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Effect of different inorganic phosphorus sources on growth performance, digestibility, retention efficiency and discharge of nutrients in rainbow trout (*Oncorhynchus mykiss)*

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Abstract

This study aims to evaluate the effect of different sources of inorganic phosphate, monosodium phosphate (MSP), monocalcium phosphate (MCP) and monoammonium phosphate (MAP), included in a diet for rainbow trout, on growth performance, nutrient digestibility, nutrient retention efficiency and discharge of nutrients to the water. Juvenile rainbow trout (*Oncorhynchus mykiss*) with an initial weight of 120 grams were fed for 56 days four experimental diets: basal diet containing 430 g kg^{-1} protein, 210 g kg^{-1} lipids and 6.7 g kg^{-1} total phosphorus (P) (Control), and three experimental diets consisting in the basal diet plus 4 g P kg^{-1} from MSP, MCP and MAP, respectively. Both MSP and MAP resulted in higher levels of P apparent digestibility (≈90%) compared to MCP (70%), increasing the dry matter digestibility of the nutrient in the feed. The retention efficiency of P in fish tissues was increased by nearly 15% units over the Control when MSP and MAP were used as P supplement, whereas in agreement with the lower digestibility observed in MCP, P retention efficiency from this phosphate source was lower than the other sources. Total P discharge to the water was similar for MSP, MCP and MAP (4.12–4.58 g kg^{-1} BW fish gain). However, fish fed with MCP showed higher solid/particulate P discharge to the water through faeces due to the lower digestibility of this P–source within the gut of the fish. Among the phosphate salts evaluated, fish fed MSP and MCP did not show differences for soluble or solid N discharged fractions. However, fish fed the diet supplemented with MAP released a higher amount of non–protein N fraction to the water, probably as undigested ammonium through the faeces released by fish.

Keywords: monosodium phosphate; monocalcium phosphate; monoammonium phosphate; phosphorus; nitrogen; rainbow trout

1. Introduction

Aquaculture has become the fastest growing food–producing sector in the world, supplying 48% of the world's food fish in 2014 (FAO, 2016). This rapid growth is explained mostly by the intensification of the activity and the utilization of both improved fish feed formulations and optimized rearing technics that allow for the production of more food. However, the environmental management of intensive aquaculture is an important issue to assure the sustainable development of the aquaculture in the coming years. The reduction of P discharge to the water by fish is considered an essential element for the long–term sustainability of aquaculture and has become imperative for fish feed industries.

In the past, the retention of dietary P by fish was around 20%, and most dietary P was discharged to the environment (Holby and Hall, 1991; Ketola and Harland, 1993). This was largely explained because fish feeds were based on fish meal (FM) as the main protein source in the diet, providing a total P content that surpasses the minimal requirements needed to obtain optimum growth and nutrient body retention. Moreover, FM is associated with a high bone content, where a considerable fraction of the total P is located mainly in the form of tricalcium phosphate or hydroxyapatite, which remains almost inaccessible for many of the farmed fish species (Ketola and Richmond, 1994; Sugiura et al., 2000b).

Despite the aquaculture feed industry reliance on FM, its limited supply and rising price restrict the use of this protein commodity for sustainable fish farming (Baruah et al., 2004; New and Wijkström, 2002), leading to an increased use of alternative plant protein ingredients in aquafeeds. However, a complete substitution or reduced amount of FM,

combined with the use of alternate plant protein sources that have lower total P content (mainly as non–bioavailable inositol phosphate salts) can result in P deficiencies in fish.

Modern formulations include a lower content of FM, higher inclusion levels of plant protein ingredients, enzymatic additives such as phytases to improve the bioavailability of the endogenous phytate P, and inorganic P sources to reach a specific nutrient requirement by the fish. Thus, currently farmed fish can retain about 30–40% of P in typical commercial feeds (Green et al., 2002a,b) or even more, which is a considerable improvement over historic figures.

Despite this fact, the use of phytases can partially improve P bioavailability in FM–substituted diets, a necessary strategy to enhance P availability from inorganic P supplements used in aquafeeds is needed to accurately cover fish requirement, to minimize P waste outputs from fish farms, and to prevent P deficiencies, such as skeletal deformities and reduced growth (Lall, 2002; Sugiura et al., 2004; Tacon, 1992).

Different inorganic P sources have different solubilities and can affect the digestibility of the nutrient (Lall, 1991). When different inorganic P sources are used as a dietary supplement in farmed fish, the solubility of the P salt can be affected by the content of calcium in the diet, as well as the changes in pH under the gastrointestinal conditions of a given fish species (Hua and Bureau, 2006). These factors can affect the bioavailability of the dietary P under the neutral conditions of the intestine, and therefore, the absorption and retention efficiency of the nutrient in the fish and, consequently, its discharge to the water.

The aim of this study was to evaluate the effect of three widely used inorganic P sources, monosodium phosphate (MSP), monocalcium phosphate (MCP) and monoammonium phosphate (MAP), included in a plant–based diet for rainbow trout on growth performance, nutrient digestibility, nutrient retention efficiency and discharge of N

and P to the water.

