	10.33	4.939	52.2
11/93	9.49	4.321	0.041	2.409	1.249	16.58	46.0	6.804	5.126	2.717	47.0
12/93	9.22	4.150	0.195	2.243	1.181	18.79	47.1	4.218	4.874	2.631	46.0
1/94	18.36	0.000	6.128	3.258	2.013	21.19	0.00	8.859	18.36	8.974	17.7
2/94	32.02	10.97	3.962	4.982	3.437	23.96	46.6	9.780	17.09	12.11	29.2
3/94	51.68	26.54	6.028	7.341	5.238	31.31	63.0	9.984	19.11	11.77	38.4
4/94	42.03	16.96	4.918	6.674	4.722	13.62	52.1	5.988	20.15	13.48	33.1
5/94	56.97	36.81	0.401	8.098	6.554	12.55	65.3	8.718	19.76	11.66	41.0
Sum	699	240	23	147				161	442	289	

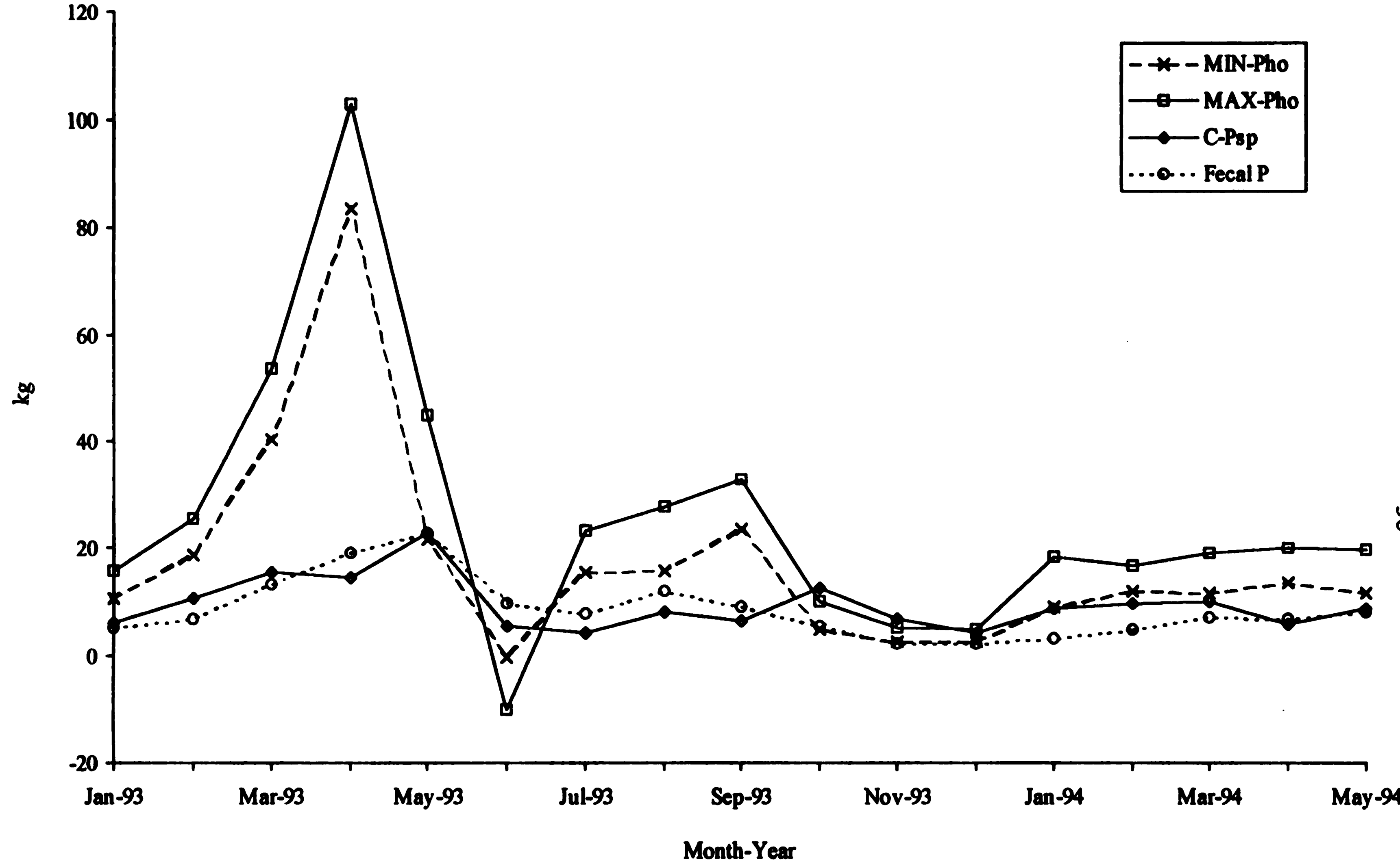

Figure 2. Monthly P loading and fecal P production estimates for the Platte River Anadromous State Fish Hatchery for the period January 1, 1993 through May 31, 1994.

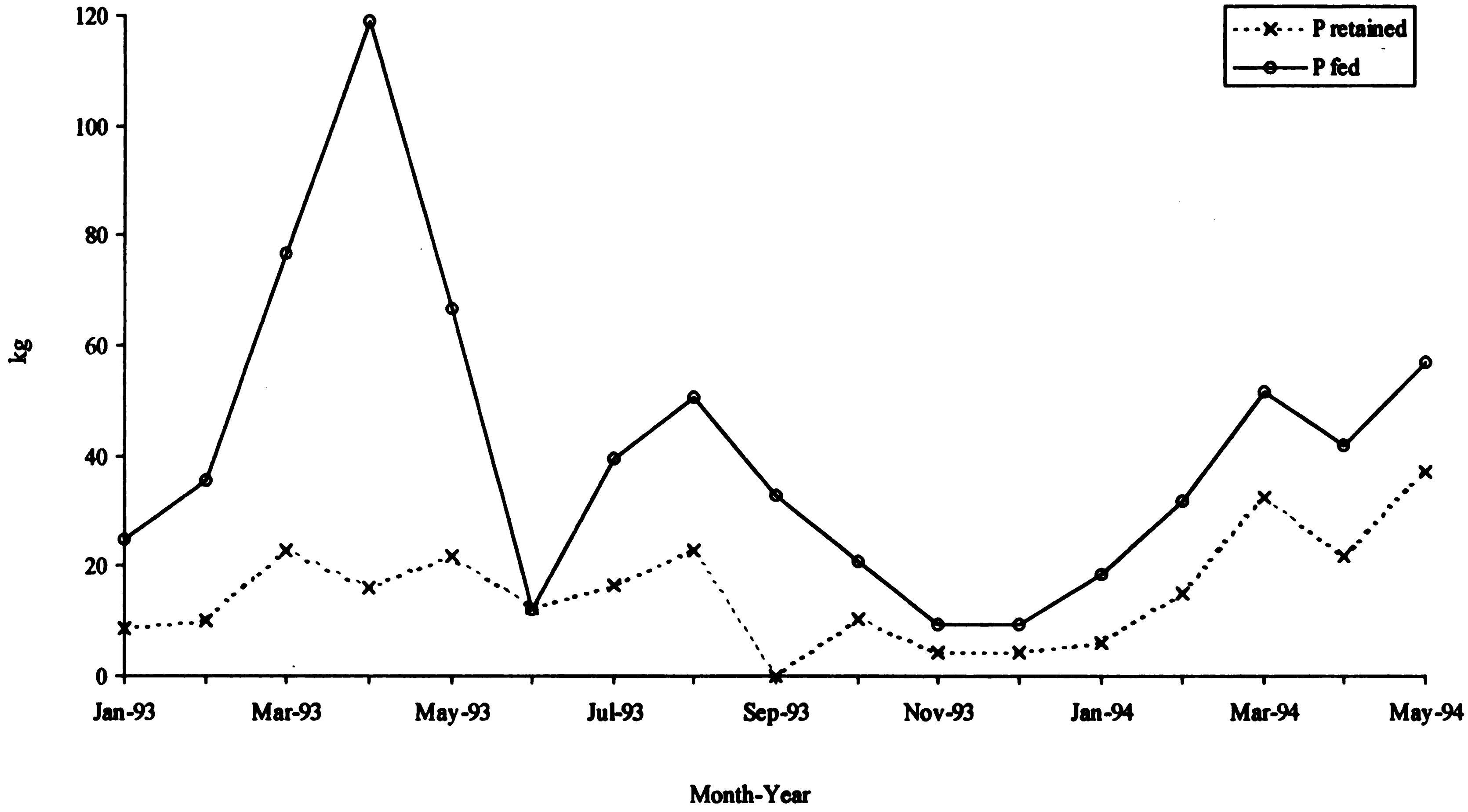

Figure 3. Relationship between P fed and P retained by coho and chinook salmon at the Platte River Anadromous State Fish Hatchery for the period January 1, 1993 through May 31, 1994.

calculated by difference (62.3% - 21.0% = 41.3%, Table 5). The ratio of P lost as dissolved waste and fecal waste was 2:1 (41.3%:21.0%).

Expressing total P losses on a production basis, a maximum of 2.8 kg P·metric ton^{-1} of fish produced was discharged into the stabilization pond. If all fecal P could have been recovered through solids management, the loading rate could have been as low as 1.8 kg P lost·metric ton^{-1} of fish produced.

Table 5. Phosphorus mass balance results for the experimental period of January 1993 through May 1994 at the Platte River Anadromous State Fish Hatchery.

Variable	kg	% feed P
P fed	698.67	100
P retained in live fish	240.15	34.4
P retained in dead fish	23.01	3.3
P in feces	146.75	21.0
P in dissolved waste	288.76†	41.3†‡
$MAXP_{ho}$	441.63	62.3
$MINP_{ho}$	288.76	
CP_{sp}	161.14	

† Calculated by difference: 100 - (34.4 + 3.3 + 21.0) = 41.3.
‡ Represents the best case scenario if 100% fecal P could be recovered by raceway solids removal.

DISCUSSION

Incomplete hatchery records prevented the development of nutritional mass balance models for the Platte Hatchery based solely on collected data. Hatchery feed and mortality records were meticulously kept, but fish inventory, weight distribution and collected solids information was partially or totally unavailable. For example, 344,418 Coho '92 were unaccounted for in the Platte Hatchery inventory records June 30, 1993. Loss of fish from the records resulted in negative values for $MAXP_{ho}$ and $MINP_{ho}$ in June 1993 (Figure 2). There has been little incentive for hatchery personnel to conduct fish size inventories because handling stress has increased the risk of disease, and the criterion for release of salmon from the Platte Hatchery has been smoltification rather than size. Consequently, certain assumptions had to be made to facilitate mass balance calculations. It was assumed that average fish weights estimated from water temperature history were accurate even though they were not calibrated by sampling. A normal distribution of fish weight was also assumed. Both of these assumptions could be easily addressed in the future by a small scale, relatively non-disruptive sampling program. Ewing et al. (1994) reported that 7-9 small (2.5 kg) random samples of trout from a raceway provided estimates of fish size with only 2-5% error.

Estimates of dietary dry matter digestibility were used to calculate total fecal P production. Implicit in this calculation was the assumption that 98-99% of feed fed was usually consumed by fish. This assumption may have been invalid March-May 1993, when values for P fed and P released from the Platte Hatchery spiked (Figures 2 and 3). During that period, unusually high amounts of a higher-P medicated feed (14.8 g P·kg^{-1} as fed) were fed to Coho '91 as a treatment for cold water disease. The production of feces was probably overestimated since more feed was used in the calculation of feces production than was actually consumed. Further, dissolved P losses were probably underestimated since they were calculated by subtracting P in feces and P retained in fish from P fed.

A 37.7% retention of dietary P by coho and chinook salmon at the Platte Hatchery compared favorably with retention of P by fish observed in other studies, especially in light of the P lost from overfeeding in March-May 1993. P retention values reported for salmonids have ranged from 13-50% (Håkanson 1986; Wiesmann et al. 1988; Cho et al. 1991; Foy and Rosell 1991a; Gomes et al. 1995). In an earlier experiment at the Platte Hatchery, Ketola et al. (1991) reported that 28.4% and 42.4% dietary P was retained by coho salmon fed a standard fish meal-based diet (Oregon Moist Pellets) and a vegetable-based diet (T2M), respectively. Both diets used by Ketola et al. (1991) contained 10 g P·kg^{-1} (dry matter basis). The wide range in % P retained by fish probably resulted from differences in feeding rates and the digestibility and concentration of dietary P (Foy and Rosell 1991a; Ketola et al. 1991; Ketola and Harland 1993).

The maximum P loading rate of 2.8 kg P·metric ton^{-1} fish produced during the experimental period was low compared to P loading rates of 4.8-12.2 kg·metric ton^{-1}

reported for coho salmon (Ketola et al. 1991) and 4.8 kg·metric ton^{-1} as the average for Nordic fish farms (Enell 1995). Wiesmann et al. (1988) fed 90 g rainbow trout an experimental feed similar in composition to the commercial "low P" feeds used at the Platte Hatchery[5]. They reported a loading rate of 3.9 kg·metric ton^{-1} fish produced. In contrast, Foy and Rosell (1991a) reported a loss of 25.6 kg P·metric ton^{-1} rainbow trout produced, and attributed the high loading rate to overfeeding.

