

Bioavailability of feed grade calcium phosphate incorporated into practical diets for *Penaeus vannamei*

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Abstract

Practical diets designed for penaeid shrimp are commonly supplemented with phosphorus, which may lead to unnecessary nutrient loading of the culture system and effluent waters as well as unnecessary investments in a nutrient that is not utilized by the culture species. To facilitate the optimization of phosphorus levels in practical shrimp feeds, research was conducted with *Penaeus vannamei* juveniles to determine the biological availability of two feed-grade calcium phosphate sources. A practical basal diet containing 350 g protein kg⁻¹ diet and 9.8 g P kg⁻¹ diet was formulated using anchovy and soybean meal as the primary protein and phosphorus sources. The basal diet was supplemented with graded levels of phosphorus and offered to juvenile shrimp (0.57 ± 0.017 g) over a 10-week period. Weight gain and estimated feed efficiency values increased with phosphorus supplements, indicating a dietary deficiency of the basal diet. Under the reported conditions, a dietary supplement of 1.4 or 2.3 g P kg⁻¹ diet was required for maximum growth and estimated feed efficiency values if Cefkaphos (primarily monobasic calcium phosphate) or Dynafos (primarily dibasic calcium phosphate) was utilized. Dynafos was determined to have a relative biological value (RBV) of 63.8% of Cefkaphos based on final weights of the shrimp offered diets containing 1.25 g supplemental P kg⁻¹ diet. A similar RBV of 60.9% was estimated based on broken-line analyses of growth data. There were no significant differences in apparent net phosphorus retention (ANPR) for the basal diet (23.1%) or diets supplemented with 1.25 g P kg⁻¹ diet originating from Cefkaphos (25.7%) or Dynafos (17.9%). However, shifts in ANPR values of the diets corresponded to biological availability of the two phosphorus sources.

KEY WORDS: *Penaeus vannamei*, phosphorus, nutrition

Received 18 August 1997, accepted 5 March 1998

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Introduction

The optimization of dietary phosphorus levels in aquaculture feeds has received considerable attention in recent years. From an economic view, phosphorus accounts for the greatest expense in the mineral supplement. Over-supplementation of the feed not only results in an unnecessary investment in a nutrient that will not be efficiently utilized by the cultured species but it also adds to the nutrient loading of the culture systems and effluent waters, possibly increasing the pollution load of receiving waters. As the initial source of aquaculture pollutants, manipulation of the feed added is the most direct and effective means to reduce pollutants (Cho *et al.* 1991, 1994). Optimization of nutrient levels consequently will facilitate reduction in feed costs as well as reductions in nutrient loading.

The need for a phosphorus supplement in practical diet formulations for fish is well established (Watanabe *et al.* 1988; Satoh *et al.* 1992). However, determinations of phosphorus availability and the need for supplements in practical shrimp feed formulations have received little attention. Several studies have evaluated the dietary phosphorus requirements of shrimp (Kitabayashi *et al.* 1971; Deshimaru & Yone 1978; Cheng 1984; Kanazawa *et al.* 1984; Civera & Guillaume 1989; Davis *et al.* 1993) utilizing semi-purified diets. Determinations of phosphorus bioavailability for *Penaeus vannamei* are limited to one study utilizing purified phosphorus sources incorporated into semi-purified diets (Davis & Arnold 1994). Owing to a poor understanding of dietary phosphorus requirements, particularly in practical diet formulations, and limited data on the biological availability of feed-grade phosphorus sources, commercial shrimp feeds tend to be over-supplemented with phosphorus. The objectives of this research were to evaluate if a phosphorus supplement is required in a practical diet formulation and determine the relative biological value of two feed-grade phosphorus sources for *P. vannamei*.