2. Materials and Methods

2.1. Fish and rearing conditions

Rainbow trout (*Oncorhynchus mykiss*) with an initial weight of 120 grams were used (25 fish per tank, 3,000 g biomass per tank), aiming at duplicating the weight during the experimental period (56 days). Fish were provided by a local farmer (Patagonia Harvest S.A., Argentina) and classified to assure equal start weights among tanks. Fish were manually fed, three times per day, 7 days per week. Uneaten feed was collected daily from the outlet water of each tank and stored at −22 °C before dry matter analysis. Feed intake was monitored according to the method described by Helland et al. (1996). Fish were reared under natural photoperiod conditions 14(L):10(D). The fish were held in twelve 250 L tanks with 3 replicates per treatment. The source of water used was taken from a floodgate located on the wall of the hydroelectric dam of Piedra del Águila (Limay River Basin, province of Río Negro, Argentina). Each tank was supplied with a flow rate of 6 l min^{-1}. Temperature and oxygen levels were monitored daily; both parameters varied in the range of 12–14 °C and 7–9 mg l^{-1} OD.

2.2. Experimental diets

Experimental treatments were a basal diet (Control), basal diet plus 4 mg P kg^{-1} from monosodium phosphate (MSP; NaH_2PO_4), basal diet plus 4 mg P kg^{-1} from monocalcium

phosphate (MCP; $Ca(H2PO_4)_2$) and basal diet plus 4 mg P kg^{-1} from monoammonium phosphate (MAP; $NH_4H_2PO_4$). The four experimental diets were produced using gelatine and pre–gelatinized corn starch as binders. Chromium (III) oxide (Cr_2O_3) was used as inert consumption marker to determine apparent nutrient digestibility (Austreng et al., 2000). All dry ingredients except gelatine were mixed in a 63 L feed mixer machine 250w 0.33 HP (Mc8070, Neo, Argentina). Phosphate sources were added into the micro–ingredients premix, and then fish oil and vegetable oil were added to the final dry mixture. The gelatine was mixed in water at 90 ºC and the dry mix was added to make a firm dough, which was homogenized and pelletized with a machine Freire 1.5 HP Nº 32 (Freire, Argentina) equipped with a 4–mm die and cutting blade. The pellets were dried using a drying chamber with a forced air flow with a temperature of 45 ºC until the pellets reached ≈900 g kg^{-1} of dry matter. The formulation and chemical composition of the experimental diets are detailed in Table 1.

2.3. Sampling and chemical analysis

All sampling was carried out on randomly captured fish from each tank. At the beginning of the experiment, 12 fish were taken to determine the proximate composition. Fish were euthanized by an overdosing and overexposure in anaesthetic (100 mg l^{-1} benzocaine). The gut was opened to remove the digestive contents. Samples of whole fish were kept frozen until analysis. At the end of the experiment (12 hours after the last feeding), all fish were anaesthetized with 50 mg l^{-1} benzocaine and faeces were stripped as described by Austreng (1978). The faecal and whole fish samples from each tank were pooled and stored in a freezer for subsequent analysis.

The ingredients and the completed diets were analyzed for dry matter, crude protein, lipids, total P and Ca. Also, the diets were analyzed for chromium marker. A pooled faeces sample from each tank was analyzed for chromium marker, dry matter, crude protein, total P and Ca to calculate apparent digestibilities. Four fish per tank were taken at the end of the trial, for analysis of whole body composition, and compared with the fish composition at the beginning of the trial to calculate nutrients retention efficiency.

The dry matter of the diets, freeze–dried faeces and homogenates of whole fish were determined by drying at 105 °C for 24 h. Nitrogen was determined by Kjeldahl method and crude lipid was determined by Soxhlet method. Gross energy was measured by bomb calorimetry. Chromium in the samples of digesta was determined by the method of Williams and David (1962) with minor modifications. In brief, after homogenization and centrifugation (17,000 x g, 15 min) of the digestive contents, the precipitate was dried at 105 °C for 24 h. Dry samples were hydrolysed with concentrated nitric acid during 6 h at 150 °C. The mixture was cooled to ambient temperature and a second hydrolysis (150 °C, 12 h) was carried out after addition of 4 ml of a mixture comprising sodium molybdate, sulphuric acid and perchloric acid. The sample was afterwards diluted in 50 mL distilled water and the content of chromium determined colorimetrically at 350 nm in (UV–Visible Spectrophotometer Perkin Elmer Lambda 20). To determine Ca and P content in diets, fish and faeces, a mixture of nitric acid and perchloric acid (2:1, v/v) was added to the samples and the mixture was maintained 3 hours at 235 ºC (Johnson and Ulrich, 1959). Following cooling to room temperature, the samples were analyzed for elements using an ICP–AES (Shimadzu 9000 model III).

To evaluate the P solubility of the different inorganic P sources, an *in vitro* assay was conducted. An equivalent amount of 0.35 mg P from MSP, MCP and MAP was dissolved

in distilled H_2O plus HCl or NaOH to adjust the desired pH (pH 3.0 and 7.0). The mixtures were constantly stirred with a magnetic multi–stirrer (Cimarec I Poly, Thermo Scientific) at room temperature. After 90 min, soluble P was determined in the supernatant solution previous centrifugation of the mixture (17,000 x g, 15 min) with a microcentrifuge (Sorvall Legend Micro 17, Thermo Scientific).

2.4. Calculations and statistical analysis

The response parameters measured were initial body weight and final body weight to calculate fish growth rate. Daily feed intake and the amount of uneaten feed were recorded to calculate the effective feed intake and feed conversion ratio.