The ratio of P lost in dissolved vs. fecal form (d:f ratio) was 2:1 for the present experiment. Data of Ketola et al. (1991) and Ketola and Harland (1993) have shown that the d:f ratio could be altered through dietary formulation. D:f ratios (calculated using P in raceway solids instead of feces) for coho salmon fed the T2M diet and Oregon Moist Pellets at the Platte Hatchery were 2:1 and 5:1, respectively. Ketola and Harland (1993) reduced the d:f ratio from 3:1 to 1:1 by replacing the highly soluble P supplement, $CaHPO_4 \cdot 2H_2O$, with less soluble defluorinated rock phosphate in rainbow trout diets.

The P mass balance analysis provided maximum and minimum possible values for P discharged into the stabilization pond. However, the immediate pollutive potential of discharged P depends on its chemical form. Orthophosphate, or soluble reactive P, is the P form most available to aquatic plants, phytoplankton and bacteria (Wetzel 1983). Foy and Rosell (1991b) found that approximately 60% of total P released from a rainbow trout farm was orthophosphate. Studies to determine the affect of dietary formulation on the

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[5] Diets used by Wiesmann et al. (1988) contained low-ash fish meal, hydrolyzed feather meal, and hydrolyzed wheat. Dry diet contained 8.9 g P·kg^{-1}.

d:f ratio should also consider how diet affects the proportion of orthophosphate in dissolved waste.

CP_{sp} (P leaving the stabilization pond as determined by the chemical method) approached or exceeded $MAXP_{ho}$ (P entering the stabilization pond) for October, November and December 1993. During that period, P may have been released from the stabilization pond sediments into pond water. Turnover of P in the sediments of the stabilization pond has been documented during late fall and early winter (Charles Pecor, MDNR, personal communication). Alternately, flaws in the Platte Hatchery's water sampling system may have resulted in overestimates of P discharged from the stabilization pond. P losses from the hatchery were at their lowest during those three months. The average daily concentrations of P in water discharged each month from the stabilization pond October through December 1993 were 16 μg $P{\cdot}L^{-1}$, 8 μg $P{\cdot}L^{-1}$ and 5 μg $P{\cdot}L^{-1}$, respectively. The November and December concentrations were close to the minimum detection limit of 3 μg $P{\cdot}L^{-1}$, suggesting that water P concentrations may have been too low to accurately measure during those months. The frequent malfunctioning of the water collection devices used by the Platte Hatchery also prevented the collection of representative 24 h samples.

Representative water samples could be gathered using a more robust device which continuously delivered water into an acid washed receptacle containing acid and a preservative. A preservative would prevent the growth of P-absorbing algae or bacteria in the water sample. Acid treatment of the receptacle would prevent the transfer of P between the sample and the inner surface of the receptacle (APHA et al. 1985). During periods when water P concentrations were near the lower detection limit (e.g., October and December 1993), relatively large volumes of water would need to be digested for P to be accurately measured.

Whole body P in Platte Hatchery coho and chinook salmon was comparable to whole body P in juvenile salmonids fed P-replete diets (Ketola 1975; Watanabe et al. 1980; Shearer 1984; Shearer et al. 1994; Rodehutscord 1996). However, regressions of whole fish P on fish weight differed for chinook salmon reared at the Platte Hatchery and at MSU. This may have resulted from the limited range of fish sizes reared at the Platte Hatchery (0.3-3.2 g) or from different water temperature regimes, feeds, feeding frequencies and feed efficiencies at the Platte Hatchery and MSU (Rodehutscord 1996; Åsgård and Shearer 1997). Consequently, regressions of whole fish P on fish weight developed for coho and chinook salmon at the Platte Hatchery should be used cautiously to estimate P retention in salmon reared elsewhere.

SUMMARY AND CONCLUSIONS

Based on mass balance estimates of maximum possible P losses over a 17 month period, the Platte River Anadromous State Fish Hatchery was one of the least-polluting salmonid hatcheries described. During the experimental period, the hatchery discharged 1.8-2.8 kg P·metric ton^{-1} fish produced into the stabilization pond. More precise estimates of P discharged into the stabilization pond could not be calculated without data on the P content of sludge removed from the raceways.

Although the P concentration was relatively low in most feeds used at the Platte Hatchery, the P content of whole coho and chinook salmon was comparable to whole body P content reported for other salmonids fed diets containing higher concentrations of P. However, regressions of whole fish P on fish weight for coho and chinook salmon from the Platte Hatchery should be used cautiously to estimate the P content of whole fish from other locations.

CHAPTER 2

Effect of dietary zinc supplementation and phytase pre-treatment of soybean meal or corn gluten meal on growth, zinc bioavailability and protein utilization in rainbow trout

INTRODUCTION

Plant feedstuffs like soybean meal have been important protein sources in fish feeds (NRC 1993). However, many plant feedstuffs contain phytate, a P storage molecule which is not digestible by fish (Cain and Garling 1995). Phytate has been shown to bind Zn and other cations in the gut, forming indigestible molecules (Cosgrove 1980; Spinelli et al. 1983). Reduced protein, P and Zn utilization has been observed in fish, pigs and poultry fed diets containing phytate (Gatlin and Phillips 1989; Lei et al. 1993a,b,c; Cain and Garling 1995; Qian et al. 1997). If dietary Zn is bound in an indigestible phytate complex, a Zn deficiency could result. Insulin secretion and the activity proteases and phosphatases have been shown to be Zn-dependent (Prasad and Oberleas 1971; Prasad 1993). Consequently, reduced protein and P utilization by fish fed diets containing phytate could be caused by a Zn deficiency.

In this experiment, juvenile rainbow trout were fed a combination of diets containing soybean meal and corn gluten meal either untreated or pre-treated with phytase with or without Zn supplements for 170 d.

Objectives

1. Determine if rainbow trout fed a plant-based diet high in phytate become Zn deficient based on growth, protein utilization, plasma insulin concentration, the activity of carboxypeptidase B and intestinal alkaline phopshatase, and whole fish and bone Zn, P or ash content.
2. Determine if dephytinized plant ingredients or a dietary Zn supplement is more effective in providing Zn to fish.

LITERATURE REVIEW

I. Effect of dietary phytate and zinc on protein, Zn and P digestibility

Reduced protein (Spinelli et al. 1983), P (Cain and Garling 1995; Riche and Brown 1996) and Zn (Richardson et al. 1985; Gatlin and Phillips 1989) digestibility has been observed in fish fed purified diets containing phytic acid, and in practical diets containing untreated soybean meal. Protein, P or Zn retention were improved for rainbow trout and Atlantic salmon when dietary soy products were pre-treated with phytase (Cain and Garling 1995; Storebakken et al. In press), or when phytase was added directly to the diet (Rodehutscord and Pfeffer 1995; Riche and Brown 1996).

Reduced protein digestibility was observed by Ogino and Yang (1978) in rainbow trout fed Zn-deficient diets. They reported a protein digestibility of 66% in fish fed a semipurified diet containing 1 mg $Zn \cdot kg^{-1}$ diet for 7 weeks, and hypothesized that Zn deficiency had caused a reduction in carboxypeptidase activity. However, fish fed diets with 5 mg $Zn \cdot kg^{-1}$ diet exhibited a normal protein digestibility of 97%. Protein efficiency increased significantly in juvenile chinook salmon when dietary Zn was raised from approximately 50 to 400 mg $Zn \cdot kg^{-1}$ in diets containing 25.8 g phytic acid$\cdot kg^{-1}$ dry diet (Richardson et al. 1985). Spry et al. (1988) found that severely Zn-deficient rainbow trout exhibited depressed plasma protein concentrations (2.1 $g \cdot dL^{-1}$) compared to Zn-replete fish (3.4 $g \cdot dL^{-1}$), even though Zn-deficient fish appeared to have normal feed intakes.

The possible additive interaction of phytic acid and low dietary Zn concentration on protein utilization has not been directly addressed for fish. Gatlin and Phillips (1989) reported a significant interaction between dietary Zn and phytate on bone Zn concentration in channel catfish, but not on weight gain or feed efficiency.

Oberleas and Prasad (1976) and Oberleas and Harland (1981) suggested that the bioavailability of dietary Zn could be predicted using dietary phytic acid:Zn and (phytic acid × Ca):Zn molar ratios. Reduced Zn absorption and marginal Zn deficiency was found in rats fed diets with phytic acid:Zn molar ratios of 10 or above (Davies and Olpin 1979; Lo et al. 1981) or with (phytic acid × Ca):Zn molar ratios above 3.5 (Fordyce et al. 1987). Dietary phytic acid:Zn and (phytic acid × Ca):Zn molar ratios have not been tested as predictors of Zn bioavailability in fish.

II. Metabolic role of zinc

Zn has been identified as a structural component or cofactor for over 70 enzymes, including some required for the digestion and synthesis of protein and nucleic acids and for P digestion (Wallwork and Duerre 1985; Prasad 1993). Zinc is also a structural component in nucleoproteins and insulin storage crystals (Scott 1934; Greider et al. 1969).

A. Zinc requirement

Ogino and Yang (1978) reported that 1.5-3.5 g rainbow trout fed a diet based on egg albumin, starch and dextrin required 15-30 mg $Zn \cdot kg^{-1}$ dry diet for normal growth rate (treatment effects were not evaluated statistically). Maage and Julshamn (1993) fed diets based on cod fillet meal and corn meal to 40 g Atlantic salmon for 12 weeks. Based on whole body Zn and serum Zn concentrations, they concluded that the dietary Zn requirement of juvenile Atlantic salmon was 37-67 mg $Zn \cdot kg^{-1}$ dry diet. Channel catfish, blue tilapia and common carp fed semipurified diets have been reported to require 20 mg $Zn \cdot kg^{-1}$ dry diet based on serum or bone saturation with Zn (Ogino and Yang 1979; Gatlin and Wilson 1983; McClain and Gatlin 1988). Clinical signs of Zn deficiency in fish include lens cataracts, short-body dwarfism, fin erosion, growth suppression and mortality (Ogino and Yang 1979; Satoh et al. 1983, NRC 1993).

The diet Maage and Julshamn (1993) fed to Atlantic salmon probably contained less than 1 g phytic acid$\cdot kg^{-1}$ dry diet, which was approximately 8 times lower than the phytic acid concentration in a commonly used salmonid diet (Guelph diet, NRC 1993)[6]. The semipurified diet fed to rainbow trout by Ogino and Yang (1978) was probably free of phytate. The dietary Zn requirements of juvenile salmonids fed most commercial feeds manufactured in North America may be higher than the values reported by Ogino and Yang (1978) and Maage and Julshamn (1993). The higher concentrations of phytic acid and Ca often found in North American feeds has been shown to reduce Zn availability (Gatlin and Wilson 1984; Hardy and Shearer 1985; Satoh et al. 1989).

Holcombe et al. (1979) found that fish absorbed Zn directly from water, probably via the gills (Joyner 1961; Spry et al. 1988). However, Spry et al. (1988) found that Zn uptake from the diet was independent of Zn uptake from the water, and that the concentrations of Zn commonly found in unpolluted water (approximately 10 $\mu g\ Zn \cdot L^{-1}$) was not likely to mitigate a dietary Zn deficiency. Gatlin and Wilson (1983, 1984)

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The Guelph salmonid diet (NRC 1993) contains approximately 8 g phytic acid$\cdot kg^{-1}$ dry diet. Estimates of phytic acid concentrations in the Guelph salmonid diet and the diet used by Maage and Julshamn (1993) were based on the data of Nelson et al. (1968) and Harland and Oberleas (1987).

reported that channel catfish reared in water containing 10-25 $\mu g\ Zn \cdot L^{-1}$ were not able to obtain adequate Zn from water for normal growth and mineralization.

Fish sequester Zn in skeletal tissue in amounts relative to the dietary Zn concentration up to a saturation point (Ogino and Yang 1978; Satoh et al. 1987; McClain and Gatlin 1988). Adult sea trout were reported to use Zn deposited in scales during spawning migration (O'Grady 1981), and it is conceivable that bone Zn may also be available during such a major physiological event. However, O'Grady and Abdullah (1985) concluded that Zn deposited in bone was not available to juvenile brown trout during periods of low food (Zn) intake. Hatchery-phase rainbow trout are juveniles and do not undergo the physiological changes associated with maturation.

B. <u>Zinc-dependent enzymes</u>

Carboxypeptidase B [EC 3.4.17.2] is a pancreatic exopeptidase which contains Zn as an essential structural element (Folk and Schirmer 1963; Yoshinaka et al. 1985). Carboxypeptidase B (CPB) has been shown to hydrolyze the peptide bond adjacent to the carboxy terminal of a peptide chain, preferentially cleaving the basic amino acids arginine, lysine and ornithine (Folk and Gladner 1958). Since trypsin is an endopeptidase with a specificity for arginine and lysine, CPB is important in the degradation of products of tryptic digestion (REF).