Materials and methods

A feeding trial and a digestibility trial were conducted with

Table 1 Composition of the test diets, expressed as g kg⁻¹ dry weight

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Anchovy meal ¹	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Soybean meal ²	330.0	330.0	330.0	330.0	330.0	330.0	330.0
Fish solubles ³	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Menhaden fish oil ⁴	38.0	38.0	38.0	38.0	38.0	38.0	38.0
Wheat flour ⁵	359.0	359.0	359.0	359.0	359.0	359.0	359.0
Soy lecithin ⁶	13.0	13.0	13.0	13.0	13.0	13.0	13.0
Trace mineral premix ⁷	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix ⁸	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin C (15% active) ⁹	1.0	1.0	1.0	1.0	1.0	1.0	1.0
NaCl ⁵	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Cefkaphos ¹⁰	2.75	5.50	11.00				
Dynafos ¹¹	6.75	13.50	20.25				
Diatomaceous earth ⁵	21.0	18.25	15.50	10.00	14.25	7.50	0.75
Total phosphorus	9.8	10.4	11.0	12.3	11.0	12.3	13.5

¹Ralston Purina International, Checkerboard Square, St Louis, MO, USA.

²Solvent extracted, Jupe Mills, Inc., West, TX, USA.

³Zapata Haynie Corporation, Hammond, LA, USA.

⁴Zapata Haynie Corporation, Reedville, VA, USA.

⁵United States Biochemical Corporation, Cleveland, OH, USA.

⁶Lecithin, Aqualipid 95, Central Soya Chemurgy Division, Fort Wayne, IN, USA.

⁷Composition of trace mineral premix (g kg⁻¹ mix): cobalt chloride, 0.04; cupric sulphate pentahydrate, 5.50; ferrous sulphate, 20.00; magnesium sulphate heptahydrate, 283.98; manganous sulphate monohydrate, 6.50; potassium iodide, 0.67; sodium selenite, 0.10; zinc sulphate heptahydrate, 131.93; α -cellulose, 551.28.

⁸Composition of vitamin premix (g kg⁻¹ mix): thiamin-HCl, 4.95; riboflavin, 3.83; pyridoxine-HCl, 4.00; Ca-pantothenate, 10.00; nicotinic acid, 10.00; biotin, 0.50; folic acid, 4.00; vitamin B₁₂, 0.05; choline chloride, 100.00; inositol, 25.00; vitamin A acetate, 8.00(20 000 IU g⁻¹); vitamin D, 0.50(400 000 IU g⁻¹); vitamin E acetate, 80.00 (250 IU g⁻¹); menadione, 0.5; α -cellulose, 748.68.

⁹Stay C, L-ascorbyl-2-polyphosphate, Hoffman-La Roche, Inc., Nutley, NJ, USA.

¹⁰BASF Corporation, Mount Olive, NJ, USA.

¹¹Mallinckrodt Feed Ingredients, Mundelein, IL, USA.

Penaeus vannamei juveniles to determine the biological value of two commercial feed-grade phosphorus sources. A practical diet formulation (Table 1) containing anchovy meal and soybean meal as the primary protein and phosphorus sources was utilized. Prior to use, the anchovy and soybean meals were ground with a laboratory type hammer mill using a size 40 screen (1.02 mm diameter hole) and the phosphorus sources ground using a size 24 screen (0.609 mm diameter hole). Characterization of the anchovy meal, soybean meal and phosphorus supplements are presented in Table 2. After processing, the dry ingredients and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 20 min. Hot water was then blended into the mixture to a consistency appropriate for pelleting. Each diet was pressure pelleted using a meat grinder and a 3-mm die. After pelleting, the diets were dried to a moisture content of 8–10% and then stored at 4°C. The unsupplemented basal diet contained 350 g protein kg⁻¹ and 9.8 g total P kg⁻¹.