Thermal growth coefficients (TGC) was calculated as:

$$TGC = 1000 \times (BW_{final}^{1/3} - BW_{initial}^{1/3}) / \Sigma d°$$

where: BW represents the body weight and Σd° represents thermal sum (mean daily temperature in ºC × days of the period).

Feed conversion ratio (FCR) was calculated as:

$$FCR = DM_{feed} \times (BW_{final} - BW_{initial})^{-1}$$

Apparent digestibility coefficients (ADC) of individual nutrients was calculated as follows:

$$ADC = 100 \times [1 - (Di \times Fi^{-1} \times Fn \times Dn^{-1})]$$

where: Di and Fi represent the concentration of inert marker in diet and faeces, and Dn and Fn represent the concentration of nutrients in diet and faeces, respectively.

The ADC for P from phosphates sources were determined by the following formula:

$$ADC_{source} = [((x+y) \times ADC\ P_{diet}) - ((x) \times ADC_{control})] \times y^{-1}$$

where: ADC_{source} is the apparent digestibility coefficient of P in the phosphate source; $ADC\ P_{diet}$ is the apparent digestibility coefficient of P in test diet; $ADC_{control}$ is the apparent digestibility coefficient of P in the control diet; x represent the nutrient contribution from control diet to the P content of the combined diet (level of P in control diet × (1–I); y is the P contribution of test phosphate source to P content of test diet (level of P in test phosphate source × I); and I represent the proportion of phosphate source in test diet.

Nutrient Retention Efficiency (RE) was calculated as:

$$RE = 100 \times ((BW_{final} \times N_{final}) - (BW_{initial} \times N_{initial})) \times (FCR \times (BW_{final} - BW_{initial}) \times N_{diet})^{-1}$$

where: N_{diet} is the content of nutrient in the diet, and $N_{initial}$ and N_{final} represent the initial

and final concentration of nutrient in whole minced fish.

Solid nitrogen (N) or phosphorus (P) discharge was calculated as:

Solid Nutrient Discharge = N or P intake × (100 − ADC of N or P) × 100^{-1}.

Dissolved N or P discharge was calculated as:

Dissolved Nutrient Discharge = (N or P intake × ADC × 100^{-1}) – (N or P retained in whole body fish)

The mean values are reported ± the standard deviation of mean (SD) from three replicates per treatment. After verification of the assumptions of normality and homoscedasticity, data were subjected to ANOVA and Student's t multiple comparison test was used to compare means between treatments. Differences between means are significant at $P<0.05$. All the analyses were performed using the JMP 9 software package (SAS, Cary, NC, USA).

3. Results

3.1 Diets, growth and feed utilization

Diets manufactured were isonitrogenous (42.5 – 44.0 g kg^{-1} crude protein) and isoenergetic (21.0 – 21.7 g kg^{-1} lipids; 24.3 – 24.7 MJ kg^{-1} gross energy. The content of

total P in control diet was 6.7 g kg^{-1}, whereas the content of total P in MCP, MSP and MAP ranged between 10.3 and 10.6 g kg^{-1}. These values confirmed that phosphate inclusion in the diets resulted in an increment in total P of 4 g kg^{-1} with respect to the Control (Table 1). After 56 days of treatment, fish fed diets containing inorganic P sources showed a higher growth rate ($P<0.0095$) and final body weight ($P<0.0005$) compared to those fed the Control diet (Table 2). Feed intake was not significantly affected by the addition of phosphate sources at the level of 4 g P kg^{-1} diet. However, the addition of phosphates to diets improved the FCR compared to the diets without the inclusion of the supplement ($P<0.0027$).

3.2 In vitro solubility of the inorganic P sources and in vivo apparent digestibility trials

The results obtained in the *in vitro* P solubility assay for the three sources showed a significant lower solubility in MCP (66.3±1.2% and 65.1±0.1%, for pH 3.0 and pH 7.0, respectively), compared to MSP (74.6±1.3% and 76.2±2.6%) and MAP (79.4±3.0% and 75.8±1.1% for pH 3.0 and pH 7.0, respectively) under the assayed conditions; $P=0.0006$ (pH 3.0) and $P=0.0002$ (pH 7.0).

The effect of phosphate supplementation of the Control diet on nutrients apparent digestibility (ADC) is shown in Table 2. The addition of inorganic P did not significantly affect the digestibility of the dry matter and protein. The digestibility of total dietary P was higher compared than the Control when fish were fed with MSP and MAP supplementation ($P=0.0013$). However, the P ADC in the MCP diet was slightly higher (48.1%) than the value observed in the Control diet (43.5%), this difference was not significant at the level of $P=0.05$. The P apparent digestibility from phosphate products differed significantly

(P=0.0066) among the sources evaluated. MSP and MAP showed the highest and similar P ADC with values of 91.1% and 92.9%, respectively; while MCP P ADC was lower than MSP and MAP treatments with a mean value of 70.4%. Calcium digestibility was not different between the phosphate sources.

3.3 Nutrient retention and discharge to the water

The influence of phosphate sources supplementation on fish whole body nutrient content is detailed in Table 3. The use of either phosphate sources resulted in a significant (P<0.0023) increment in the P content in whole body of fish (2.23–2.40 g kg^{-1}) with respect to the P body level measured in fish fed Control diet (1.79 g kg^{-1}). Calcium body levels did not show variation between treatments, hence Ca:P ratios in whole body fish fed supplemented inorganic P were lower (P=0.046) than that observed in Control fish.