Carboxypeptidases have been identified, isolated and characterized for several fishes including chum salmon (Uchida 1970), catfish (Yoshinaka et al. 1984a, 1985), lungfish (Reeck and Neurath 1972), dogfish (Prahl and Neurath (1966), cod (Overnell 1973), and Atlantic halibut (Glass et al. 1987). Fish CPB was thought to be a metalloenzyme because it was strongly inhibited by the metal chelators EDTA and 1, 10-phenanthrolin (Kishimura and Hayashi 1992). Yoshinaka et al. (1985) hypothesized that Zn is the integral metal ion in fish carboxypeptidases since chelator-induced inhibition was reversed by the addition of $ZnCl_2$ to a carboxypeptidase A preparation. Mammalian carboxypeptidases contain one Zn atom per molecule (Folk 1971).

Ogino and Yang (1978) hypothesized that the decreased protein digestibility they observed in Zn-deficient rainbow trout was a result of reduced carboxypeptidase activity. However, the effect of Zn deficiency on carboxypeptidase activity has not been determined in fish. Significant reductions in the activity of pancreatic carboxypeptidase in Zn-deficient rats have been reported (Hsu et al. 1966; Prasad and Oberleas 1971).

Intestinal alkaline phosphatase (ALP) [EC 3.1.3.1] has been identified in fish as a non-specific phosphomonoesterase catalyzing transphosphorylation reactions and the hydrolysis of phosphomonoesters and pyrophosphates (Whitmore and Goldberg 1972a). Based on the observation that ALP spanned the apical membrane in rainbow trout, Gasser and Kirschner (1987a) suggested that it functioned as a transport mechanism between the intestinal lumen and cytoplasm for an undetermined substance.

Fosset et al. (1974) reported that mammalian and microbial ALP contained four Zn atoms per dimer. Each subunit contained one Zn atom for structural integrity and another for catalytic activity (Trotman and Greenwood 1971). Yora and Sakagishi (1986) found that fish intestinal ALP was inhibited by EDTA, and considered this as evidence that fish intestinal ALP was also a metalloenzyme. Methods of isolation and characteristics of fish intestinal ALP have been described for carp and eel (Yora and Sakagishi 1986), rainbow trout (Prakash 1960; Noda and Tachino 1965a,b; Noda 1967a,b,c; Colin et al. 1985; Yora and Sakagishi 1986; Gasser and Kirschner 1987a,b; Whitmore and Goldberg 1972a,b), Atlantic salmon (Johnston et al. 1994), pike, bream and roach (Kuz'mina and Smirnova 1991), sea bass (Bogé et al. 1993), tilapia (Gelman et al. 1992) and sterlet (Kuz'mina and Kuz'mina 1991).

ALP activity was reduced in the pancreas and intestine of Zn-deficient rats and mice (Prasad et al. 1967; Prasad and Oberleas 1971; Taneja and Arya 1992). However, Taneja and Arya (1992) have questioned whether the changes in enzyme activity were caused by insufficient Zn for enzyme activity or by general malnutrition associated with Zn deficiency. The effect of Zn deficiency on fish intestinal ALP activity has not previously been addressed. Serum ALP has been used as an indicator of Zn status or physiological state in channel catfish (Gatlin and Wilson 1983), rainbow trout (Hille 1984) and Atlantic salmon (Maage and Julshamn 1993; Johnston et al. 1994).

C. Insulin

Zn status may affect protein (amino acid) utilization in fish through its relationship with insulin. Ince and Thorpe (1977) reported that insulin secretion was stimulated in fish by increases in plasma amino acids. Insulin has been shown to facilitate the uptake of amino acids by fish tissues (Ince and Thorpe 1978) and stimulate muscle protein synthesis (Millward 1989). Zn has been shown to have a role in insulin storage (Scott 1934; Greider et al. 1969) and insulin sensitivity (Park et al. 1986). Droke et al. (1993) reported that serum insulin concentrations were reduced in growing lambs fed a severely Zn-deficient diet (1 mg $Zn \cdot kg^{-1}$ dry diet) but were normal in lambs fed a Zn-marginal diet (5 mg $Zn \cdot kg^{-1}$ dry diet).

MATERIALS AND METHODS

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from the Michigan Department of Natural Resources Wolf Lake Hatchery. The fish had been artificially spawned from mature rainbow trout returning to the Little Manistee River, Michigan. From first feeding in June 1995 to the start of the experiment in December 1995, fish were fed Biodry 1000[7] (Bioproducts, Inc., Warrenton, Oregon). One day prior to the beginning of the experiment, 9-11 fish were randomly distributed to tanks. Initial fish weight (± SD) was 11.8 ± 1.1 g. Total weight of fish in a tank was 115 ± 12 g.

Culture practices

Triplicate groups of rainbow trout were reared in 125 L rectangular plastic tanks for 170 d. Tanks were supplied with aerated well water at 12 $\pm$ 1 °C. Well water contained 10 μg $Zn \cdot L^{-1}$. Flow rates for the single pass, flow-through system were 2.5 $L \cdot m^{-1}$. Fish were maintained on the natural light:dark photoperiod with no supplemental lighting. Fish were periodically treated for fungal infection with static formalin baths (250 ppm formalin for 25 min followed by 50% water replacement).

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7 Biodry 1000 met NRC (1993) recommendations for protein (450 $g \cdot kg^{-1}$ diet as fed), fat (165 $g \cdot kg^{-1}$ diet as fed) and Zn (133 $mg \cdot kg^{-1}$ dry diet).

Phytase activity and application to feedstuffs

The activity of fungal phytase (Finase lot # 500033, Alko Ltd., Rajamaki, Finland) was determined colorimetrically (Han et al. 1987). Enzyme activity was approximately 500,000 PU·g^{-1}. One phytase unit (PU) was defined as the amount of enzyme which liberated 1 nmol of inorganic P from sodium phytate per minute at pH 5 and 37°C.

Defatted soybean meal (49% protein) and corn gluten meal (60% protein) were dephytinized using a procedure modified from Cain and Garling (1995). Three g phytase was dissolved in 1.5 L 50mM citrate buffer, pH 5.0. The buffered phytase was mixed with 1.5 kg soybean meal or corn gluten meal, loosely covered, and allowed to stand for 24 h at 25°C. Treated meal was dried in a forced-air oven at 27 ± 1°C for 28 h. The dried products were stored in plastic containers at room temperature until used. Soybean meal not treated with phytase was sham-treated in buffer.

Phytate analysis

Feed ingredients and a commercial salmonid feed used as the reference diet (Biodry 1000, Bioproducts, Inc., Warrenton, Oregon) were analyzed for phytic acid according to Latta and Eskin (1980). Approximately 0.1 g sample was placed in a scintillation vial with 5 mL of 2.4% HCl. Vials were gently agitated on a shaker table overnight. The samples and 20 mL deionized distilled (DDI) water, used to rinse the vial, were filtered through Whatman #1 filter paper and the liquid was collected in a flask. A column containing 200-400 mesh AG1-X4 anion exchange resin was prepared with 0.5 g resin in approximately 5 mL 0.05 NaCl, charged with 15 mL 0.7 M NaCl and rinsed with

15 mL DDI water. After the liquid sample was added to the column, it was washed with 15 mL DDI water, then with 15 mL 0.05 M NaCl. The column was then eluted with 15 mL 0.7 M NaCl, and the eluate was collected into a beaker for colorimetric analysis using sodium phytate as the standard. One mL Wade Reagent (0.03 g $FeCl_3 \cdot 6H_2O$ and 0.3 g sulfosalicylic acid diluted to 100 mL with DDI water) was added to 3 mL of standard or sample within 30 min of elution from the column. Absorbance was read at 500 nm using DDI water as a blank.

The phytic acid content of individual ingredients was used to calculate the phytic acid content of the experimental diets. Dietary phytic acid:Zn molar ratios were calculated based on molecular weights of 660.08 g phytic acid$\cdot$mole^{-1} and 65.38 g Zn$\cdot$mole^{-1}.

Feeds and feeding

The composition of the experimental diets is presented in Table 6. Diets contained 530 g protein$\cdot$kg^{-1} dry diet. Diets designated NT ('not treated') and T ('treated') contained sham-treated and dephytinized soybean meal, respectively. The numbers 0 and 50 in the diet labels indicated mg supplemental Zn$\cdot$kg^{-1} dry diet added as $ZnSO_4 \cdot 7H_2O$. Diets labeled TC contained dephytinized corn gluten meal as well as dephytinized soybean meal. The mineral and phytic acid composition of the reference and experimental diets are presented in Table 7. Diets containing sham-treated soybean meal were supplemented with P from defluorinated rock phosphate (18% P) to equal total P in soybean meal (0.74% P by analysis).

Table 6. Composition of experimental diets[1] fed to rainbow trout to determine the effects of feedstuff dephytinization and Zn supplementation on fish Zn status and Zn bioavailability. Ingredients listed on as-fed basis.

	$g \cdot kg^{-1}$				
Ingredient	NT0[4]	NT50	T0	T50	TC0
soybean meal	310	310	--	--	--
dephytinized soybean meal	--	--	310	310	310
corn gluten meal	300	300	300	300	--
dephytinized corn gluten meal	--	--	--	--	300
blood flour	100	100	100	100	100
herring meal	100	100	100	100	100
fish oil (by volume)	110	110	110	110	110
L-lysine HCl	4	4	4	4	4
vitamin mix[2]	10	10	10	10	10
choline chloride	0.14	0.14	0.14	0.14	0.14
ascorbic acid	0.28	0.28	0.28	0.28	0.28
mineral mix[3]	15	15	15	15	15
defluorinated rock phosphate	12.7	12.7	--	--	--
zinc sulfate	--	0.22	--	0.22	--
α cellulose	37.88	37.66	50.58	50.36	50.58

Table 6 (cont'd)

[1] The basal diet was based on the modified T2M diet described by Ketola et al. (1991).

[2] Vitamin mix #30 supplied by United States Fish and Wildlife Service contained ($mg \cdot g^{-1}$ premix): D calcium pantothenate, 26.5; pyridoxine, 7.7; riboflavin, 13.2; niacinamide, 55.1; folic acid, 2.2; thiamine, 8.8; biotin, 0.0882; vitamin B_{12}, 0.0055; menadione sodium bisulfite complex, 2.76; ($IU \cdot g^{-1}$): vitamin E, 88.2; vitamin D_3, 110.25; vitamin A, 1,653,750 USP.

[3] Zinc- and phosphorus-free mineral mixture ($g \cdot kg^{-1}$ premix): $MgSO_4 \cdot 7H_2O$, 660; $FeSO_4 \cdot 7H_2O$, 33.2; $MnSO_4 \cdot H_2O$, 20.5; $CuSO_4 \cdot 5H_2O$, 3.9; $NaMoO_4$, 0.55; KIO_3, 0.43; Na_2SeO_3, 0.044; α-cellulose, 281.376.

[4] Diets designated NT ('not treated') and T ('treated') contained sham-treated and dephytinized soybean meal, respectively. The numbers 0 and 50 in the diet labels indicated mg supplemental $Zn \cdot kg^{-1}$ dry diet (as $ZnSO_4$). Diets labeled TC contained dephytinized corn gluten meal as well as dephytinized soybean meal.

Table 7. Mineral and phytic acid composition of experimental diets[1]. All values are on a dry weight basis.

		Diet[2]					
Dietary component	Unit	R	NT0	NT50	T0	T50	TC0
Zn	$\mu g \cdot g^{-1}$ dry diet	133-157	51.1	115	51.3	109	54.1
P	$mg \cdot g^{-1}$ dry diet	10.5-11.1	9.87	9.10	7.15	6.71	7.19
Phytic acid	$g \cdot kg^{-1}$ dry diet	2.56	8.53	8.53	4.71	4.71	0.272
Phytic acid:Zn molar ratio		1.77	16.0	7.34	9.10	4.28	0.498

1 Mineral concentrations were determined for finished diets. Phytic acid concentrations of experimental diets were calculated based on the phytic acid concentration of feedstuffs. The phytic acid concentration of the reference feed R (Biodry 1000) was determined on the finished feed.

2 Diets designated NT ('not treated') and T ('treated') contained sham-treated and dephytinized soybean meal, respectively. The numbers 0 and 50 in the diet labels indicated mg supplemental $Zn \cdot kg^{-1}$ dry diet (as $ZnSO_4$). Diets labeled TC contained dephytinized corn gluten meal as well as dephytinized soybean meal. Diet R (Biodry 1000, Bioproducts, Inc., Warrenton, Oregon) was the reference feed.

Diets were made in 2-kg batches. Dry ingredients for individual batches were premixed in a liquid-solids blender for 2 hours (Patterson-Kelley Co., Inc., East Stroudsburg, Pennsylvania). Fish oil was blended into the premixed dry ingredients using a Univex mixer (Univex Corp., Salem, New Hampshire), then DDI water was added to create a moist dough. Dough was extruded without heat through a 3 mm die. The resulting spaghetti-like strands were dried at ambient temperatures in a forced-air oven. After drying, the dry matter content of diets was 89 - 94%. Dry matter of feed was determined at 2 wk intervals throughout the experimental period in order to calculate daily rations on a dry matter basis. Dried strands were chopped in a Waring blender (Waring, New Hartford, Connecticut) and screened to retain pellets 1-2 mm in diameter. Pellets were stored frozen in plastic containers until the day of use.

