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***In situ* chelation of phosphorus using microencapsulated aluminum and iron sulfate to bind intestinal phosphorus in rainbow trout (*Oncorhynchus mykiss*)**

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Running title: Intestinal binding of soluble phosphorus

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Highlights

- Incorporation of encapsulated P-chelating agents into fish reduces phosphorous release by feces.
- Using encapsulated Al and Fe does not induce a decrease in growth performance and does not alter the retention of phosphorus in fish.
- This encapsulation process limits the action of chelating agents (Al, Fe) in the stomach and proximal intestinal regions.

Abstract

Excess phosphorus (P) in freshwater ecosystems increases primary production which, left uncontrolled, may lead to eutrophication, accelerating the ageing process of receiving water bodies. To limit phosphorus release resulting from freshwater aquaculture, we propose to incorporate microencapsulated P-chelating agents into fish diets. In a first trial, alum (Al_2SO_4) and ferrous sulfate (FeSO_4) were encapsulated by spray-chilling in a hydrogenated lipid matrix. Two practical diets incorporating one of these two chelating elements (6 g/kg) were fed to fish for five weeks (w), and P release from resulting feces was compared. In a second trial, a similar approach was used to evaluate the impact of increasing supplementation of encapsulated alum (3, 6, 15 g/kg of diet). Feces from the fish fed with the diets incorporating alum and ferrous sulfate released 62% and 54% respectively less P than feces from fish fed with control diets. The second experiment revealed a negative correlation between the level of encapsulated Al_2SO_4 included in the diet and phosphorus released by the feces ($y = 0.18x^2 - 4.78x + 62.7$; $R^2 = 0.93$). Feces from feed incorporating Al_2SO_4 at 0, 3, 6 and 15 g/kg released 62%, 52%, 39%, and 32% of the total fecal P after 14 days respectively. Fish fed encapsulated Al_2SO_4 have similar growth performance and mineral status. Incorporation of encapsulated P-chelating agents into fish feed offers an opportunity to manage P release from fish feces. Long-term feeding studies are required for validation of dietary Al_2SO_4 and FeSO_4 impacts on potential toxicity and growth/environmental performance following chronic feeding of encapsulated P chelating agents.

Keywords: Fish farming - pollution - phosphorus solubilization – encapsulating - ferrous sulfate - Alum.

Abbreviations

AIA, Acid Insoluble Ash; AOAC, Association of Official Analytical Chemists; APHA, American Public Health Association; ADC, Apparent Digestibility Coefficient, Ctrl, Control; Ctrl+, Positive control positive; FCR, Feed Conversion Ratio; FI, Feed intake; HSI, Hepatosomatic Index; GRIPHA, Groupe de Recherche Intégrée en Physiologie et Sciences Animales; LARSA, Laboratoire de Recherche des Sciences Aquatiques; NRC, National Research Council; o- PO_4 , Inorganic phosphorus; OP, Organic phosphorus; P, Phosphorus; PCBF, Programme Canadien des Bourses de la Francophonie; RAQ, Ressources Aquatiques Québec; SGR, Standard Growth Rate; TGC, Thermal-unit Growth Coefficient; TP, Total Phosphorus

Introduction

Eutrophication is a slow, natural process by which water bodies receiving excess nutrients, notably phosphorus (P) and nitrogen (N) leads to the growth of algae and aquatic plants (Elser et al. 2007). In freshwater systems, it is typically the enrichment of P that accelerates this process (Correll 1998; Elser et al., 2007). In streams and lakes, P is found in dissolved and particulate forms; in dissolved form, soluble reactive phosphorus (SRP) is the amount of phosphorus directly available for plants. This phosphorus fraction consists mainly of the inorganic orthophosphates (o-PO_4 , H_3PO_4 , H_2PO_4^- and HPO_4^{2-}) (Maruo et al., 2016). Particulate forms (organic or mineral) were in permanent exchange with dissolved forms under the action of microorganisms and adsorption/desorption mechanisms. The SRP from these above processes in the oligotrophic zone diffuses into the eutrophic zone (Khan and Ansari, 2005).