The 70-day feeding trial used dietary treatments that included the basal diet without supplements and diets with the following feed-grade phosphorus supplements: Cefkaphos (primarily monobasic calcium phosphate, BASF Corporation, Mount Olive,

NJ, USA) at 2.75, 5.50, 11.00 g kg⁻¹ diet and Dynafos (primarily dibasic calcium phosphate, Mallinckrodt Feed Ingredients, Mundelein, IL, USA) at 6.75, 13.50 and 20.25 g kg⁻¹ diet. These inclusion levels produced three levels of supplemental phosphorus originating from Cefkaphos (0.62, 1.25, 2.50 g P kg⁻¹ diet) and three levels of supplemental phosphorus originating from Dynafos (1.25, 2.50, 3.75 g P kg⁻¹ diet). The test diets were offered to four replicate groups of shrimp (eight shrimp per tank; mean weight \pm standard deviation, 0.57 \pm 0.017 g) maintained in a common semi-closed recirculating system consisting of a series of rectangular tanks containing 68 L of seawater, a circulation pump, rapid-rate sand filter, supplemental aeration and biological filter. The shrimp were weighed at day 0, day 42 and day 70. The shrimp were fed a fixed feeding rate designed to be in slight excess. Feed rations were adjusted for expected growth and observed mortalities resulting in a total of 16.2 g (dry wt) of feed per shrimp being offered over the course of the growth trial. Shrimp were fed four times per day at approximately 0800, 1100, 1300 and 1600 h. Photoperiod was set for a 12 h light/12 h dark cycle. Water quality was maintained by biological filtration, by removing uneaten feed in the morning before the first feeding,

Table 2 Analysis¹ of selected feed ingredients

Assay (g kg ⁻¹)	Anchovy fish meal	Soybean meal	Cefkaphos	Dynafos
Moisture	85.0	138.0	72.7	5.37
Protein	633.0	451.0		
Fat (ether extract)	91.9	15.5		
Phytate		15.2		
Ash	165.0	64.3		
Calcium	35.3	2.6 ²	176.0	205.0
Phosphorus (P)	25.6	6.2	230.0	187.0
Water soluble (g kg ⁻¹ of P)	5.6	0.9	172.0	75.4
2% citric acid soluble (g kg ⁻¹ of P)	816.0	2.5	939.0	810.0
Tyler Screen Size (g kg ⁻¹ retained)				
Held on Pan			368	276
Held on size 60			84	196
Held on size 100			244	268
Held on size 200			304	260

¹Analyses of ingredients were conducted by Ralston Analytical Laboratories, St Louis, MO, USA.

²As reported by the National Research Council (1983).

and by replacing the systems make-up water at a rate of 4 L min⁻¹ with prefiltered ozone-treated seawater. Water temperature, dissolved oxygen and salinity were maintained at 28.5 ± 1.6°C, 6.2 ± 1.0 mg L⁻¹ and 33 ± 1.6 mg L⁻¹, respectively. Total ammonia-nitrogen, nitrite-nitrogen and pH were measured twice weekly by the methods of Spotte (1979) and were maintained at 0.03 ± 0.05 mg L⁻¹, 0.03 ± 0.05 mg L⁻¹ and 8.0 ± 0.11, respectively.

At the conclusion of the growth trial, weight gain, survival, estimated feed efficiency (EFE) and apparent phosphorus retention (APR) were determined. Samples of shrimp from the initial population, and from each tank at the termination of the feeding trial, were collected and frozen for subsequent analysis. Three shrimp from each tank were minced, oven dried (90°C) to a constant weight and ground prior to biochemical analysis. Diets and whole body samples were wet-ashed based on the methods described by the Association of Official Analytical Chemists (1984) and the phosphorus levels were determined photometrically (Fiske & Subbarow 1925) on duplicate samples.