A commercial salmonid feed (Biodry 1000; Bioproducts, Inc., Warrenton, Oregon) from a single manufacturing lot was used as the reference feed. Biodry 1000 was formulated with fish meal from which bones, skin and scales had been removed, feather meal, cereal tailings (cooked oats, wheat, barley or rice), spray dried blood meal, herring meal, processed deboned fish, vitamin and mineral supplements and preservatives.

The daily ration (dry matter basis) was calculated as a percentage of total weight of fish per tank determined every 14 d (Piper et al. 1982). Daily rations were divided into two feedings fed at least 4 h apart. Minor adjustments to the daily ration were made between tank weighings to account for fish that died. Feed was fed by hand. To prevent gut contents from affecting fish weights, feed was withheld one day prior to weighing. Feeding resumed the day after weighing.

Tissue collection

On the final day of the experiment (day 170), morning feed was offered to one randomly selected tank every 30 min. Sampling began approximately 1 h after feeding. Elapsed time between feeding and blood sampling (60-80 min) was recorded to the nearest minute.

Fish were emersed in a tank containing 15-30 $mg \cdot L^{-1}$ of the anaesthesia MS-222 (tricaine methane sulfonate). After each fish was screened for clinical signs of Zn deficiency (opaque lens of the eye and eroded fins), a 1 mL blood sample was drawn from the caudal vein with a 1 mL tuberculin syringe which had been rinsed in a 200 $U \cdot mL^{-1}$ solution of Zn-free sodium heparin. Blood samples were placed into tubes containing 20 units of Zn-free sodium heparin. Whole blood was kept in an ice bath until all samples were collected. Whole blood was centrifuged for 10 min at room temperature and 13,600 $\times$ g, and the plasma pipetted into microtubes and stored at -20°C.

After blood sampling, fish weight was recorded, and the pyloric caeca and intestine were dissected and weighed. The pyloric caeca was the portion of the digestive tract found between the pyloric region of the stomach and the posterior-most cecal sac, and contained the duodenum. The 'intestine' consisted of the remainder of the intestine, including anterior and posterior intestine and the rectum. Because pancreatic cells are diffused throughout the mesentery surrounding the pyloric caeca in rainbow trout (Yasutake and Wales 1983), tissue homogenates for CPB analysis were prepared from the entire pyloric caeca and related connective and fatty tissue. Dissected tissues and

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Carcasses were later thawed, and a 2-cm section of the spinal column from between the base of the head and the anterior edge of the dorsal fin was removed for bone mineral analysis. The spinal sample was lightly scraped of tissue using a stainless steel scalpel, and only the centra were retained for bone mineral analysis. Bone samples were dried at 105°C for 24 h and stored in Whirl-Paks.

Mineral analysis

After the removal of bone samples, carcasses were sliced into 1-cm sections and lyophilized 72 h. Lyophilized fish were ground in liquid nitrogen using a stainless steel Waring blender, then frozen in Whirl-Paks. Ground fish, feedstuff, feed samples and a bovine liver standard (National Institute of Standards and Technology, United States Department of Commerce) were dried in a forced-air oven 24 h at 105°C in pre-weighted aluminum pans, then cooled in a desiccator. Dry matter content was determined by subtracting the initial weight of the pan from the weight of the pan containing the dried sample. Samples were wet ashed using nitric and perchloric acids (AOAC 1984) as described in Appendix A. Mineral residues were dissolved in DDI water.

Oven-dried bone samples were individually wrapped in Whatman #1 filter paper, dehydrated in 500 mL absolute ethyl alcohol for 8 h and de-fatted in ethyl ether for 8 h. The resulting samples of fat-free dry bone were weighed, then ashed at 600°C for 16 h in a muffle furnace. Bone ash was dissolved in concentrated (69-70%) nitric acid prior to dilution and analysis.

Fish, feedstuff and feed digests and bone ash were assayed for P using visible light spectroscopy (Gomori 1942) as described in Appendix B. Zn in sample digests and rearing water was determined by atomic absorption spectroscopy. Instrument parameters were verified by analysis of the bovine liver standard and confirmed with an atomic spectral standard (Baker Instra-Analyzed®, J. T. Baker Chemical Co., Phillipsburg, New Jersey). Carcass Zn values were corrected for Zn removed in bone samples. Zn concentrations were not determined for digestive tissues removed for enzyme analysis. The carcass constituted approximately 91% total body weight.

Total bone weight per fish was estimated using a total wet bone weight vs.fish weight regression for Atlantic salmon (Rottiers 1993). Total bone Zn was calculated for each fish as (μg Zn$\cdot$g^{-1} wet bone) × (g wet bone$\cdot$fish^{-1}).

The N content of whole fish and diets was determined using the micro Kjeldahl method (AOAC 1984). Crude protein was calculated as N×6.25.

Enzyme and insulin assays

Detailed ALP and CPB assay protocols are presented in Appendices D and E. Briefly, ALP and CPB activities were determined spectrophotometrically in the supernatants of tissue homogenates using the artificial substrates p-nitrophenyl phosphate and hippurl-L-arg, respectively (SIGMA Chemical Co., St. Louis, Missouri). Enzyme activity is reported based on supernatant protein content (specific activity), which was determined according to Bradford (1976) using a Sigma Total Protein Kit 610-A. Porcine or bovine enzymes (SIGMA Chemical Co., St Louis, Missouri) were used as standards in all assays.

The concentration of insulin in plasma was determined by radioimmunoassay with skipjack tuna (class Osteichthyes, family Scombridae, *Katsuwonas pelamis*) insulin as the standard and a rabbit anti-skipjack insulin antibody (Gutiérrez et al. 1984).

Zinc and protein efficiency

Percent Zn retained (PZR) was calculated as (mg Zn gained$\cdot$mg^{-1} Zn fed) × 100. Percent protein retained (PPR) was calculated as (g protein gained$\cdot$g^{-1} protein fed) × 100.

Statistical analysis

Weight gain of all fish in a tank, PZR, PPR and mortality data were subjected to an analysis of variance (ANOVA). ALP and total bone Zn were subjected to a nested ANOVA, where each fish per tank was considered a subsample. Analysis of covariance was used to detect treatment effects on plasma insulin concentration, whole fish Zn, P and protein content and bone Zn and P concentrations. The covariate for insulin was postprandial time (Navarro et al. 1993). The covariate for whole fish Zn, P and protein content and bone Zn and P concentration was fish weight (Shearer 1994).

Significant differences between means ($P < 0.05$) were identified in nonorthogonal contrasts using the Bonferroni t statistic (Gill 1978). Statistical tests were performed using the SAS software Mixed procedure, with tank(treatment) as a random variable (SAS Institute 1997).

The nonorthogonal contrasts were designed to answer the following questions:

1. Did dephytinized soybean meal improve fish performance (contrast NT0 v T0)?
2. Does a diet containing both dephytinized soybean meal and dephytinized corn gluten meal improve fish performance (contrast NT0 v TC0)?
3. Did dephytinized soybean meal with 50 mg $Zn \cdot kg^{-1}$ diet improve fish performance (contrast NT0 v T50)?
4. Is fish performance improved by using dephytinized corn gluten meal in a diet which already contains dephytinized soybean meal (contrast T0 v TC0)?
5. Do fish fed a diet containing dephytinized soybean meal plus 50 mg $Zn \cdot kg^{-1}$ diet perform as well as fish fed a diet containing both dephytinized soybean meal and dephytinized corn gluten meal (contrast T50 v TC0)?
6. Did the addition of 50 mg $Zn \cdot kg^{-1}$ to the basal diet improve fish performance (contrast NT0 v NT50)?
7. Do 50 mg $Zn \cdot kg^{-1}$ diet and dephytinized soybean meal equally affect fish performance (contrast NT50 v T0)?
8. Does adding 50 mg $Zn \cdot kg^{-1}$ diet to a diet containing dephytinized soybean meal improve fish performance (contrast T0 v T50)?
9. Did fish fed the basal diet perform as well as fish fed the reference feed (contrast NT0 v R)?

RESULTS

Final values for weight gain, whole fish protein, Zn and P content, bone Zn, P and ash concentration, PZR, PPR, specific activity of ALP and CPB and plasma insulin concentration are summarized for each diet in Table 8. Results of the nonorthogonal contrasts are summarized in Table 9. Dietary treatment significantly affected all variables except whole fish protein and PPR. Significant differences between treatments were not found for weight gain or plasma insulin concentration using the pre-planned contrasts (Table 9). Fish fed the experimental diets averaged a 4.5-fold weight increase during the experimental period. No clinical signs of Zn deficiency were observed.

Substituting dephytinized soybean meal for untreated soybean meal in the basal diet did not affect fish performance (contrast NT0 v T0; Table 9). Fish fed diets containing both dephytinized soybean meal and dephytinized corn gluten meal had higher bone Zn concentrations and total bone Zn than fish fed the basal diet (contrast NT0 v TC0). Bone Zn and ash concentrations and total bone Zn were also increased when fish were fed the diet containing dephytinized soybean meal and 50 mg $Zn \cdot kg^{-1}$ diet (contrast NT0 v T50).

Using dephytinized corn gluten meal in diets containing dephytinized soybean meal increased whole fish P, bone ash concentration and/or bone P concentration, but did not affect whole fish or bone Zn (contrast T0 v TC0 and T50 v TC0).

Table 8. Weight gain, protein and mineral composition of whole fish and fat-free dry bone, percent Zn retained (PZR)[1], percent protein retained (PPR)[2], specific activity of alkaline phosphatase (ALP) and carboxypeptidase B (CPB) and plasma insulin concentrations of rainbow trout[3] fed experimental diets[4] containing untreated or dephytinized ingredients and/or supplemental Zn. Values ± SE.

		Diet					
Variable	Unit	R	NT0	NT50	T0	T50	TC0
Weight gain	g	75.0 ± 18	54.7 ± 6.1	52.8 ± 10	47.2 ± 10	42.6 ± 10	64.1 ± 8.6
Whole fish							
Protein	$g \cdot g^{-1}$ fish	16.9 ± 0.28	16.7 ± 0.29	16.7 ± 0.24	16.6 ± 0.29	16.5 ± 0.34	16.8 ± 0.25
Zn	$mg \cdot g^{-1}$ fish	1.70 ± 0.55	0.918 ± 0.46	1.08 ± 0.28	0.986 ± 0.44	1.01 ± 0.40	1.35 ± 0.90
P	$mg \cdot g^{-1}$ fish	4.23 ± 0.23	4.22 ± 0.37	4.14 ± 0.27	3.96 ± 0.26	3.89 ± 0.30	4.22 ± 0.18
Bone							
Zn	$\mu g \cdot g^{-1}$ bone	188 ± 37	120 ± 32	194 ± 42	150 ± 38	165 ± 48	173 ± 38
P	$mg \cdot g^{-1}$ bone	59.8 ± 5.4	55.3 ± 5.0	56.9 ± 7.0	57.0 ± 5.8	50.6 ± 6.9	59.5 ± 3.7
Ash	% bone	33.3 ± 2.0	31.5 ± 2.4	31.2 ± 2.7	31.1 ± 2.4	27.3 ± 4.0	34.2 ± 1.7
Total bone Zn	μg	443 ± 157	231 ± 91	361 ± 105	252 ± 90	246 ± 108	363 ± 120
PZR	%	5 ± 2	10 ± 8	6 ± 1	13 ± 1	4 ± 4	21 ± 5
PPR	%	39 ± 14	21 ± 7	24 ± 5	16 ± 1	15 ± 5	33 ± 1

Table 8 (cont'd)

ALP	$U \cdot mg^{-1}$ protein	0.291 ± 0.08	0.256 ± 0.05	0.260 ± 0.07	0.272 ± 0.08	0.348 ± 0.09	0.285 ± 0.04
CPB	$U \cdot mg^{-1}$ protein	0.364 ± 0.11	0.388 ± 0.12	0.485 ± 0.30	0.366 ± 0.24	0.457 ± 0.17	0.341 ± 0.15
Insulin	$ng \cdot mL^{-1}$	16.5 ± 3.5	15.7 ± 4.4	15.4 ± 4.1	16.1 ± 3.9	15.3 ± 4.3	14.9 ± 3.5

1 Percent zinc retention = (mg Zn gained$\cdot$mg^{-1} Zn fed) × 100.

2 Protein efficiency ratio = (g protein gained$\cdot$g dry matter fed) × 100.

3 Initial fish values: weight, 11.8 g; whole fish zinc, 0.579 mg$\cdot$g^{-1} fish; bone zinc, 167 μg$\cdot$g^{-1} bone; bone compartment zinc, 80μg.

4 Diets designated NT ('not treated') and T ('treated') contained sham-treated and dephytinized soybean meal, respectively. The numbers 0 and 50 in the diet labels indicated mg supplemental Zn$\cdot$kg^{-1} dry diet (as $ZnSO_4$). Diets labelled TC contained dephytinized corn gluten meal as well as dephytinized soybean meal.

Table 9. Results of nonorthogonal contrasts of treatment means for whole fish protein, Zn and P, bone Zn, P and ash, percent Zn retained (PZR), total bone Zn, percent protein retained (PPR), the specific activity of alkaline phosphatase (ALP) and carboxypeptidase B (CPB) and the concentration of plasma insulin. Significant contrasts ($P < 0.05$) are marked 'X'.