Fish farming activities constitute point sources of organic-P discharge to the environment and measures to limit its release are necessary to protect receiving water bodies. Two strategies have been implemented to control P emissions: first; limiting dietary P level by reducing nonavailable P source from raw ingredients to improve the digestible P fraction in diet and second, treating effluents by mechanic filtration followed or not by a P-removal treatment (Koko, 2007). For this former strategy, organic matter filtration and removal treatment of dissolved orthophosphates are generally used.

Effluent P-removal methods include chemical precipitation; crystallization enhanced chemical precipitation (e.g., using steel slag, Claveau-Mallet et al., 2015) and ion exchange (Morse et al., 1998). Chemical precipitation by the addition of hydrated lime to the supernatant from fish sludge storage tanks is the most widely-used method. This approach was demonstrated to be very efficient (90% reduction of SRP) but leads to an increase in effluent pH (≥ 10), which needs to be diluted into the main effluent stream. Effluent treatment techniques often take place several (≤ 6) months after feces egestion, resulting in potentially significant P (80% of total fecal P) release into the water column, making subsequent o-PO_4 to remove higher (Dosdat et al., 1992).

In this study, we hypothesized that incorporating chelating agents directly into the fish diet would reduce o-PO_4 solubilization by feces in settled ponds. The compounds used to chelate the unabsorbed P were aluminum sulfate (alum) and ferrous sulfate. These metal salts have been used since the 1950s in municipal wastewater treatment. Studies in poultry have demonstrated the efficiency of alum and ferrous sulfate to render P insoluble in broiler litter (Shreve et al., 1995; Moore et al., 1999; Codling et al., 2000; Sims and Luka-McCafferty, 2002).

Phosphorus is an essential element for fish and is uniquely obtained from ingested food. In rainbow trout (*Oncorhynchus mykiss*), available phosphorus is rapidly absorbed in the pyloric region of the proximal intestine (Avila et al., 2000; Vandenberg, 2001; Sugiura et al., 2003) with fractions of unabsorbed and unavailable P transiting to the distal intestine. To ensure adequate P absorption in the proximal intestine, chelating compounds were encapsulated in a hydrogenated lipid matrix. This approach was based on the ability of

anencapsulation process to limit the action of chelating agents in the stomach and proximal intestinal regions by avoiding early release of chelating agents. In distal intestinal regions, the action of pancreatic lipases liberates the chelating compounds, allowing complexation prior to egestion into the tank water. This study aimed to determine the efficiency of the addition of microencapsulated chelating agents in the fish diet to reduce P release from settled and undisturbed feces at 7 and 22 °C. Secondly we study the dose-reponse of incorporating alum on P release and P-status of fish.

Materials and methods

Experimental Diets

Four diets were formulated based on the *National Research Council* recommendations for rainbow trout (NRC, 2011). The four diets included the following: diet Ctrl-, the control diet without a chelating compound; diet Ctrl+, the positive control diet containing the lipid encapsulation matrix without a chelating compound; diet Al-containing the chelating compound Al_2SO_4 in the lipid matrix; diet Fe containing the chelating compound FeSO_4 in the lipid matrix. The two chelating compounds were encapsulated whereby a molten lipid matrix (using a proprietary processing technique) by spray chilling, thus entrapping the product of interest. The information about the lipid matrix (Jefo matrix) and the production process is internal to Jefo Nutrition Inc, (5020 Avenue Jefo, Saint-Hyacinthe, Québec, Canada, <https://jefo.ca/fr/innovation-developpement/technologie-jefo-matrix/>). The chelating compounds used to chelate the unabsorbed P in feces produced fine, free-flowing microbeads (between 500 -1000 μm) which were added in the feed mixture at a level of 20 g/kg before pelleting for the first feeding study (**Table 1**). The chelating compounds, Al_2SO_4 and FeSO_4 were encapsulated in a commercial-scale facility (Jefo Inc., St Hyacinthe, Québec, Canada), based on preliminary work to validate the optimal matrix composition to ensure chelating compound release in the hindgut.

For the second feeding study, encapsulated Al_2SO_4 was supplemented at four (0, 10, 20 et 50 g/kg of Al_2SO_4 -chelating compound which gives 0, 3, 6 and 15 g/kg of Al_2SO_4 in diet, respectively) dietary concentrations (**Table 2**). The nutrient digestibility of the two groups of 4 diets formulated in two experiments is indicated in **table 3**.