The relative biological value (RBV) of the two phosphorus sources were compared based on mean weights (MW) of the shrimp at the termination of the feeding trial. Two methods of calculation were employed. First, RBV was based on weight gain of the shrimp above that of the basal diet according to $(MW \text{ diet } 5 - MW \text{ diet } 1)/(MW \text{ diet } 3 - MW \text{ diet } 1) \times 100$, for which diets 3 and 5 were supplemented with 1.25 g P kg diet. The second method used broken line analysis (Robbins *et al.* 1979) to estimate the required level of each supplement to meet the dietary requirement. For this analysis the data for diets containing 2.5 g P kg⁻¹ or greater were assumed to be replete and were pooled for each analysis. The required supplement, as determined by broken

line analyses, for the two phosphorus sources was then utilized to calculate the RBV of Dynafos expressed as a percentage of Cefkaphos (required supplement of Dynafos/required supplement of Cefkaphos × 100).

To evaluate the net absorption of phosphorus or apparent phosphorus availability (APA) of the basal diet and the effects of supplementing low levels of phosphorus (1.2 g P kg⁻¹ diet), diets 1, 3 and 5 (Table 1) were modified to contain 5 g kg⁻¹ chromic oxide, replacing diatomaceous earth. The test diets were prepared as previously described. Dry matter and phosphorus losses from the test diets were evaluated. Quadruplicate samples of the diets (0.1 g) were placed in 10 mL of prefiltered seawater under light agitation (shaken at 60 cycles min⁻¹) for a 45-min immersion period. At the conclusion of the leaching period, the pellets were transferred to a 48 µm sieve, rinsed with distilled water and then dried to a constant weight. Dry matter losses were then calculated based on the differences in pellet weight. Phosphorus losses from the pellets after immersion were also evaluated by decanting a sample of seawater and determining the quantity of phosphorus in the water.

Apparent phosphorus availability was determined using the chromic oxide indicator technique (National Research Council 1983) and voluntary consumption of the test diets by the shrimp. Prior to initiation of the digestibility trials, shrimp (7.4 ± 1.6 g) were acclimatized to the experimental conditions over a 1-week holding period. The shrimp were maintained in the previously described system under similar water quality conditions. Each diet was offered to four replicate tanks of shrimp (six shrimp per tank) over a 6-day collection period. In the morning, the tanks were siphoned clean and the feeding cycle initiated. Shrimp were allowed to feed for 35–45 min, after which the faeces were

Table 3 Response of *Penaeus vannamei* after 70 days of feeding diets containing various levels of phosphorus (P) from two sources¹

Diet	Supplemental P ²	Whole-body analyses					
		Weight (g)	Survival (%)	EFE ³ (%)	APR ⁴ (%)	DM ⁵ (g kg ⁻¹)	P (g kg ⁻¹ dry wt)
1	0	7.39y	59.4z	41.1y	9.5z	250.4z	9.0z
2	0.62-C	8.43yz	62.5z	47.3yz	10.1z	242.7z	9.2z
3	1.25-C	9.13z	68.8z	51.5z	11.1z	252.4z	9.5z
4	2.50-C	9.52z	87.5z	53.9z	10.5z	248.0z	9.8z
PSE ⁶		0.44	9.15	2.66	0.63	3.35	0.18
1	0	7.39y	59.4z	41.1y	9.5z	250.4z	9.0y
5	1.25-D	8.55yz	71.9z	48.0yz	9.7z	245.1z	9.3y
6	2.50-D	9.37z	78.1z	52.9z	9.8z	251.3z	9.2y
7	3.75-D	9.31z	87.5z	52.5z	9.5z	250.0z	10.3z
PSE		0.41	9.92	2.47	0.55	3.23	0.14

¹Means of four replicates. Numbers within the same column followed by different letters are significantly different ($P < 0.05$).

²C, Cefkaphos; D, Dynafos.

³EFE, estimated feed efficiency = weight gain/feed offered*100.

⁴APR, apparent phosphorus retention = phosphorus gain/phosphorus offered \times 100.

⁵DM, dry matter.