The findings observed for nutrient retention efficiency in fish are detailed in Table 3. A significant increase in N and P retention efficiency was found in fish fed diets supplemented phosphates compared to those fed Control diet (P=0.016). This effect can be explained by the better fish growth rate and feed conversion ratio recorded with MSP, MCP and MAP supplemented diets compared to the observed response with the Control diet. It can be noted that the MAP diet resulted in a slightly lower N retention efficiency (49.7%) in comparison to MSP (54.4%) and MCP (54.2%) diets. On the other hand, a higher P retention efficiency was observed with MSP and MAP (37.0% and 36.2%, respectively) compared to Control (22.0%) (P=0.013), whereas fish fed the MCP diet showed an intermediate P retention efficiency value (31.6%) (Table 3).

The effect of the different phosphate sources in the diet on N and P discharges to the

water are shown in Table 4. All fish groups showed a lower fraction of solid N discharge (≈10 g kg^{-1} BW gain) compared with the soluble N fraction (≈15–25 g kg^{-1} BW gain). The use of inorganic P as a dietary supplement did not change the solid fraction of N loadings from fish. However, results suggest that fish fed diets with MSP and MCP inclusion had lower levels of soluble N discharges to the water (15 g kg^{-1} BW fish gain) compared to MAP (19 g kg^{-1} BW fish gain) (P=0.0001); that explains the higher total N waste output observed in fish fed MAP diet (Table 4). The Control diet resulted in the highest levels of metabolic excretions of N, which is in agreement with the lower N retention efficiency of this fish group in contrast to the fish fed supplemented diets.

Solid fraction of P discharged to the water, as faeces, was higher in those fish groups fed MCP diet (3.5 g kg^{-1} BW fish gain) than the observed with MSP and MAP (2.9 and 2.6 g kg^{-1} BW fish gain, respectively). Despite these differences, no significant differences were observed in the total amount of P discharged to the water among fish groups (values between 4.0 and 4.6 g kg^{-1} BW fish gain).

4. Discussion

Fish that received the Control diet containing 6.7 g kg^{-1} total dietary P did not show signs of P deficiency, such as depressed appetite, dark coloration and lower physical activity (Hardy et al., 1993) or anatomical symptoms of P deficiency, although the experimental period of eight weeks was too short to express clinical signs associated to P deficiency as reported Sugiura et al. (2004). However, the results suggest that the use of inorganic P sources, such as MSP, MCP and MAP, as dietary supplement in a diet for rainbow trout with an inclusion level of 4 g kg^{-1} of P plus the total P level of the Control

diet resulted in a better growth rate and feed conversion ratio.

On the other hand, the supplementation of the diet with phosphate sources increased the content of P in fish body composition, suggesting that the P content in the Control diet was insufficient to cover fish requirement for an optimal growth and maximal nutrient deposition in fish biomass as it was reported in previous studies (Åsgård and Shearer, 1997; Bureau and Cho, 1999; Rodehutscord, 1996; Skonberg et al., 1997).

Shearer (1995), stated that dietary P requirement depends on several factors, including the bioavailability of the element, feed intake, the requirement for new tissue synthesis and the amount of the endogenous loss, among others; being these factors life cycle and size dependent. In this sense, according to Rodehutscord (1996) the dietary P content for maximum growth and maximum P deposition can be estimated in 3.7 g and 5.3 g digestible P kg^{-1} diet, respectively. However, commercial feeds frequently contain from 10 to 15 total P g kg^{-1} diet with variable digestible P content, depending on the quality of the ingredients and P sources used in aquafeeds.

In the present study, the ADC of the three inorganic P sources was evaluated using diets formulated according to the general total P content that is present in commercial trout diets (10 g kg^{-1} of total P). Taking into account the P ADC coefficients observed for the assayed diets, the estimated level of ingested P was 2.9 g digestible P kg^{-1} in Control diet, suggesting that this control diet did not cover the digestible P requirement of the fish. Nevertheless, MSP, MCP and MAP diets showed 6.0, 5.1 and 6.2 g digestible P kg^{-1}, respectively, hence covering the fish requirement for maximum growth and even maximum P deposition, according to Rodehutscord (1996).

The supplementation of the diet with MSP and MAP significantly increased the apparent digestibility of the total dietary P, while MCP supplementation slightly increased the

digestibility of the nutrient when compared to the Control diet. It is recognised that P digestibility is dependent on its dietary inclusion level (Rodehutscord et al., 2000a,b; Satoh et al., 1997). Rainbow trout respond to supplements of P in a curvilinear manner, fractional P absorption approaching an upper asymptote at an inclusion level which is close to the 7 gkg^{-1} and decreasing thereafter (Sugiura et al. 2000a, 2003). In this sense, Rodehutscord (2000a) suggested the efficiency of utilization and bioavailability of P in rainbow trout is optimal when the dietary P content is slightly lower than the requirement of the fish for maximal P deposition, dropping at higher values. In this work, ADC of P was higher in diets with a higher content of P, but only because of the more soluble nature of supplemental P sources.