An indigestible marker (Sipernat 50™ as a source of insoluble acid ash (AIA)) was added to each diet at 10 g/kg to evaluate the apparent digestibility coefficient (ADC). Guar gum (3 g/kg) was added to the diets to improve feces stability. The ingredients were thoroughly mixed, and steam pelleted using a California Pellet Mill (detail in the footnote of **Table 1**). Fish oil was added to the feed in two stages; 25 g/kg of diet was first added to the mixture and the remaining (90 g/kg of diet) quantity by coating after the pellets were produced and dried (45 °C, 8 h). Then pellets (4.0 mm dia.) were dried in a forced-air oven (45 °C, 24 h), sieved and stored at -20 °C until feeding.

Fish rearing, feeding and experimental design

The feeding trials were conducted for 5 weeks in a freshwater recirculating aquaculture system (98% recirculation) at the *LABoratoire de Recherche en Sciences Aquatiques* (LARSA - Université Laval). Suspended solids were removed using a sand filter, and ammonia was converted to nitrate using a trickling biofilter. Ammonia and nitrite concentrations were monitored twice weekly to assess biofilter performance. Fish were

held at 12°C. Dissolved oxygen varied between 9.4 -10.7 mg/L and the photoperiod were adjusted to 16 h light - 8 h dark. For each experiment, all-female (n = 264) triploid rainbow trout (Exp.1: 182 ± 7 g; Exp.2: 110 ± 5 g; mean ± SEM) were transferred from a local fish farm (*Pisciculture des Monts de Bellechasse Inc., Saint-Damien-de-Buckland, Canada*) to the LARSA facilities. Fish were randomly distributed among 12 gray semi-square tanks (150 L volume; density of 24 kg/m³ at the start of the study) in a complete randomized design with four diets and three replicate tanks per diet.

Fish were acclimated during the first week and fed with the control reference diet. Fish were fed to satiation by hand on two consecutive days per week followed by restricted feeding by belt feeders on the subsequent five days of the week. Restricted feeding was defined as 80% of the average daily feed intake (FI) when fed to satiation. During satiation feeding, fish were fed by hand at 08.00 and 15.00 until no further feeding activity was observed. The experiments complied with the guidelines of the *Canadian Council on Animal Care* (Olfert et al., 1993) and approved by the *Comité de Protection des Animaux de l' Université Laval* (CPAUL 2010).

Growth measurements

Fish were weighed just before each experiment, after two weeks (w) and at the end of the experiment (5 weeks). Growth performance was evaluated based on fish tank biomass (tank biomass/number of fish = Initial Body Weight or Final Body Weight; concise respectively IBW or FBW) gain and FI using the total amount of feed given to each tank in this period divided by the number of fish. Average feed conversion ratio (FCR) standard growth rate (SGR) thermal-unit growth coefficient (TGC) and hepatosomatic index (HSI) were calculated as follows:

$$\text{Weight gain} = [(FBW - IBW) / IBW] \times 100 \%$$

$$FCR = [FI / (FBW - IBW)]$$

$$SGR = 100 \times (\ln FBW - \ln IBW) / \text{days}$$

$$TGC = 100 \times (FBW^{1/3} - IBW^{1/3}) / \text{sum of daily water temperature}$$

$$HSI = (\text{liver weight/body weight}) \times 100 \%$$

Fecal collection

Feces were collected using a modified Guelph system based on Cho et al., (1982) placed under the fish tanks. Following the last feeding of the previous day, the tanks and collection systems were thoroughly cleaned and purged immediately of any uneaten feed and feces. Feces were collected overnight; before the morning feeding, feces were decanted, excess water removed and stored at -20 °C. Feces were freeze-dried for 7 d before analysis to determine ADC. Feces used for the phosphorus release experiment were collected the same day for all tanks. For each treatment, the feces collected in the three tanks were pooled and used immediately for the experiment.