⁶PSE, pooled standard error.

collected by hand by siphoning into a collection sieve (48 μ m), followed by a rinse with distilled water. After the faeces had been collected, uneaten food was removed from the tank and the feeding process repeated. During the collection period, the first collection in the morning was discarded with the following four to five collections per day pooled by tank. Apparent availability values were determined based on the relative change in percentage chromic oxide in the feed and faeces. Chromic oxide levels were determined by the method of McGinns and Kasting (1964). The phosphorus levels were determined photometrically (Fiske & Subbarow 1925) after wet ashing (Association of Official Analytical Chemists 1984). All chemical analyses were conducted in triplicate.

Data were analysed using an analysis of variance to determine significant ($P < 0.05$) differences among treatment means. Duncan's multiple range test was utilized to determine significant differences between treatment means (Steel & Torrie 1980). All statistical analyses were conducted using the SAS Systems for Windows V6.12 (SAS Institute Inc. Cary, NC, USA).

Results and discussion

Anchovy and soybean meals were used as the primary protein and phosphorus sources for this research. Analysis of these feed ingredients and the two supplemental phosphorus sources are presented in Table 2. Cefkaphos and Dynafos were selected as examples of feed-grade phosphorus sources with potentially different phosphorus availability values. Both phosphorus sources were ground under similar conditions and had similar particle size distributions. Owing to differences in the primary

phosphorus form, the phosphorus content and water solubility were greatest for Cefkaphos, which is composed primarily of monobasic calcium phosphate.

The growth trial evaluated the response of *P. vannamei* juveniles to varying levels of phosphorus originating from Cefkaphos and Dynafos. The response of the shrimp is summarized in Table 3. Under the conditions reported, there were no significant differences in percentage survival; however, there was a general trend of increasing survival with dietary phosphorus supplements. There were no significant differences, or clear trends, observed in APR and dry matter content of whole-body samples for shrimp receiving either phosphorus source. The phosphorus content of shrimp receiving the basal diet supplemented with 3.75 g P kg⁻¹ diet was significantly higher than values for shrimp receiving the basal diet or diets supplemented with 1.25 or 2.50 g P kg⁻¹ diet originating from Dynafos. However, there were no clear trends in the mineralization data. The lack of a clear influence on tissue mineralization, which in vertebrates is considered a sensitive indicator of phosphorus status, has been reported in other studies on the phosphorus requirements of shrimp. This poor correlation is generally thought to be associated with variations due to the moult cycle (Kanazawa *et al.* 1984; Davis *et al.* 1993).

Shrimp responded positively, in terms of growth and EFE values, to increases in dietary phosphorus supplements. Significant increases in final weights, as well as EFE, values indicate that the basal diet did not have adequate levels of available phosphorus to meet physiological demands for growth and feed utilization. Similar reductions in growth rates have been reported by Davis *et al.* (1993) using semi-purified diets and would be