According to the finding observed in the present study, the bioavailability of P from MSP and MAP is higher than that observed for MCP under the digestive conditions of the rainbow trout. Both MSP and MAP resulted in high levels of P apparent digestibility (≈90%) compared to MCP (≈70%). These results agree with those obtained by Kals et al. (2012) who reported a lower apparent digestibility of the inorganic P source MCP (≈79%) compared to MAP (≈90%). In this sense, the solubility of a phosphate salt is an important criterion to select the inorganic P salt used as a supplement in aquafeeds as only dissolved phosphate is available for intestinal absorption.

The digestibility of inorganic P sources is affected by their solubility. Lall (1991) suggested that MCP is more digestible than DCP (dicalcium phosphate) because of its higher solubility. In general, monobasic phosphates of monovalent cations are the most digestible and also the most soluble phosphates, closely followed by the MCP and, far away, by tricalcium phosphates or bone apatite. This is generally true for marine and freshwater fish with a stomach such as yellowtail, catfish and rainbow trout (Eya and

Lowell, 1997; Sarker et al., 2009; Satoh et al., 1997). Nevertheless, at least one study shows that dicalcium phosphate ($CaHPO_4$) seems to be more digestible than MCP in sea bass (*Dicentrarchus labrax*) juveniles fed a diet with a P inclusion under the requirement (Pimentel–Rodrigues and Oliva–Teles, 2007). Taking into account that MCP is less soluble than MSP (Dorozhkin and Eppel, 2002), the result found by Pimentel–Rodrigues and Oliva–Teles (2007) probably points to the poorly understood interactions of dietary phosphates within the complex environment of the gastrointestinal tract. In an interesting work conducted by Hua and Bureau (2006), the authors developed a model to predict digestible P content in rainbow trout, in which the apparent digestibility coefficients expected for the inorganic P fraction such as Ca monobasic/Na/K phosphate supplements were estimated to be 89%. However, different P–compounds may affect the dynamics of P digestion and absorption in the gastrointestinal tract and, in the end, P digestibility. The results obtained in the present work suggest that MCP had a lower *in vitro* solubility (65%), both at pH 3.0 and pH 7.0, compared to MSP and MAP (≈75%), resulting in a lower apparent digestibility of the MCP salt compared to MSP and MAP, and in a more efficient P absorption and utilization by fish.

As mentioned, chemical interactions within the gastrointestinal tract are complex and the digestibility of phosphate supplements cannot be predicted simply from solubility data. For example, it is known that calcium can bind phosphate during fish digestion (NRC, 1993). This fact can be observed as negative interactive terms in the digestibility models developed by Hua and Bureau (2006, 2010). This interaction can be explained by the sudden change the gastric chyme suffers after being evacuated to the pyloric caeca and intestine. Calcium released from the diet components can interact with phosphates ions to form insoluble calcium phosphates in the intestine. Moreover, two additional factors should

be taken into account to understand P absorption: i) the availability of Na^+, necessary for Na/Pi co–transporters (Avila et al., 2000) and ii) the postprandial intestinal secretion of bicarbonate (Taylor et al., 2006) which removes Ca^{2+} by precipitation of $CaCO_3$ and increase phosphate solubility (Fernández et al., 1999). In reverse, phosphate supplementation can affect the digestibility of macro– (K, Mg) and micro–minerals (Cu, Zn) in trout (Prabhu et al., 2014). This net of ionic interactions affecting P absorption deserves further, *in vitro* and *in vivo*, research.

The results found in the present study suggest that the higher apparent digestibility of MSP and MAP compared to MCP, increased the level of soluble P into the lumen, that resulted in a more efficient absorption and retention of the nutrient in fish body. Despite the body P–content was similar for the three inorganic P sources and minimal in fish fed the diet without P supplementation, the P retention efficiency was significantly higher for diets with MSP (37%) and MAP (36%) in comparison with the diet containing MCP (32%). This last value was similar to the P retention efficiency (33%) reported by Hernández et al. (2005), where rainbow trout of a similar body weight was fed a low fish meal, MCP–supplemented diet.

The mass balance of nutrients applied to estimate the discharge of nutrients to the water indicated differences among supplement sources. Despite total P discharge to the water was similar for fish fed MSP, MCP and MAP supplemented diets, those fish fed MCP supplemented diet showed a higher solid discharge of undigested P through faeces (3.5 g kg^{-1} BW gain) in comparison with the values observed in fish fed MSP and MAP supplements (2.9 and 2.6 g kg^{-1} BW gain, respectively). These results are in agreement with those of apparent digestibility coefficients for the inorganic P sources, where MCP had 70% digestible P and MSP and MAP around 90%.