Scale and carcass collection

At the beginning and end of the experiments, six fish per tank (3 for scale and 3 for carcass collection) were sacrificed using MS-222 (150 mg/L, Syndel International Inc., Vancouver, BC, Canada), measured (fork length) and weighed. Scales were scraped from tail to head and stored in 70% ethanol solution until the ash and P determination. For carcass processing, fish were stored at -20 °C pending the determination of mineral content. Ash

content in scales and carcass was determined using the method described in Le Luyer et al. (2014). Scales were dehydrated in a graded series of ethanol (70, 90, 100 %; 24 h /bath), delipidated in acetone (two baths of 24 h), then in trichloroethylene (two baths of 24 h). Carcasses were autoclaved, homogenized then freeze-dried. Scales and carcasses were used to estimate fish bone mineral and ash whole-body content. These parameters indicate the bone mineral status of fish (P-sufficient or P-deficient).

Analytical Methods

Scales, carcasses, feces, and diet were analyzed for dry matter (drying in a vacuum oven for 18 h at 105 °C) and ashed (incinerating in a muffle furnace for 18 h at 550 °C) to the nearest 0.1 mg according to AOAC 927.05 and 930.30 methods guidelines (AOAC 1990). Phosphorus content was determined by ion chromatography (ICS-3000, Dionex Corporation, Sunnyvale, CA, USA) following ash digestion in nitric acid (18 ml of HCl 50% + 3 ml nitric acid) solution and filtering (Whatman paper #1, rinsed three times in 100 mL volumetric flask) (Naumann and Bassler, 1976). The mass of acid-insoluble ash represented mainly the mass of Sipernat 50™ (Atkinson et al., 1984).

For diet and feces, crude protein (% N×6.25) was quantified using the semi-automatic Kjeldahl method (Foss Electric, Denmark; AOAC method 7, B01-7, B04), lipid content using ethyl ether extraction without acid hydrolysis (Soxtec System HT12, Foss Tecator AB; Hoganas, Sweden), and crude energy using content was an adiabatic bomb calorimeter (Parr Instrument Co., Moline, IL, USA). The ADC for dry matter, protein, energy, lipids, and P were calculated using the following formula (Gui et al., 2010):

$$ADC = 1 - (N_{feces}/S_{feces}) \times (S_{diet}/N_{diet})$$

where S and N were the Sipernat 50 and nutrient content (dry matter, protein, lipids, energy, ash, and P) in the diet or feces, respectively.

Fecal phosphorus release

Feces use for P release trials were collected on two consecutive days, 3 weeks following feeding initiation. These feces samples were pooled according to the treatment, transferred to 50 mL conical tube (Falcon, Becton Dickinson) and centrifuged (5 min at 1300 xg) to dewater feces to a similar degree. The supernatant was removed, and the sedimented pellet was used for the P release experiments. The fecal samples used for the digestibility study were collected during the entire experiment except for these two days. These fecal samples were frozen (-20 °C) until used for analytical analysis as previously described.

Approximately 5 g of feces from each diet were placed in the bottom of a beaker (500 ml) containing 300 ml of deionized water (Hasnaoui et al., 2001). Each treatment (feces from one of the four diets) was repeated three times. Two conditions were tested: low temperature (7 °C) and room temperature (22 °C) in the absence of light. On days 1, 2, 4 and 7, duplicate water samples (5 mL) were aspirated from each beaker using a 15 mL syringe, fitted with a 0.45 µm EMD Millipore Millex filter, to measure the released inorganic P, which was determined using the molybdate vanadate *American Public Health Association* method (APHA, 1992).

Statistical analysis

Data were expressed as mean $\pm$ standard error mean (SEM) or standard deviations (SD) with tank or beaker as the experimental unit. Normality and homogeneity of variance were tested using Shapiro Wilk, and Bartlett tests and data were log-transformed when needed. When data respected the assumptions of normality ANOVA, or ANCOVA was performed. For growth performance indicators and P-status despite the initial body weight (IBW) was significantly ($P = 0.033$ and 0.007) different between diet, ANCOVA was used to compare the effect of diet on the growth indicators (FBW, FI, WG, TGC, HIS, ash, and P content). When analysis showed a significant difference, the Tukey test was performed to compare the treatments. For the o-PO₄ release, two way (feces and time) analysis of variance was performed. When significant interaction between these factors was found, the Tuckey test was used to compare treatment each time (Zar, 1999). All statistical analyses were performed using R version 3.2.3. The level of significance used in all tests was $P < 0.05$ except the scale's ash ($P < 0.01$) in experiment 2. Regression analysis was performed using the regression function of the software Microsoft Excel (Microsoft, Seattle, WA, USA).