	Contrast								
Variable	NT0 v NT50	NT0 v T0	NT0 v T50	NT0 v CGM	T0 v CGM	T0 v T50	T0 v NT50	T50 v CGM	NT0 v R
Weight gain	-	-	-	-	-	-	-	-	-
Whole fish									
Protein	-	-	-	-	-	-	-	-	-
Zn	-	-	-	-	-	-	-	-	X
P	-	-	-	-	X	-	-	X	-
Bone									
Zn	X	-	X	X	-	-	X	-	X
P	-	-	-	-	-	-	-	X	-
Ash	-	-	X	-	X	X	-	X	-
Total bone Zn	X	-	X	X	-	-	X	-	X
PZR	-	-	-	X	-	X	-	X	-
PPR	-	-	-	-	-	-	-	-	-
ALP	-	-	-	-	-	-	-	-	X
CPB	-	-	-	-	-	-	-	-	X
Insulin	-	-	-	-	-	-	-	-	-

Supplementing the basal diet with 50 mg $Zn \cdot kg^{-1}$ diet resulted in increased bone Zn concentration and total bone Zn (contrast NT0 v NT50). Adding 50 mg $Zn \cdot kg^{-1}$ basal diet was more effective than using dephytinized soybean meal in increasing the bone Zn concentration and total bone Zn (contrast NT50 v T0). Adding 50 mg $Zn \cdot kg^{-1}$ diet to a diet containing dephytinized soybean meal increased bone ash concentration, but not bone Zn concentrations (contrast T0 v T50).

Whole fish Zn, bone Zn concentration, total bone Zn and the specific activity of ALP and CPB were greater in fish fed the reference feed than in fish fed the basal diet (contrast NT0 v R).

PZR was highest in fish fed diets without supplemental Zn (Table 8 and Figure 4). The PZR of fish fed the basal diet was increased when dephytinized soybean meal and dephytinized corn gluten meal were used (contrast NT0 v TC0).

Bone Zn concentration was negatively correlated with dietary phytic acid:Zn molar ratios (Figure 5). Total bone Zn was significantly lower in fish fed diet NT0 than in fish fed NT50, T50, TC0, and R (Table 8). However, total bone Zn increased during the experiment in all fish regardless of dietary treatment. Total bone Zn in fish fed diet NT0 increased from 80.0 μg at the beginning of the experiment to 231 μg at the end, a 293% increase.

A 16% mortality rate was observed over the course of the experiment. The distribution of the mortalities was not related to dietary treatment. Examination of moribund fish by the MSU Animal Health Diagnostic Laboratory revealed that fish were infected with the fungus *Saprolegnia* spp.

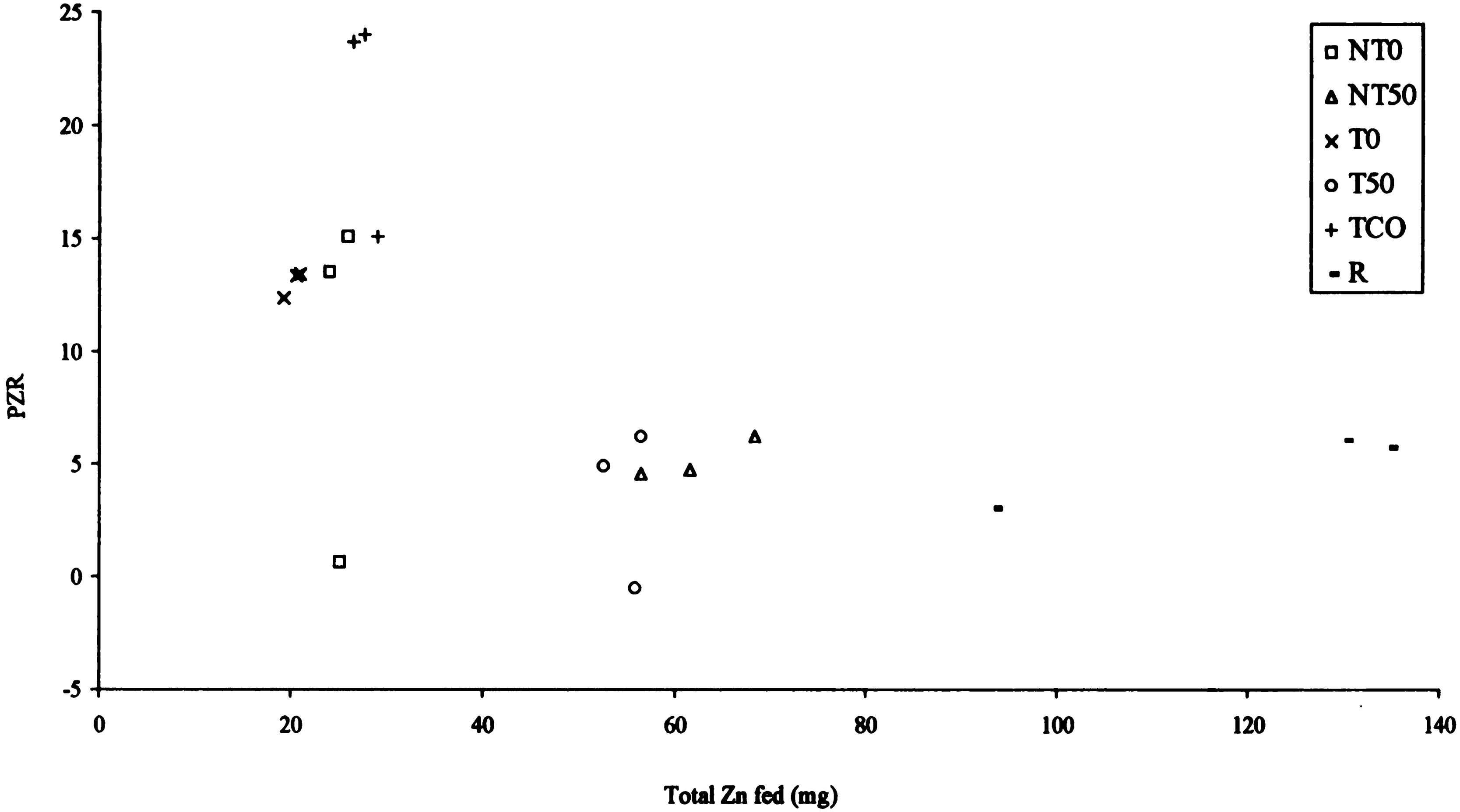

Figure 4. Relationship between percent dietary Zn retained (PZR) and total Zn fed to rainbow trout receiving experimental diets. Diets designated 'NT' and 'T' contained untreated and phytase-treated soybean meal, respectively. Diet TC0 contained phytase-treated corn gluten meal as well as phytase-treated soybean meal. The numbers 0 and 50 indicated mg supplemental Zn per kg dry diet. The reference diet 'R' was Biodry 1000.

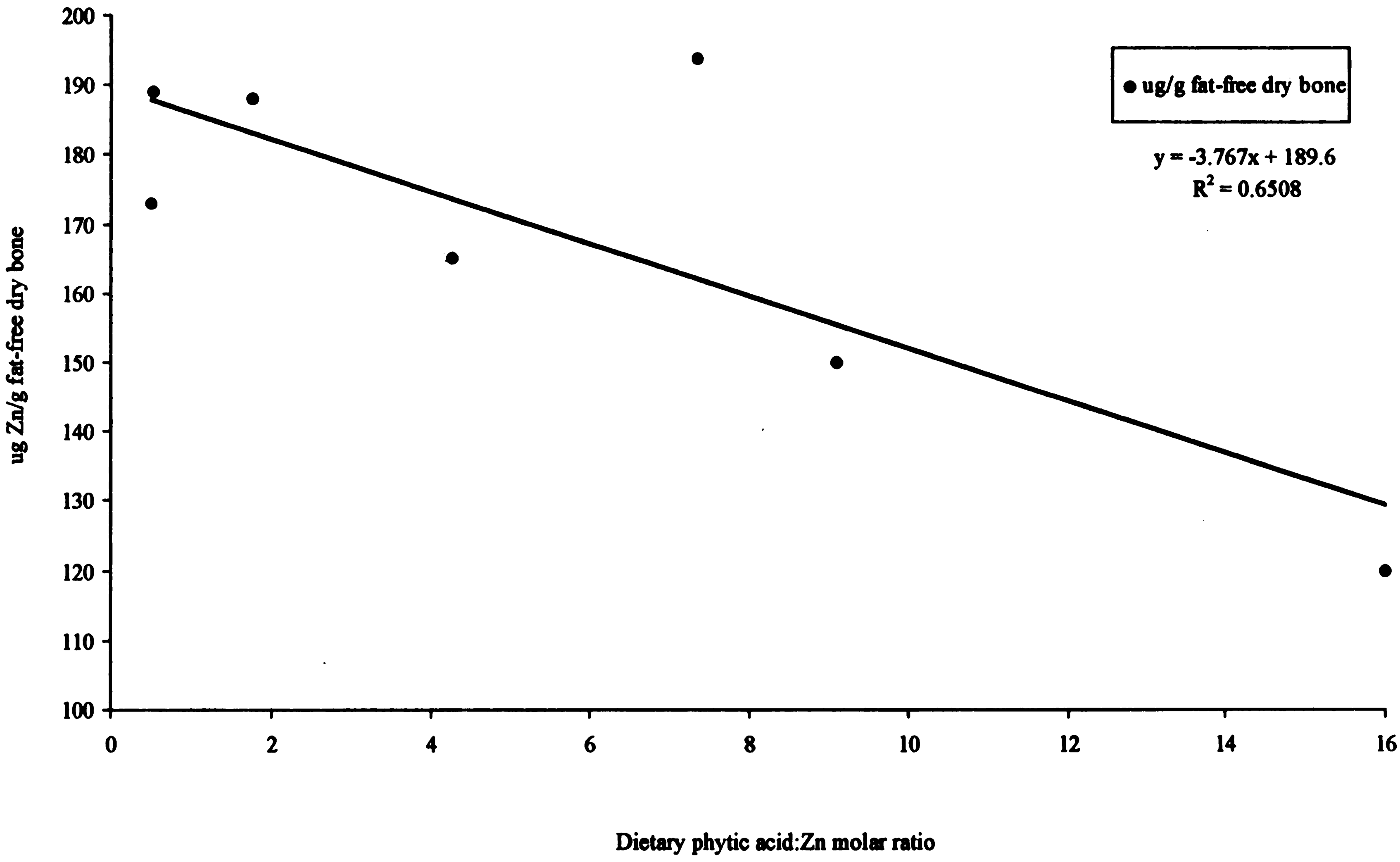

Figure 5. Relationship between the concentration of Zn in rainbow trout bone and the dietary phytic acid:Zn molar ratio.

DISCUSSION

Fish fed the basal diet were not Zn deficient after 170 d. The final concentration of Zn in bone was lower in fish fed diet NT0 than in fish fed the other diets, but normal PPR and specific activities of ALP and CPB indicated there was no functional deficiency. Clinical signs of absolute Zn deficiency such as cataracts or fin erosion (Ogino and Yang 1978) were not observed. Further, increases in total bone Zn of fish fed diet NT0 indicated that a Zn deficiency would not have developed even if fish were fed diet NT0 for an extended period. Therefore, enough Zn was available in the high-phytate, plant-based diet to meet the metabolic requirements of juvenile rainbow trout.

Weight gain and body composition of fish fed the experimental diets were similar to values reported for rainbow trout (Shearer 1984; Cain and Garling 1995). Although ALP and CPB activities were reduced in fish fed the basal diet (NT0) relative to fish fed the commercial reference diet (R), weight gain, whole body protein content and PPR were not significantly different. Possibly, phytic acid in diet NT0 directly interfered with enzyme activity in the lumen. Phytic acid has been shown to interfere with the activation of trypsinogen and reduce the stability of trypsin (Caldwell 1992).

Blood samples were drawn approximately 1 h after feeding, when concentrations of plasma insulin should have been at their highest (Navarro et al. 1993). The concentrations of plasma insulin observed in the present experiment were comparable to values observed for rainbow trout 1 h after being fed commercial feeds to satiation (Sundby et al. 1991; Navarro et al. 1993). Based on growth, ALP and CPB activity, total bone Zn, and normal concentrations of plasma insulin, fish in the present experiment were not Zn deficient. However, since decreases in insulin secretion and insulin sensitivity (i.e., clearance) have both been observed in Zn-deficient animals (Kirchgessner and Roth 1983; Park et al. 1986), it may have been possible to observe normal plasma insulin concentrations in Zn-deficient fish.

CPB activities have not been previously reported for rainbow trout. The specific activity of CPB found in the present study was similar to the specific activity of CPB in extracts of the pancreas and pyloric caeca of cod (Overnell 1973), carp (Cohen et al. 1981) and catfish (Yoshinaka et al. 1984b). The specific activity of ALP in intestinal homogenates was similar to the specific activity of ALP in scrapings of intestinal mucosa in rainbow trout (Gasser and Kirschner 1987a).