Total P discharged to the water by juveniles of trout in the present study agrees with the results obtained with trout diets reported by different authors (Cho and Bureau, 2001; Green et al., 2002b; Hernández et al., 2005; Satoh et al., 2003), where the expected amount of total P waste output is estimated over 4 g kg^{-1} BW fish gain. In this last study, the authors suggested a total discharge of 4.7, 5.7 and 7.8 g P kg^{-1} BW gain when juvenile rainbow trout were fed a low fish meal diet supplemented with 0, 5 and 10 g kg^{-1} of MCP, respectively. More recently, Morales et al. (2016a) reported a total P discharge of 4 g kg^{-1} BW gain in 120–350 g rainbow trout fed a plant–based diet with a content of total dietary P of 7.3 g kg^{-1}. However, the authors observed a 50% reduction in the P discharge to the water when phytase was used as a dietary additive. Different studies suggested that the addition of phytase in plant–based diets for fish improves the utilisation of the endogenous P present as phytate in the plant–ingredients fraction of the diet by 20–60% and decreases the total–P discharge to the environment by 30–50% in rainbow trout (Morales et al., 2016b). Many other reports have demonstrated that phytase supplementation improves the availability of dietary P in several fish species (Lemos and Tacon, 2017). In this sense, the combined use of phytase with a high digestible inorganic P source, such as MSP and MAP to cover the available dietary P requirement of a given fish species could be an accurate choice to optimize the dietary P retention efficiency in fish and hence reducing the discharge of P to the environment.

In the present study, fish fed MSP and MCP did not show differences for soluble or solid N discharged fractions. Nevertheless, fish fed the diet supplemented with MAP released a higher amount of N (around 4 g kg^{-1} BW fish gain) in its dissolved form to the water in comparison with MSP and MCP (Table 4). Although the mass balance model used to determine the fractions of nutrients discharged to the water cannot discriminate between the

metabolic N fraction excreted via urine/gills and the soluble, but unabsorbed, ammonium ion from MAP that pass through the digestive tract, the results of this study suggest that MAP could produce more N wastes than the other inorganic P sources evaluated (Figure 1).

To our knowledge, there are no records about the higher N waste output of an inorganic P dietary supplement for fish containing non–digestible N such as MAP with respect to other phosphate sources. In this sense, a higher discharge of ammonium ions directly through the faeces when MAP is used as dietary supplement, could lead to an overload of non–desirable reduced–N dissolved fraction in recirculation aquaculture systems or other high–intensity systems. On the other hand, taking into account that the aquaculture sector is facing increasing pressure concerning the discharges of nutrients into the surrounding ecosystems, and that both N and P are considered limiting nutrients for phytoplankton growth in marine environments (Bristow et al., 2017), the optimization of the dietary P utilization by fish is crucial. In the same way, the use of diets with an optimum level of dietary digestible N will become another key for sustainable open–water aquaculture, such as salmon and trout marine cages established in fjords as well as in more exposed offshore marine areas.

5. Conclusion

The findings obtained in the present work suggest that the three inorganic P sources evaluated are useful to cover the requirement of P in rainbow trout and to assure an optimal growth, feed conversion ratio and nutrient retention efficiency in fish tissues. However, it was demonstrated that MSP and MAP release more bioavailable P for the fish. On the other hand, equivalent to the expected response of MSP, the use of MAP as dietary inorganic P

supplement results in a high P retention efficiency by fish, although associated with a higher discharge of N, as undigested ammonium, directly through the faeces to the environment. The results obtained in this work suggest that depending on the inorganic P source used as dietary supplement, a different amount of N waste output could be expected from farmed fish. This point could be taken into account by feed manufacturing companies in the estimation of the environmental declaration of N and P discharge to the water by fish fed with a given diet.

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Tables

Table 1. Diet formulation and analyzed chemical composition (based on dry matter).

Diets	Control	MSP	MCP	MAP
Ingredients, g kg^{-1}				
Corn gluten meal (60% CP)[1]	300	300	300	300
Soybean expeller (44% CP)[2]	250	250	250	250
Fish meal (68% CP)[3]	80	80	80	80
Poultry by–product meal (60% CP)[4]	60	60	60	60
Fish oil[5]	100	100	100	100
Sunflower oil[6]	50	50	50	50
Gelatine[7]	50	50	50	50
Pre–gelatinized wheat starch[8]	87.1	70.4	76.4	71.7
$CaCO_3$[9]	6.9	6.9	0.0	6.9
Vit/min premix[1]	5	5	5	5
L−Lysine HCl[1]	8	8	8	8
Chromium sesquioxide[10]	3	3	3	3
MSP[11]	0	16.7	0	0
MCP[11]	0	0	17.6	0
MAP[11]	0	0	0	15.4
Analyzed content, Kg^{-1}				
Dry matter, g	913.9	900.0	904.0	897.5
In dry matter				
Crude protein, g	424.3	440.7	434.0	436.5
Crude fat, g	209.9	206.8	215.8	216.8
Phosphorus	6.7	10.5	10.6	10.3
Calcium	6.7	6.4	6.3	6.1
Gross energy, MJ	24.7	24.3	24.3	24.7

1 Brascorp, Argentina; 2 Aceitina, Argentina; 3 Apolo Fish, Argentina; 4 Conidia, Argentina, 5 Mundo Branco, Argentina; 6 Molino Cañuelas, Argentina; 7 Xantana, Argentina; 8 Semino, Argentina; 9 Anedra, Argentina; 10 Sigma-Aldrich, USA; 11 Yara Animal Nutrition, Sweden.

Table 2. Growth, feed intake, feed conversion ratio of rainbow trout; and apparent digestibility of dry matter, protein, total phosphorus in feed (P feed), P in feed phosphates (P source) and calcium (Ca) in rainbow trout fed the experimental diets. Results expressed as the mean ± SD; n = 3 tanks diet^{-1}.