Results

Effect of feeding encapsulated alum and iron sulfate

At day 0, individual average body mass for fish fed with Fe was lower than fed with Ctrl+. However, the initial individual body mass of these two groups does not individually differ with the other two groups (Ctrl and Al). These differences were found to impact FI, mass gain, and TGC during the first two weeks. At the end of the experiment these effects disappeared. Indeed, no significant effect of diet were found on FCR, TGC and HSI, but remain for FI and FBW (**Table 4**) .

The ash content in fish scales (30.6 ± 2.2 %) was similar at the beginning and end of the experiment. However, the ash content of carcasses was significantly higher at the beginning (9.4 ± 1.0 %) than the end (7.8 ± 0.4 %) of the experiment. At the end of the experiment, carcass ash was similar regardless of the dietary treatment. These same variations were found in carcass P (**Table 6**).

The growth performance the differences at the end of the experiment were not statistically significant but we note that the fish fed with a diet incorporating Al have numerically lower weight gains than the other treatments (40.7 vs. 51.9, 54.4, 54.1; see **Table 4**). Therefore, FCR was numerically (1.65 vs. 1.40, 1.46 and 1.19) higher.. These results were correlated with a lower scale P ($P = 0.026$) for a fish fed diet containing Al (3.0 vs. 3.9, 3.2 and 4.4). These results (weight gain, FCF, scale P) suggested lower availability of P dietary due to the presence of Al in this diet. These results weren't confirmed in experiment 2 where higher levels of Al were incorporated in the diet (**Figure 1**). Scale P, FCR, TGC, FI, FBW were not significantly different at the end of experiment 2 (**Table 5**).

P content in fecal matter used for the P-release experiment was 18 ± 0.75 g/kg (mean $\pm$ sd; dry basis). P-release was calculated using total P in feed taking in account ADC of total P (TP). The release of o-PO₄ from the feces of fish fed different diets was higher at room temperature (**Figure 2**). Indeed, the minimum and maximum values, after seven days, were 1.2 ± 0.7 and 40.7 ± 2.5 % of TP in feces at 7 °C and 0.7 ± 0.5 and 70.4 ± 2.2 % of TP in feces at 22 °C; the effect of chelating compounds was more pronounced at room temperature. After seven days, the o-PO₄ released from feces in Ctrl and Ctrl+

groups was significantly higher than released by feces from diets with chelating compounds (Fe and Al). The feces from the diet including encapsulated Al released the lowest quantity of o-PO₄.

Experiment 2: increasing Al₂SO₄ concentration

At the end of the five-week feeding study, the level of alum incorporation did not affect scale mineralization of fish fed increasing encapsulated Al (**Figure 1**). Scale mineralization differed between fish at the beginning, and the fish fed after five weeks with the diet having the highest inclusion of alum (15 g/kg)..

The release of o-PO₄ from the feces increased significantly during the incubation period (0-14 d). The level of encapsulated Al incorporated in diets influenced fecal o-PO₄ release over time. The interactions between the level of encapsulated Al and time were highly significant ($P < 0.001$). Thus, after 14 days, the amount of o-PO₄ released was highest from the feces of fish consuming the control diets (62.0 ± 6.3 % of TP in feces, $P < 0.01$).

At the end of the feces incubation period (d 14), there was a strong relationship ($R^2 = 0.81$ and $R^2 = 0.93$ for linear and polynomial models; respectively; $P < 0.001$; **Figure 3**) between fecal o-PO₄ release and encapsulated Al concentration fed to fish. At the end of the P release experiment, feces from fish fed with a diet having 15 g/kg of encapsulated alum demonstrated a significantly reduced rate of P solubilisation/release.

Discussion

Numerous developments have been made in the field of encapsulated food ingredients (Gibbs, 1999). In this study, o-PO₄ chelating compounds were dispersed within a molten hydrogenated vegetable fat matrix and lipid microcapsules produced by spray-chilling (Champagne and Fustier, 2007). Lipid-based microcapsules resulting from this process were assumed to remain mostly intact through gastric and proximal intestinal transit, with chelating compounds being released into the intestinal lumen as intestinal lipases degrade the lipid matrix.