Adding 50 mg $Zn \cdot kg^{-1}$ diet was a more effective way of increasing bone Zn concentration and total bone Zn than using dephytinized soybean meal (contrast NT50 v T0, Table 9). Both dephytinized soybean meal and dephytinized corn gluten meal were required in the diet to increase bone Zn concentration and total bone Zn relative to values in fish fed the basal diet. The soybean meal used in this experiment contained 40 mg $Zn \cdot kg^{-1}$. Consequently, dephytinized soybean meal only contributed 12 mg $Zn \cdot kg^{-1}$ diet. Dephytinized soybean meal together with dephytinized corn gluten meal (29 mg $Zn \cdot kg^{-1}$) contributed a total of 21 mg $Zn \cdot kg^{-1}$ diet.

PZR decreased as total Zn fed increased (Figure 4). The observed trend supported an earlier finding by Spry et al. (1988). They reported that as dietary Zn concentration increased, the concentration of Zn in whole rainbow trout also increased, but at a diminishing rate.

Zn status in fish has been assessed using many criteria including growth, protein efficiency, feed efficiency, and the concentration of Zn in liver, kidney, plasma, scales, bone, or the whole fish. On rare occasions, serum ALP activity or protein digestibility have been used as indicators of Zn status (Ogino and Yang 1978; Gatlin and Wilson 1983; Maage and Julshamn 1993). The dietary Zn requirements of fish have been based on the dietary Zn concentration resulting in bone Zn saturation because bone Zn concentration was very sensitive to increasing dietary Zn concentrations (Gatlin and Wilson 1983; Maage and Julshamn 1993). For most fish fed purified diets, 15-30 mg Zn·kg^{-1} dry diet was the minimum concentration which resulted in bone Zn saturation (NRC 1993). Although normal growth, feed efficiency, protein digestibility, and serum ALP activity were observed in fish fed diets containing 5-10 mg Zn·kg^{-1} dry diet, decreased bone Zn concentrations in fish fed the lower-Zn diets was interpreted as a trend toward deficiency (Gatlin and Wilson 1983; Maage and Jushsamn 1993).

The dietary Zn requirement may be overestimated if it is equated with the dietary Zn concentration that saturates bone. Since bone Zn is probably not available for metabolic use in juvenile fish (O'Grady and Abdullah 1985), a high concentration of bone Zn indicates excessive Zn intake. The present study demonstrated that it was possible for diets to provide adequate Zn for metabolic requirements without maximum Zn deposition in bone. Similar results have been reported for rats fed low-Zn diets (Zhou et al. 1992).

To determine if decreased concentrations of Zn in fish bone really represents a deficiency or a trend toward deficiency, changes in total bone Zn should be measured (Zhou et al. 1992; Bobilya et al. 1994). For example, Maage and Julshamn (1993) fed Atlantic salmon a practical diet (17 mg native Zn·kg^{-1})[8] supplemented with 0-80 mg Zn·kg^{-1} dry diet. Based on dietary Zn concentrations that saturated bone and the whole body with Zn, they recommended supplements of 20-40 mg Zn·kg^{-1} for that diet. However, when their bone Zn concentration data was recalculated and expressed as total bone Zn, it was revealed that total bone Zn increased in all fish, including those fed the unsupplemented diet. Increases in total bone Zn implied that the diets provided Zn in excess of metabolic requirements. Assessing Zn status using total bone Zn may have lead to the conclusion that native dietary Zn was adequate.

The phytic acid:Zn molar ratio has not been used as an indicator of Zn availability in fish diets. However, data from the present study and recalculated data from Gatlin and Phillips (1989) and Satoh et al. (1989) suggested that the concept could be applicable to fish (Figure 6). The concentration of Zn in fish bone had an apparent negative relationship with the dietary phytic acid:Zn molar ratio. The dietary phytic acid:Zn molar ratio could be an important tool for formulating diets once its impact on fish performance is more clearly defined.

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8 Diets of Maage and Julshamn (1993) contained 150 g corn meal·kg^{-1} dry diet. Based on approximately 9 g phytic acid·kg^{-1} corn meal (Harland and Oberleas 1987), the diets contained 1.4 g phytic acid·kg^{-1} dry diet.

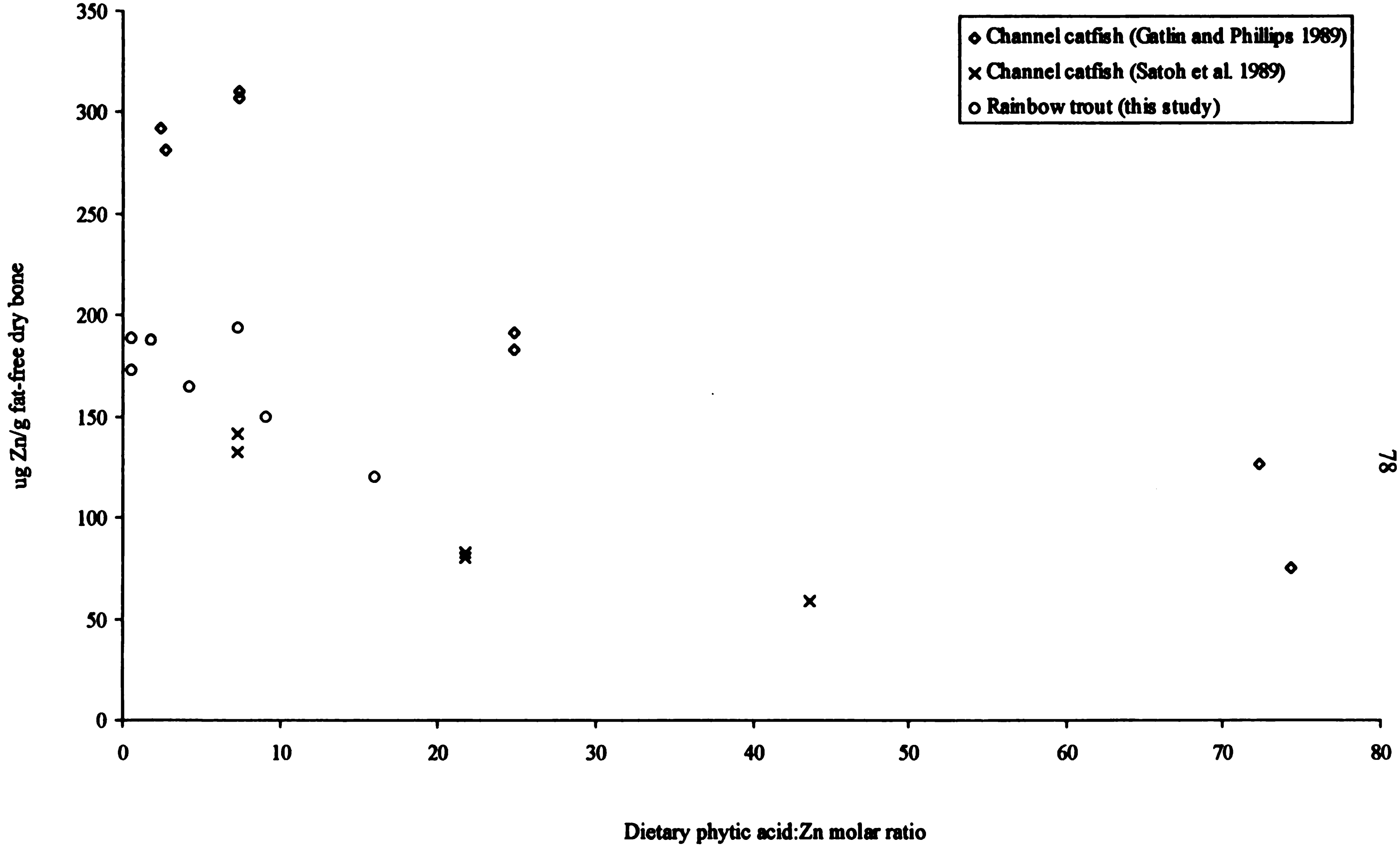

Figure 6. Relationship between bond Zn concnetration and dietary phytic acid:Zn molar ratio for channel catfish and rainbow trout.

SUMMARY AND CONCLUSIONS

Feeding plant-based diets containing untreated soybean meal and no supplemental Zn to rainbow trout for 170 d did not result in a Zn deficiency based on growth, whole fish Zn and protein composition, total bone Zn, and the activity of ALP and CPB. The opposite conclusion may have been drawn if Zn status had been assessed using the traditional indicator, the concentration of Zn in bone.

No benefit in terms of weight gain or fish protein content was realized from dephytinizing dietary ingredients or adding supplemental Zn to the plant-based diets. However, some Zn supplementation may be required for other feed formulations. The dietary Zn requirements of salmonids fed standard commercial formulations need to be reassessed based on changes in Zn-dependent metabolism or total bone Zn, possibly in relation to the dietary phytic acid:Zn molar ratio.

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APPENDICES

APPENDIX A

Sample Digestion Procedures

The following procedures were used to digest fish, feed, fecal and water samples in experiments described in Chapters 1 and 2.

I. Nitric-perchloric acid method - for fish, feed and fecal samples.

A. Each sample was placed in a 250 mL Phillips beaker. Feeds and feces were digested in 10 mL concentrated nitric acid and 3 mL perchloric acid. Fish samples were digested in 20-40 mL concentrated nitric acid and 5-10 mL perchloric acid. Blanks were digested in the same manner as the samples. Beakers were covered with watch glasses. Additional nitric acid was added as needed.

B. Samples were boiled on a hot plate until chemical reactions were complete and the perchloric acid created a thick white smoke. Watch glasses were then removed to allow excess acid to fume until about 1 mL remained.

C. Beakers were allowed to cool, and the inside beaker walls were rinsed down with deionized distilled water (DDI). Cooled samples were covered with plastic wrap if not immediately stored in test tubes.

D. Bovine liver and citrus leaves (Standard Reference Materials 1577a and 1572, respectively, National Institute of Standards and Technology, United States Department of Commerce) were digested as internal standards.

E. Reference:

AOAC (Association of Official Analytical Chemists). 1984. Official methods of analysis, 14th edition. Association of Official Analytical Chemists, Arlington, Virginia, USA.

II. Sulfuric acid-ammonium persulfate method - for water samples.

A. One mL 11N H_2SO_4 and 0.4 g ammonium persulfate were added to a 125 mL Erlenmeyer flask containing 50 mL unfiltered water or standard. Samples were boiled down to about 10 mL on a hot plate in a fume hood.

B. Samples were allowed to cool. Flasks were capped with aluminum foil if samples were not processed immediately.

C. Reference:

APHA (American Public Health Association), American Water Works Association, and Water Pollution Control Federation. 1985. Standard methods for the examination of water and wastewater, 16th edition. American Public Health Association, Washington, D.C.

III. Notes: Preliminary experimentation revealed that fecal samples and the bovine liver standard were not completely digested using the sulfuric acid-ammonium persulfate digestion method (APHA et al. 1985). Experimental fecal, feed and fish samples were therefore digested using nitric and perchloric acids (AOAC 1984). Sample sizes for dry fish and feed were approximately 1 g. Because fecal samples were very small (0.2-28 mg dry matter), the entire sample was digested.

Glassware used in water P analysis was soaked in a 6 N HCl bath for at least 15 min. Glassware used in the analysis of P in feed, feces and fish was soaked in a 5 HNO_3 acid bath for 12 h. Acid-washed glassware was rinsed in DDI water, dried in an inverted position, and capped with plastic wrap or aluminum foil.

APPENDIX B

Phosphorus determination procedures

These procedures were used to determine the P content of digested fish, feed, fecal and water samples from experiments described in Chapters 1 and 2.

I. Gomori (1942) method - for whole fish, feed and bone samples.

A. Reagents

MS solution: Dissolve 5.0 g $Na_2MoO_4 \cdot H_2O$ into 200 mL DDI water. Add 14 mL concentrated H_2SO_4. Bring volume to 1 L.

Elon solution: Dissolve 3 g $NaHSO_3$ into 50 mL DDI water. Dissolve 1 g Elon[9] into the $NaHSO_3$ solution. Bring volume up to 100 mL.

B. Standards

Phosphate standard: A 60 mg $P \cdot L^{-1}$ certified commercial phosphate standard (Banco™ phosphate standard, Anderson Laboratories, Inc., Ft. Worth, TX) was diluted to the following concentrations for whole fish, feed and bone samples (mg $P \cdot L^{-1}$): 0, 10, 20, 30, 40, 50, 60.

C. Sample treatment

Five mL MS and 0.5 mL Elon solution were added to 1 mL sample or standard. Color was allowed to develop for 45 minutes. Absorbance was read using visible light at 700 nm on a Beckman spectrophotometer using a 1 cm light path. Note:

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[9] Elon™ (Kodak Co.) is a commercial name for methyl-p-aminophenol sulfate.

sample and reagent volumes could be divided in half without altering results. Phosphorus was expressed as elemental P.

D. Final dilution

Final concentrations were approximately: 97.3% water, 1.4% $Na_2MoO_4 \cdot H_2O$, 1% H_2SO_4, 0.2% $NaHSO_3$, and 0.1% Elon.

E. Reference: Gomori (1942).

II. APHA et al. (1985) method - for fecal and water samples.

A. Reagents

5N sulfuric acid: 140 mL concentrated H_2SO_4 to was added to approximately 500 mL DDI water. After cooling, the solution was brought to a final volume of 1 L.