Diets	Control	MSP	MCP	MAP	*P*–value
Growth, feed intake and feed conversion					
Initial weight (g fish^{-1})	125.6 ±1.91	128.9 ± 2.68	127.3 ± 0.32	126.4 ± 1.39	0.214
Final weight (g fish^{-1})	237.5 ± 3.28 [b]	259.2 ± 3.73 [a]	252.0 ± 3.97 [a]	256.3 ± 4.32 [a]	0.0005*
TGC x 1000 [1]	1.73 ± 0.03 [b]	1.93 ± 0.08 [a]	1.88 ± 0.05 [a]	1.95 ± 0.07 [a]	0.0095*
Feed intake (g fish^{-1}) [2]	104.0 ± 1.8	105.9 ± 2.2	104.2 ± 3.3	103.8 ± 4.8	0.836
Feed conversion ratio [3]	0.93 ± 0.03 [a]	0.81 ± 0.02 [b]	0.84 ± 0.03 [b]	0.80 ± 0.03 [b]	0.0027*
Apparent digestibility (%)					
Dry matter	70.6 ± 0.9	68.5 ± 1.4	70.0 ± 1.2	68.5 ± 0.7	0.08
Protein	83.7 ± 1.2	81.3 ± 0.3	83.5 ± 1.4	82.6 ± 0.8	0.14
P feed	43.5 ± 3.2 [b]	57.2 ± 4.2 [a]	48.1 ± 2.2 [b]	59.8 ± 3.5 [a]	0.0013*
P source		91.1 ± 7.1 [a]	70.4 ± 4.6 [b]	92.9 ± 6.0 [a]	0.0066 *
Ca	47.3 ± 1.1	44.0 ± 3.8	39.2 ± 3.6	43.7 ± 5.1	0.07

[1] Thermal growth coefficient (TGC); [2] As dry matter bases; [3] Feed intake (as dry matter basis) / body weight gain; Different letters indicate significant differences (P<0.05) when the treatments were applied to Student's t multiple comparison test.

Table 3. Content of dry matter (DM), nitrogen (N), phosphorus (P) and calcium (Ca) in whole body rainbow trout (Kg^{-1}) at the start and after being fed experimental diets; and N, P and Ca retention efficiencies (% of nutrient intake) in fish fed the experimental diets. Results expressed as the mean ± SD; n = 3 tanks $diet^{-1}$.

Diets	Initial levels	Control	MSP	MCP	MAP	*P*–value
Whole body fish, kg^{-1}						
DM, g	244.2	315 ± 8	323 ± 3	324 ± 13	322 ± 16	0.775
N, g	23.9	25.2 ± 0.8	27.1 ± 0.8	27.4 ± 1.5	25.6 ± 0.6	0.064
P, g	2.40	1.79 ± 0.06 ^{b}	2.40 ± 0.03 ^{a}	2.23 ± 0.08 ^{a}	2.35 ± 0.25 ^{a}	0.0023*
Ca, g	1.64	2.74 ± 0.46	2.63 ± 0.39	2.10 ± 0.18	2.77 ± 0.45	0.199
Ca:P ratio	0.68	1.53 ± 0.24 ^{a}	1.10 ± 0.16 ^{b}	0.94 ± 0.09 ^{b}	1.20 ± 0.29 ^{b}	0.0463*
Retention efficiency (%)						
N		42.5 ± 2.4 ^{b}	54.4 ± 2.3 ^{a}	54.2 ± 6.0 ^{a}	49.7 ± 3.5 ^{a}	0.016*
P		22.0 ± 3.6 ^{b}	37.0 ± 1.1 ^{a}	31.6 ± 3.3 ^{ab}	36.2 ± 7.6 ^{a}	0.013*
Ca		64.7 ± 16.1	71.0 ± 15.7	49.5 ± 8.7	80.2 ± 20.4	0.193

Different letters indicate significant differences (P<0.05) when the treatments were applied to Student's t multiple comparison test.

Table 4. Solid and dissolved nitrogen (N) and phosphorus (P) waste loading (g kg^{-1} BW fish gain) of rainbow trout fed the experimental diets (mean ± SD, n = 3 tanks diet−1). Values in parentheses indicate the percentage of solid and dissolved fractions respect to the total waste loading.

Diets	Control	MSP	MCP	MAP	*P*–value
Total solids	273 ± 3.0	257 ± 16	251 ± 19	252 ± 11	0.256
Solid N	10.3 ± 0.4 (28.4)	10.7 ± 0.2 (41.1)	9.6 ± 1.1 (38.7)	9.7 ± 0.7 (33.4)	0.226
Dissolved N	26.0 ± 1.9 [a] (71.6)	15.4 ± 1.3 [c] (58.9)	15.1 ± 0.9 [c] (61.3)	19.3 ± 1.1 [b] (66.6)	0.0001*
Total N	36.3 ± 1.6 [a] (100)	26.2 ± 1.4 [c] (100)	24.7 ± 1.6 [c] (100)	29.0 ± 1.7 [b] (100)	0.0001*
Solid P	2.89 ± 0.19 [b] (72.4)	2.88 ± 0.30 [b] (67.9)	3.48 ± 0.28 [a] (76.0)	2.59 ± 0.20 [b] (63.3)	0.014*
Dissolved P	1.11 ± 0.21 (27.6)	1.35 ± 0.20 (32.1)	1.11 ± 0.26 (24.0)	1.53 ± 0.40 (36.7)	0.260
Total P	4.00 ± 0.31 (100)	4.23 ± 0.13 (100)	4.58 ± 0.38 (100)	4.12 ± 0.55 (100)	0.334

Different letters indicate significant differences (P<0.05) when the treatments were applied to Student's t multiple comparison test.