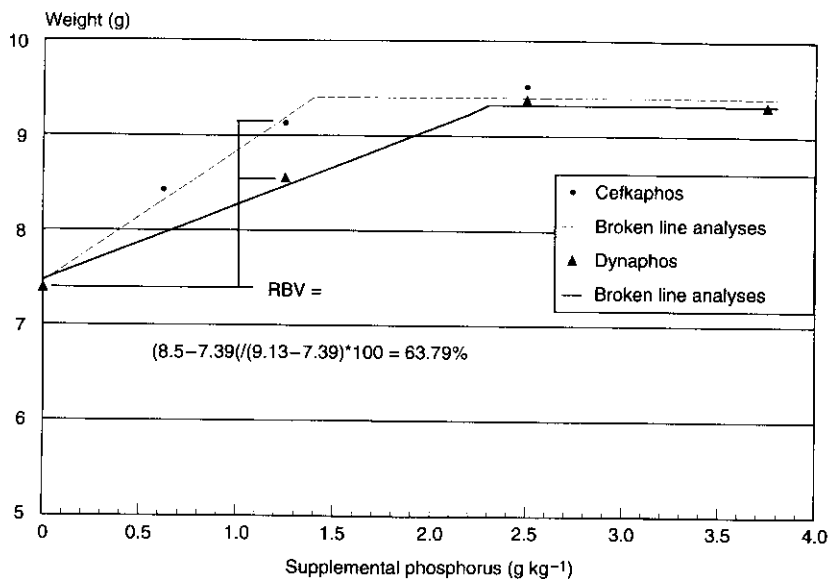


Figure 1 Response of *Penaeus vannamei* to practical diets containing varying levels of phosphorus supplements originating from Cefkaphos or Dynafos. Broken line analysis for the determination of the level of supplemental phosphorus required for maximum growth and the determination of relative biological values (RBV) for Dynafos expressed as a percentage of Cefkaphos is presented.

considered indications of a phosphorus deficiency. For shrimp receiving diets supplemented with Cefkaphos the supplementation of 1.25–2.50 g P kg⁻¹ diet resulted in maximum growth. Similarly, the supplementation of Dynafos at 2.5–3.75 g P kg⁻¹ diet resulted in maximum final weights of the shrimp.

Research with purified sources of calcium phosphate incorporated into semi-purified diets has indicated differences in the APA values of monobasic and dibasic forms (Davis & Arnold 1994). Similar results would be expected with feed-grade sources, resulting in differences in the biological availability of the phosphorus. Shrimp offered the basal diet supplemented with 1.25 g P kg⁻¹ diet originating from Cefkaphos grew better than shrimp offered diets containing the same quantity of phosphorus supplemented using Dynafos (9.13 vs. 8.55 g, respectively), indicating differences in the biological availability of the two phosphorus sources. Since shrimp offered diets containing 0.62 g P kg⁻¹ diet originating from Cefkaphos grew similarly to shrimp receiving 1.25 g P kg⁻¹ diet originating from Dynafos it is apparent that the relative biological value of Dynafos is about 50% that of Cefkaphos.

Although a number of variations exist in determining the relative biological value of minerals, the basic procedure is common among studies. These methods compare the response to a test source with that of a reference source. The measured response, whether it be growth, mineralization or enzyme activity, must produce a linear increase over the area of comparison. Based on plots of the final weights of shrimp offered varying levels of phosphorus (Fig. 1), it would appear that diets containing 2.5 g P kg⁻¹ diet or greater supported maximum growth. This area clearly represents a plateau in the growth res-

ponse, indicating that the phosphorus requirement has been met. It would also appear that the growth response to dietary supplements at or below 1.25 g P kg⁻¹ diet is linear. Consequently, the response can be compared at this level of inclusion.

While it is not clear whether the diet supplemented with 1.25 g P kg⁻¹ diet originating from Cefkaphos is marginally deficient, this level of inclusion was utilized to estimate the RBV for Dynafos expressed as a percentage of Cefkaphos. Using the equation, $(8.5 - 7.39) / (9.13 - 7.39) \times 100$, an RBV of 63.79% was calculated.

Similarly, one can use the growth data and broken line analysis to estimate the level of each phosphorus source required. Ideally, more data points should be used for this method; however, if one assumes that the dietary requirement has been met by the supplementation of 2.5 g P kg⁻¹ diet, this data can be pooled and a line fitted to the data. Based on broken line analysis, 1.4 ± 0.18 g P kg⁻¹ and 2.3 ± 0.40 g P kg⁻¹ would be required from Cefkaphos and Dynafos, respectively. These values would indicate that Dynafos has an RBV $(1.4/2.4 \times 100)$ equal to 60.9% of Cefkaphos, which is similar to the value calculated using weight gain of shrimp offered the basal diet supplemented with 1.25 g P kg⁻¹ diet.