Potassium antimonyl tartrate solution: 2.743 g $K(SbO)C_4H_4O_6 \cdot 1/2H_2O$ was dissolved in DDI water, brought to a final volume of 1 L and stored in dark glass bottle at 4°C.

Ammonium molybdate solution: 40 g $(NH_4)6Mo_7O_{27} \cdot 4H_20$ was dissolved in DDI water, brought to a final volume of 1 L and stored in polypropylene bottle at 4°C.

Ascorbic acid solution: 8.80 g $C_6H_8O_6$ was dissolved in DDI water, brought to a final volume of 500 mL, and stored at 4°C. Solution was stable for about 1 week.

Combined reagent: Reagents were mixed in the following order for a total volume of 500 mL: 250 mL 5N sulfuric acid, 25 mL potassium antimonyl tartrate solution, 75 mL ammonium molybdate solution, 150 mL ascorbic acid solution. Concentrated reagent was stored in a dark glass bottle for up to 4 h.

B. Standards

Phosphorus stock solution (50 mg·L^{-1}): 0.2197 g potassium phosphate monobasic (KH_2PO_4 dried at 105°C) was dissolved in DDI water. One mL concentrated H_2SO_4 and 1 mL chloroform ($CHCl_3$) were added as preservatives. The solution was brought to a final volume of 1 L and stored in a glass bottle at 4°C.

Phosphorus standard solution (1 mg·L^{-1}): 20 mL of phosphorus stock solution was diluted to 1 L with DDI water. The solution was prepared fresh daily.

Working standards: The 1 ppm standard solution was diluted to concentrations of 0.01, 0.03, 0.05, 0.10, 0.30, 0.50 mg·L^{-1}. Working standards were made fresh daily.

C. Sample treatment

One drop of phenolphthalein indicator solution was added to each sample and standard digest. While swirling the digest, 6N NaOH was added dropwise until the solution just turned pink. The solution was brought to 50 mL final volume with DDI water using a volumetric flask, then transferred back to the 125 mL digestion flask. Eight mL of combined reagent was added. Color was allowed to develop for at least 10 min but no longer than 30 min. Absorbance was measured at 880 nm using a spectrophotometer. A reagent blank was the reference solution. P was expressed as elemental P.

D. References:

APHA (American Public Health Association), American Water Works Association, and Water Pollution Control Federation. 1985. Standard methods for the examination of water and wastewater, 16th edition. American Public Health Association, Washington, D.C.

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III. Notes: Total phosphorus was determined in whole fish and feed digests according to Gomori (1942) and in fecal and water digests according to APHA et al. (1985). The P determination method was selected based on laboratory availability at MSU. Based on preliminary tests using digested fecal matter, bovine liver and phosphate standards, the two P determination procedures provided identical results.

APPENDIX C

Predicting the Nitrogen and Phosphorus content of whole fish from fish weight

INTRODUCTION

The maximum possible amount feed N and P discharged into fish hatchery effluents can be calculated if the quantities of N and P fed to and retained by fish are known (Chapter 1). To calculate the amount of N and P retained by fish, the N and P composition of fish at the beginning and end of the growth period must be known. Collecting body composition data for each fish species cultured and for every production period represents an enormous task. For more commonly cultured species, a growing amount of published body composition data is available. If whole fish N- or P vs. fish weight regression equations were established for commonly cultured species, considerable resources could be saved by predicting these constituents from body weight.

The objective of this study was to describe the mathematical relationships between whole fish N and P and fish weight for several species of fish important to the aquaculture industry.

METHODS

Data sets containing whole fish N, whole fish P and corresponding fish wet weight values were located using the *Aquatic Biology, Aquaculture, and Fisheries Resources* NISC database (National Information Services Corp., Baltimore, Maryland) using combinations of the key words proximate composition, body composition, nitrogen, phosphorus and protein. Database entries covered 1974 through 1996. Additional data sets were found through citations within papers, personal communication with researchers and by manually searching the journals *Aquaculture* and *Progressive Fish-Culturist* from approximately 1987-1996. The literature search was not exhaustive. Any omission of pertinent papers was accidental and in no way constituted a judgement of their merits. Tabulated data sets with at least a 10-fold weight difference between the smallest and largest fish were preferred. However, in the interest of gathering data on as many species as possible, smaller data sets or data from figures was occasionally used. Nitrogen was calculated from crude protein values as: (crude protein ÷ 6.25). Data sets, including those from this dissertation, were collected for bluegill sunfish, green sunfish, longear sunfish, yellow perch, muskellunge, tiger muskellunge, largemouth bass, seabass, red drum, sunshine bass, rainbow trout, brook trout, chinook salmon, coho salmon and sockeye salmon.

Linear regression relationships were calculated using the SAS software GLM procedure (SAS Institute 1997). Data sets for a species or genus were combined if evidence against homogeneous regression was not strong ($P \geq 0.05$). Regression relationships were considered significant if $P \leq 0.05$.

RESULTS

Whole fish N data were collected for 14 fish species (Table 10). Whole fish N content was significantly correlated with fish wet weight in all cases ($r^2 \geq .89$). Values from individual data sets could be combined into a single data set for each species except yellow perch. Data could also be combined to produce equations for the genera *Lepomis* spp. (sunfishes) and *Oncorhynchus* spp. (salmonids). Although the assumption of homogeneous regression was not met for yellow perch data sets or all species combined, regressions were computed for those combinations for illustrative purposes. Table 11 contains details on individual data sets.

Whole fish P and fish wet weight data were found for 4 species of salmonids (Table 12). All data sets regressed homogeneously. Data were combined to create regression equations for trout, salmon and trout+salmon (Table 12). The degree of data scatter (Y response to an X) increased with increasing fish size (Figure 7).

Table 10. Regression relationship between log whole fish N (g) and log wet fish weight (g) of selected fish species. Values ± SE.

Species or group	slope	intercept	r^2	n	fish wet weight range (g)
trout and salmon	1.04 ± 0.00	-1.65 ± 0.01	1.00	128	1.39-289
sunfishes	1.02 ± 0.03	-1.64 ± 0.04	0.98	36	14.8-106
yellow perch [1]	1.07 ± 0.02	-1.68 ± 0.02	0.99	66	3.50-48.1
sunshine bass	1.02 ± 0.01	-1.60 ± 0.01	1.00	39	16.9-565
seabass	1.18 ± 0.10	-1.89 ± 0.15	0.95	9	22.8-40.8
largemouth bass	1.17 ± 0.15	-1.80 ± 0.22	0.89	10	24.3-31.1
red drum	1.01 ± 0.02	-1.56 ± 0.02	0.99	40	10.3-138
muskellunges	1.03 ± 0.05	-1.55 ± 0.08	0.98	10	21.0-99.0
combined species [1]	1.05 ± 0.00	-1.66 ± 0.01	0.99	343	1.39-565

[1] Individual data sets were combined despite non-homogeneous regression.

Table 11. Sources of whole fish N and fish wet weight data, sample sizes (n) and fish wet weight ranges used in the study of whole fish N content.

Species	reference	n	weight range (g)
brook trout	Phillips et al. (1960)[1]	7	2.31-22.0
rainbow trout	Reinitz (1983a)	6	18.0-149.4
rainbow trout	Reinitz (1983b)	66	2.80-149.4
rainbow trout	Kaushik et al. (1995)	9	83.1-224.2
rainbow trout	Heinen et al. (1993)	4	137.2-185.9
sockeye salmon	Groves (1970)	12	2.86-289.0
sockeye salmon	Brett et al. (1969)	24	1.39-29.28
bluegill sunfish	Gerking (1955)	27	18.5-51.4
green sunfish	Gerking (1952)	4	14.8-62.7
longear sunfish	Gerking (1952)	5	15.3-106
yellow perch	Tanasichuk and MacKay (1989)	10	3.50-25.3
yellow perch	Ramseyer (unpublished data)	33	24.6-48.1
yellow perch	Rodgers and Qadri (1982)	10	6.22-18.3
yellow perch	Garber (1981)	12	5.50-30.1

Table 11 (cont'd).

Species	reference	n	weight range (g)
sunshine bass	Brown et al. (1993)	31	16.9-124
sunshine bass	Webster et al. (1995)	8	417-565
seabass	Catacutan and Coloso (1995)	9	22.8-40.8
largemouth bass	Brecka et al. (1996)	10	24.3-31.1
red drum	Bai et al. (1994)	40	10.3-138.1
muskellunge	Brecka et al. (1995)	5	21.0-47.0
tiger muskellunge	Brecka et al. (1995)	5	28.0-99.0

[1] Values for fasting fish omitted.

Table 12. Regression relationships between whole fish P (mg) and wet fish weight (g) for salmon and trout. Values ± SE.

species	slope	intercept	r^2	n	x range
coho and chinook salmon	4.20 ± 0.03	-1.01 ± 0.50	0.99	224	0.278-46.79 g
brook and rainbow trout	4.01 ± 0.05	4.21 ± 3.61	0.97	208	0.08-166 g
salmon and trout combined	4.06 ± 0.02	0.66 ± 1.10	0.99	432	0.08-166 g

Species	reference	n	x range (g)
rainbow trout	Cain and Garling (1995)	15	1.90-63.7
rainbow trout	Chapter 2, this dissertation	184	15.57-166
brook trout	McCay et al. (1936)	8	0.08-5.50
coho salmon	Chapter 1, this dissertation	142	0.27-46.79
chinook salmon	Chapter 1, this dissertation	82	0.28-4.07

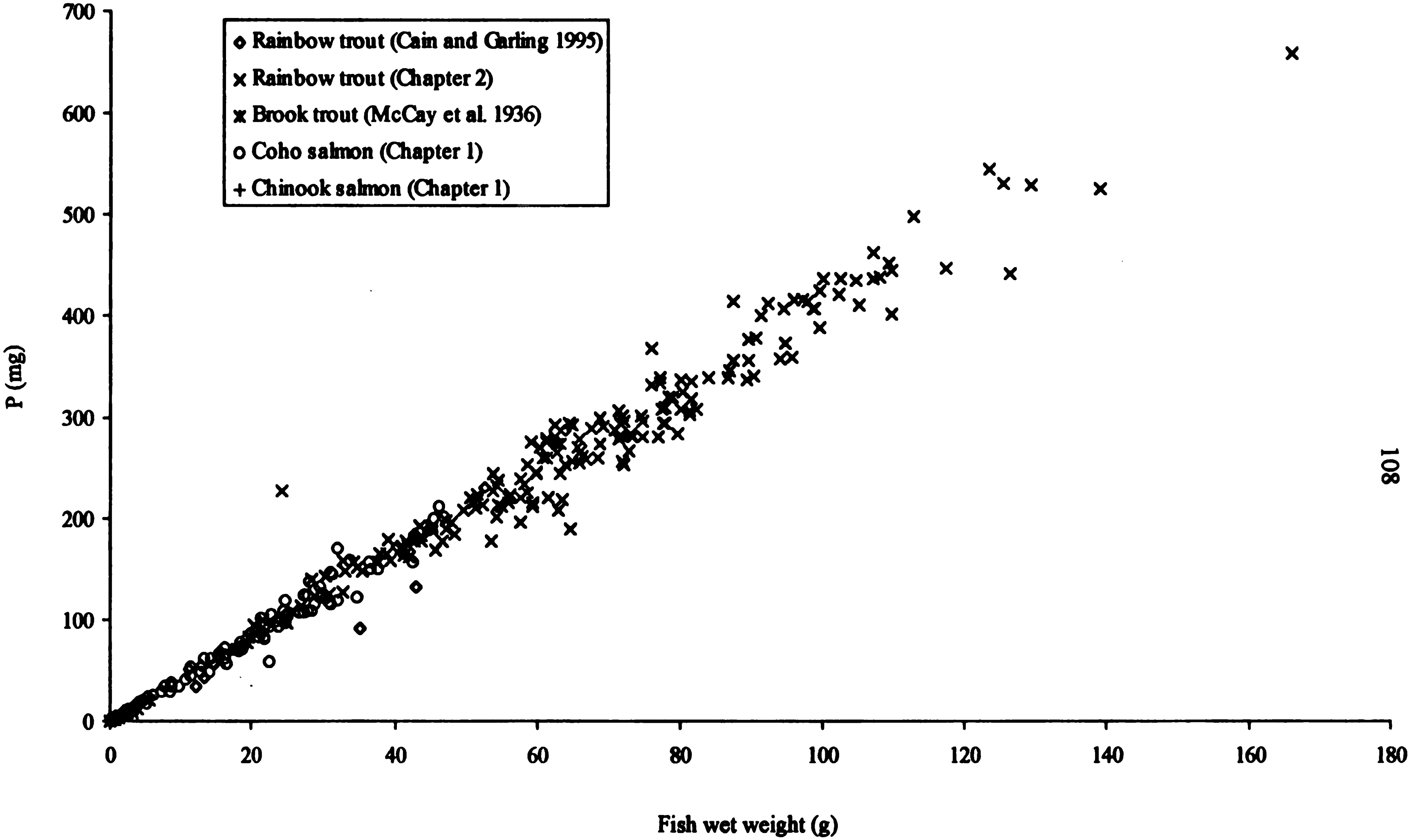

Figure 7. Relationship between whole fish P and fish weight for trout and salmon.