Figure captions

Figure 1. Nutrient mass balance for N and P expressed as percentage of the amount of dietary nutrient provided to fish.

Highlights

1. MSP and MAP showed a higher *in vitro* P solubility and higher *in vivo* P apparent digestibility compared to MCP.
2. The retention efficiency of P in fish tissues was higher when MSP and MAP were used as P supplement, whereas P retention efficiency from MCP was lower than the other sources.
3. The use of MAP as dietary inorganic P supplement resulted in a higher discharge of N to the water.

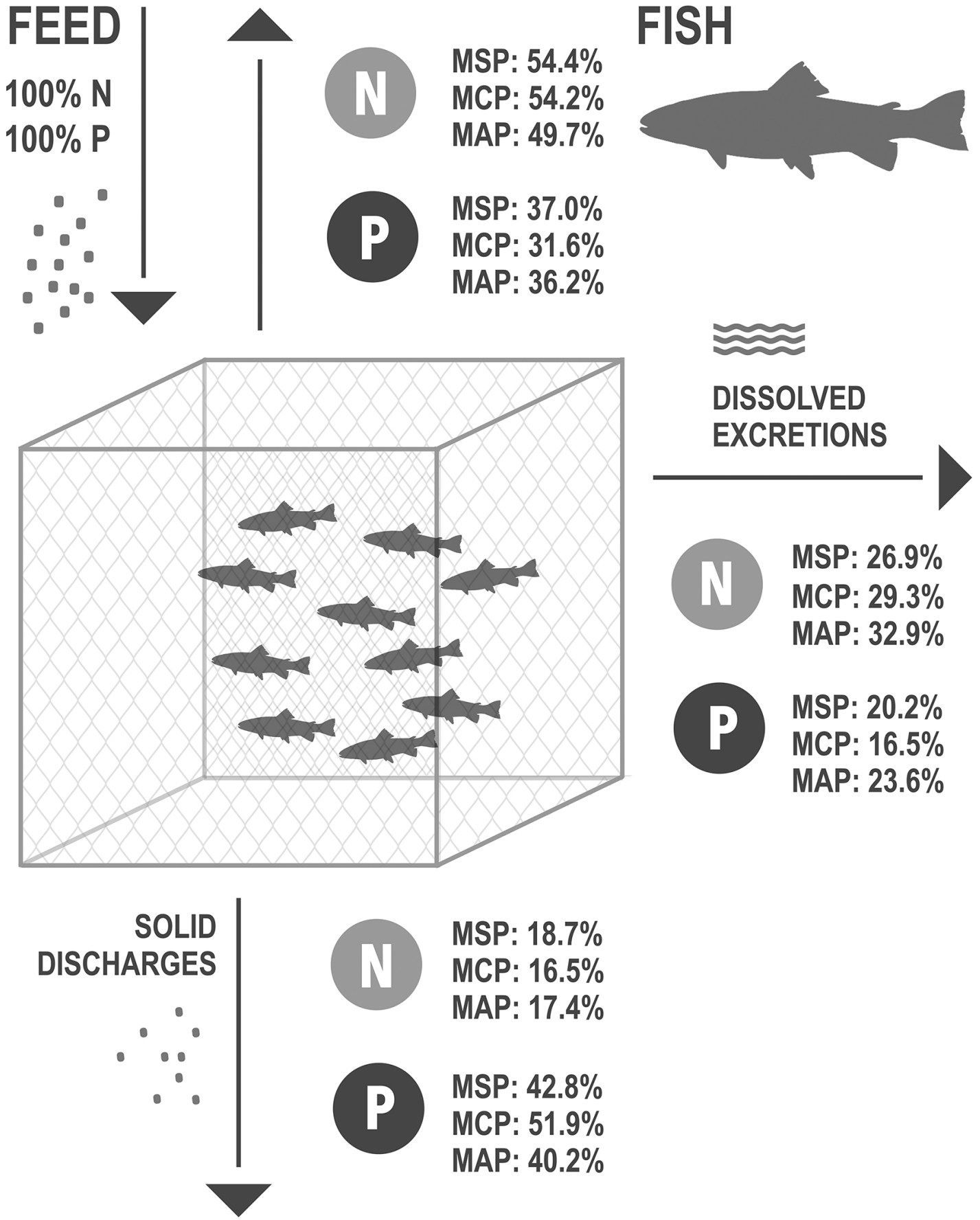
FEED
100% N
100% P
FISH
N
MSP: 54.4%
MCP: 54.2%
MAP: 49.7%
P
MSP: 37.0%
MCP: 31.6%
MAP: 36.2%
DISSOLVED
EXCRETIONS
N
MSP: 26.9%
MCP: 29.3%
MAP: 32.9%
P
MSP: 20.2%
MCP: 16.5%
MAP: 23.6%
SOLID
DISCHARGES
N
MSP: 18.7%
MCP: 16.5%
MAP: 17.4%
P
MSP: 42.8%
MCP: 51.9%
MAP: 40.2%

Figure 1