The second component of this research sought to confirm the net absorption of phosphorus, or APA, of the basal diets and the effects of the inorganic sources. Diets 1, 3 and 5 were modified to contain chromic oxide as an inert marker and evaluated using standard digestibility techniques. The stability of the test diets was confirmed by determining the dry matter and phosphorus losses after 45 min of submersion in seawater. Dry matter losses ranged from 4.7 to 6.1% and phosphorus losses ranged from

Table 4 Nutrient losses and apparent digestibility coefficients from selected diets¹

Diet	Supplemental P	DM loss	P loss (mg P g ⁻¹ diet)	% Loss ²	ADMD ³	ANPR ⁴
1	0	6.1	0.169		71.5	23.1
3	Cefkaphos	4.7	0.282	9.0	71.3	25.7
5	Dynafos	4.7	0.251	6.6	70.8	17.9
PSE		0.5	0.058		0.7	3.4

¹Analysis of variance indicated no significant differences among dietary treatments. Diets 3 and 5 were supplemented with 1.25 g P kg⁻¹ diet.

²Percentage loss = (P loss from diet – P loss from basal diet) × 100/0.125.

³ADMD, apparent dry matter digestibility.

⁴ANPR, apparent net phosphorus retention.

0.169 to 0.282 g P kg⁻¹ diet with no significant differences occurring between dietary treatments (Table 4). Although phosphorus did leach from the diets, the observed values were considered acceptable, within the range seen with previous research (Davis *et al.* 1993) and were not expected to affect the results significantly.

No significant differences were observed for apparent dry matter digestibility (ADMD) and APA values resulting from the dietary treatments (Table 4). The ADMD values observed are similar to values for other practical diets that have been evaluated in our laboratory. The basal diet (diet 1) had an APA value of 23.1%, which would indicate that the availability of phosphorus from fish meal and soybean meal is considerably lower than the values presented by Akiyama *et al.* (1992) (46.5 and 39.9, respectively). Owing to the large variation within treatments and the relatively low contribution of the phosphorus supplement as a percentage of the total phosphorus content of the diet (about 11%) APA values for the supplements could not be calculated. However, the supplementation of low levels (0.125 g P per 100 g of diet) of phosphorus originating from Cefkaphos or Dynafos to the basal diet resulted in shifts in the APA values that corresponded to the biological values of the phosphorus source. Based on the APA values determined in this study, the basal diet would contain 0.22% available phosphorus.

Conclusion

Under the conditions reported, the basal diet, containing 9.8 g P kg⁻¹ diet, was estimated to contain 2.2 g kg⁻¹ available phosphorus, which was not adequate to meet the physiological requirement of the shrimp for maximum growth and survival. The supplementation of phosphorus to the basal diet resulted in significant increases in final weights of the shrimp and EFE values. Differences in biological availability of the two phosphorus sources tested were observed and compared based on their relative biological value. Based on broken line analysis, the supplementation of 1.4 or 2.3 g P kg⁻¹ diet was required for maximum growth if Cefkaphos or Dynafos, respectively, were used as the phosphorus

sources. Based on this analysis, Dynafos was determined to have an RBA of 60.87% of Cefkaphos. This value is comparable to the RBV of 63.79% which was calculated based on final weights of the shrimp offered diets containing 1.25 g supplemental P kg⁻¹ diet. The differences in biological availability of the two phosphorus sources was also confirmed by shifts in APA values of diets supplemented with the two phosphorus sources.

The data presented should help feed manufacturers to optimize phosphorus levels in practical diet formulations and facilitate better economic comparisons between feed-grade monobasic and dibasic calcium phosphate. Because of both environmental and economic considerations, continued research designed to determine the biological availability of phosphorus from major feed ingredients is recommended.

Acknowledgements

The authors extend their thanks to those who have taken the time to critically review this manuscripts as well as students and staff who help support research at the Marine Science Institute. This research was supported in part by the Sid W. Richardson Foundation and Ralston Purina International. Mention of trade names or proprietary products does not constitute an endorsement by The University of Texas at Austin and does not imply its approval to the exclusion of other products that may also be suitable. This is The University of Texas at Austin Marine Science Institute Contribution no. 1054.

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