DISCUSSION

The high degree of correlation found between whole fish N and fish weight indicated that whole fish N analyses are probably not necessary for most species already characterized. Considerable resources could be saved by estimating whole fish N with regression equations calculated from existing data.

Although not identical, parameters from the whole fish N vs. fish weight regressions were similar for the different species. Parsons (1888) discovered over a century ago that various species of fish and aquatic mammals are strikingly similar in three-dimensional morphology. Hecht (1916) further reported a "general similarity of form" for seven marine fishes based on the regression relationships of several body dimension combinations. He attributed the regularity of form to the demands of life in water, which is dense relative to air. Since approximately 70% of wet fish mass was muscle (Krumholz 1956), and fish share similarities in form, it was not surprising that the fish species summarized here, which were all from the taxonomic subdivision Teleostei, shared similarities in N content.

In 1956, Krumholz found that eight species of freshwater fish contained approximately the same percentage of muscle. When Krumholz' data is expressed as g fish muscle vs. g fish weight, the $r^2 = 1.00$. Groves (1970) reported that the relationship between fish length and protein content was predictable and nearly identical for several salmonid species, provided that the fish were not fasting or starving. After a non-statistical comparison of protein-fish weight relationships for several fish species, Shearer (1994) hypothesized that the protein-body weight relationship was species specific. To our knowledge, the present study was the first statistical analysis of whole fish N vs. fish weight data for several species.

Certain limitations of the whole fish N vs.fish weight regressions should be addressed. First, equations based on narrow fish weight ranges should be used with caution. Error is greater in parameters calculated from somewhat clustered data. This was demonstrated in the parameter estimates for seabass and largemouth bass, which had the narrowest fish weight ranges, the lowest r^2 values, and the highest standard errors of parameter estimates. Secondly, differences between slopes or intercepts for most species were small yet significant. The importance of even minor differences can be seen through the following example. If the combined equation was used to predict whole fish N for salmonids, with salmonid data making up 37% of the combined data, whole fish N estimates would be off by about 1%. If this small difference was summed across a standing stock of several tons, the cumulative error could be quite significant. Despite these limitations, even rough estimates calculated from the combined equation or an equation for a related species could be of value in production situations where no other means of estimating N retention or loss are available.

Most literature values for whole fish N content were determined on pooled samples of 2 or more fish of approximately the same size. Pooling could artificially reduce data scatter, thereby falsely inflating the coefficient of determination (r^2). However, regressions of whole fish N values determined for individual fish by Groves (1970), Bai et al. (1994) and Brown et al. (1993) yielded r^2 values of 0.99-1.00. Thus it seems fair to conclude that the coefficients reported were not inflated by sample pooling.

The variability in whole fish P between individuals of a given weight increased with fish size. Phosphorus concentrations in growing salmonids have been shown to vary with dietary P concentration (Ketola and Richmond 1994; Rodehutscord 1996), dietary P form (Cain and Garling 1995), feed efficiency (Åsgård and Shearer 1997), and feed intake. Any differences in individual fish P caused by these factors could be compounded by time.

Under normal circumstances, bone P but not flesh P varies with P intake. Bone and whole fish P plateau in most growing salmonids when at least 5-6 g available P·kg^{-1} dry diet is fed (Ketola 1975; Watanabe et al. 1980; Ketola and Richmond 1994; Rodehutscord 1996; Åsgård and Shearer 1997). Depending on dietary P intake, a fish may contain a range of whole fish P concentrations up to and including an amount resulting from bone P saturation. Therefore, the increased scatter seen in whole fish P with increasing fish weight may have been due to the increasing contribution of bone P to whole fish P with increasing fish size. Skeletal P accounted for 10% whole fish P in 5 g chum salmon fed a 1.15% P diet for 7 weeks (Watanabe et al. 1980). However, bone may account for 35-44% of whole fish P in 0.4-2.8 kg rainbow trout[10]. Therefore, any variability in dietary P intake should have less of a measurable effect on small fish whole body P than on large fish whole body P.

Because whole fish P was responsive to dietary P independent of fish weight, regressions should be used cautiously for fish fed diets with very low concentrations (< 5

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10 Contribution of bone P to whole fish P was estimated using dry bone % P data for 0.4-2.8 kg rainbow trout from Persson (1988, cited in Lall 1991), whole Atlantic salmon bone compartment data from Rottiers (1993) and whole rainbow trout P content data from Shearer (1984). Since ribs and other bones are less mineralized than vertebral centra in some fish (Fraser and Harvey 1982), the figures presented should be considered maximum estimates.

$g \cdot kg^{-1}$) of available P. Finally, whole fish N or P should only be predicted for fish within the weight range specified for each species in Tables 10 through 12.

CONCLUSIONS

Whole fish N and P were highly predictable from fish wet weight for all species. Regression parameters were similar for all fish species examined. With the exception of yellow perch, individual data sets for each species could be combined. The regression equations presented for predicting whole fish N and P from fish weight may be used to estimate fish N and P retention when direct measurements are not possible.

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APPENDIX D

Alkaline Phosphatase Assay Protocol

I. Tissue preparation

A. The intestine was slit longitudenally to expose the mucosa, rinsed in approximately 10 mL ice-cold 0.7% NaCl (fish physiological saline), blotted dry and weighed to the nearest 0.1 mg. The blotted tissue was chopped with scisors and homoginized for 30 s in 3 mL ice-cold deionized distilled (DDI) water using a Turtox tissue homogenizer on minimum speed. The homogenizer probe was pre-chilled in ice water.

B. Tissue homogenates were centrifuged at 17,300 × g for 20 min at 4°C. Supernatants were aspirated and stored on ice until assayed. Supernatants were diluted 1:1 with DDI water prior to analysis. A 100 μL aliquot of the final supernatant dilution was reserved for protein analysis.

II. Principle

p-Nitrophenyl phosphate+H_2O+alkaline phosphatase →

p-Nitrophelon+inorganic phosphate

III. Conditions

T=37°C, pH=9.2, λ=410, light path=1 cm

IV. Method

Stopped spectrophotometric rate determination

V. Reagents

A. 100 mM glycine buffer with 1.0 mM magnesium chloride, pH 9.2 at 37°C.

To prepare 50 mL, combine 0.375 g glycine and 0.010 g magnesium chloride in approximately 30 mL DDI water. Heat to 37°C and adjust pH with 0.5 M NaOH. Bring volume up to 50 mL with DDI water. Prepare fresh daily. Glycine, free base, FW 75.07. $MgCl_2 \cdot 6H_20$, FW 203.3.

B. 15.2 mM p-Nitrophenyl phosphate solution (PNPP).

To prepare 5 mL, dissolve 0.020 g PNPP in 5 mL DDI water. Prepare fresh daily. PNPP Sigma phosphatase substrate stock no. 104-0, FW 263.1.

C. 20 mM sodium hydroxide solution (NaOH).

To prepare 1 L, dissolve 0.8 g NaOH in 1 L DDI water. NaOH, FW 40.0

VI. <u>Procedure</u>

Pipette (in mL) the following reagents into 14 mL tubes:

Reagent	Test	Blank
glycine buffer	0.5	0.5
PNPP	0.5	0.5

Mix and equilibrate to 37°C. Then add (mL):

Reagent	Test	Blank
supernatant solution	0.1	---
deionized distilled water	---	0.1

Add supernatant samples and blanks at 30 s intervals using a stop watch. Vortex after each addition. After exactally 10 min add 10 mL 20 mM NaOH to tube and vortex. Record the absorbance at λ 410 nm using the visible lamp.

VII. Calculations

Based on the Bouguer-Lambert-Beer law:

$$Abs = (\varepsilon)(c)(d)$$

where: ε=extinction coefficient; c=concentration; d=light path (cm). Rearranged, $c=(Abs)\cdot(\varepsilon \times d)^{-1}$. Accounting for the absorbance of the blank, dilution factors, assay time and volume, and sample volume:

A. Units$\cdot$mL^{-1} supernatant solution =

$(Abs_{test} - Abs_{blank})(11.1 \times d.f.)(18.3 \times 0.1 \times 10 \times 1)^{-1}$.

where: 11.1 = assay final volume in mL; df = dilution factor, in the case of most supernatants, 2; 18.3 = millimolar extinction coefficient of PNPP at 410 nm; 0.1 = volume of supernatant solution used in mL; 10 = time of assay (in min) as per Unit definition; 1 = light path (cm).

B. Units$\cdot$tissue^{-1} = (U$\cdot$mL^{-1} supernatant solution) $\times$ 3

where: 3 = mL water the intestine was homogenized in.

C. Units$\cdot$g^{-1} tissue = (U$\cdot$tissue^{-1})$\cdot$(tissue weight)$^{-1}$

D. Specific activity =

(U$\cdot$mL^{-1} supernatant solution)$\cdot$(mg protein$\cdot$mL^{-1} supernatant solution)$^{-1}$

VIII. Unit definition

One unit will hydrolyze 1.0 µmole of p=nitrophenyl phosphate per min at pH 9.2 at 37°C.

IX. References:

Anonymous. 1994. SIGMA quality control test procedure. Enzymatic assay of phosphatase, alkaline (EC 3.1.3.1). Glycine assay. SIGMA Chemical Company, St. Louis, Missouri.

Noda, H. and S. Tachino. 1965. Studies on various phosphatases of the fishes-I. Enzyme measurements. Journal of Faculty of Fisheries, Prefectural University of Mie 6:291-301.

APPENDIX E

Carboxypeptidase B Assay Protocol

I. Tissue preparation

A. Pyloric caecae were thawed at 4°C. Tissues were chopped with a scisors and homoginized in an ice bath for 30 sec in DDI water. The homogenizer probe was pre-chilled in an ice bath.

B. Tissue homogenates were centrifuged at 5495 × g for 60 min at 4°C. Supernatants were aspirated and stored on ice until assayed. Supernatants were diluted with DDI water prior to analysis. A 100μl aliquot of the final dilution was reserved for protein analysis.

II. Principle

Hippuryl-L-arginine+H_2O+carboxypeptidase A → hippuric acid+L-arginine

III. Conditions: T=25°C, pH=7.65, λ=254 , light path=1 cm

IV. Method: Continuous spectrophotometric rate determination

V. Reagents:

A. 25 mM Tris HCl buffer with 100 mM sodium chloride, pH 7.65 at 25°C. To prepare 1L, add 3.94 g Trizma-HCl and 5.844 g NaCl to water. Adjust pH to 7.65 using NaOH. Transfer to a 1L volumetric flask and bring to volume with water.

B. 1.0 mM Hippuryl-L-arginine solution.

To prepare 50 mL, dissolve 16.77 mg H-L-Arg in 50 mL water using a volumetric flask. Must be prepared fresh daily. SIGMA Hipputyl-L-Arginine, product No. H-2508.

VI. Procedure: The following volumes (mL) were added to 3-mL cuvettes

Reagent	Test	Blank
H-L-A solution	2.9	2.9
Deionized distilled water	---	0.10
Sample or standard	0.10	---

Equilibrate H-L-A solution to 25°C. Add sample, blank, or standard and mix immediately by iversion. Record changes in absorbance.

VII. Calculations:

Based on the Bouguer-Lambert-Beer law:

$$Abs = (\varepsilon)(c)(d)$$

where: ε=extinction coefficient; c=concentration; d=light path (cm)

Rearranged, $c = (Abs)\cdot(\varepsilon \times d)^{-1}$

Accounting for the absorbance of the blank, dilution factors, assay time and volume, and sample volume:

A. Units·mL^{-1} supernatant solution =

$$[(\Delta\ Abs\cdot min^{-1}{}_{test} - \Delta\ Abs\cdot min^{-1}{}_{blank})\ (3\times df)]\cdot(0.36\times 0.1\times 1)$$

where: 3 = assay final volume (mL), df = dilution factor, 0.36 = millimolar extinction coefficient of H-L-A at 254 nm, 0.1 = volume of sample or standard used (mL), 1 = light path (cm)

B. Units·tissue^{-1} = (U·mL^{-1} supernatant solution) x VB

where: VB = volume of buffer in which pyloric caeca was homogenized (mL)

C. Units·g tissue^{-1} = (U·tissue^{-1})·(tissue weight)$^{-1}$

D. Specific activity =

(U·mL^{-1} sample solution)·(mg protein·mL^{-1} supernatant solution)$^{-1}$

VIII. Unit definition: One unit will hydrolyze 1.0 μmole of hippuryl-L arginine per min at pH 7.65 at 25°C.

IX. References:

Anonymous. 1994b. SIGMA quality control test procedure. Enzymatic assay of carboxypeptidase B (EC 3.4.17.2). SIGMA Chemical Company, St. Louis, Missouri.

Yoshinaka, J., Sato, M., Morishita, J. and S. Ikeda. 1985. Enzymatic characterization of carboxypeptidase A from the catfish pancreas. Bulletin of the Japanese Society of Scientific Fisheries 51:113-116.

Yoshinaka, J., Sato, M., Morishita, J. Itoh, Y., Hujita, M. and S. Ikeda. 1984. Purification and some properties of two carboxypeptidases B from the catfish pancreas. Bulletin of the Japanese Society of Scientific Fisheries 50:1717-1722.