The anatomical region where chelating compounds are released is a key component determining the effectiveness of this technique. Ideally, the chelating compounds should be released after P absorption sites, those being within the pyloric caecae and regions immediately distal thereof (Avila et al., 2000; Sugiura et al., 2003). Release occurring before these regions may lead to inadequate P absorption inducing P deficiency, with negative impacts on adequate tissue mineralization and growth performance (NRC, 2011). Preliminary work from our laboratory demonstrated that inclusion of Al and Fe directly in the diet, without encapsulation, led to a significant decrease in feed intake and growth performance in trout (Fournier, 2008). Considering this, we proposed the use of micro-encapsulation to reduce the negative impacts on feed intake and growth performance, and control the release and the action of these two compounds towards the hindgut.

The kinetics of chelating compound release from lipid microcapsules depends on the activity of the lipases in the different regions (stomach, pyloric caeca, midgut, and hindgut) of the gastrointestinal (GI) tract. Few studies have been conducted to determine the difference in lipase activity between these GI tract regions in rainbow trout. One relevant study only considered the total lipase activity (Furné et al., 2005). We previously

determined that lipase activity is significantly higher in the pyloric caeca/midgut versus the stomach and hindgut in rainbow trout of 120 and 800 g (Ndiaye, 2018 unpublished data).

The results of scale mineralization demonstrate no signs of P deficiency in fish fed diets with encapsulated chelating compounds. This supports the underlying assumption that the lipid microspheres were degraded distal to the sites of P absorption. Deschamps et al. (2014) and Le Luyer et al. (2014) revealed a rapid decrease (within 2 weeks) of scale mineralization in rainbow trout fed a P-deficient diet. The levels of ash in scales of fish used in the two feeding experiments (30.6-32.0%) were similar to the values found in trout fed with sufficient dietary phosphorus (Le Luyer et al., 2014). In the current studies, neither scale nor carcass mineralization was altered following feeding encapsulated P-chelating compounds.

The level of dietary total P was higher compared to commercial rainbow trout diets (11.2-13.7 vs. 10 g/kg). Apparent P digestibility was relatively low, and not affected by dietary inclusion of encapsulated chelating compounds in a consistent manner. (**Table 3**). Difference in P availability is not correlated to a different levels of scale ash. The method of digestibility evaluation (settling columns) can result in significant leaching into the supernatant (data not shown) during feces collection and partially explain this difference on P apparent digestibility between the diets. The release of chelating compounds in the proximal intestinal tract of fish would lead to either a P-deficiency or significant absorption of Fe or Al. In both cases, we did not notice any significant difference in mineral status (**Table 3** and **Figure 2**) or Al or Fe digestibility (**Table 3**), when compared to the other treatments, thus confirming our underlying assumptions.

Another observation that confirms liberation in the distal part of the intestine is the fact that we do not detect any sign of short-term toxicity despite the increased dietary inclusion of Fe and Al. For fish, iron is more toxic than Al (Desjardins et al., 1987; Handy and Poxton, 1993; Bury et al., 2003;) and it is required in small quantities in the feed (0.1-0.3 g of Fe/kg of feed). At higher concentrations (0.2-6.3 g of Fe/kg of diet), fish develop signs of toxicity from this element (Desjardins et al., 1987; Baker et al., 1997;). Iron toxicity causes a decrease in FI and growth, diarrhea, and liver damage (an increase of HSI) that can lead to fish death. Despite the high level of iron (2.4 g of Fe/kg of feed) inclusion in Experiment 1, no short-term signs of toxicity were observed (**Table 3**). Long-term growth studies are required to specifically address potential toxicity issues of chronic feeding of encapsulated Al and Fe.

Few studies have been performed to highlight the effect of adding alum or iron on the insolubilization of P from egested fish feces. Preliminary studies on the use of encapsulated alum in the diet of rainbow trout were aimed to reduce feces friability (Fournier, 2012). This study demonstrated that the addition of alum reduced the suspension of o-PO₄ by up to 85% (10.5 g of Al/kg of feed) over a one-week sampling period. In experiment 1, we observed reductions of 54% and 38% of o-PO₄ with the inclusion of 0.9 g/kg of Al and 2.4 g/kg of Fe, respectively, which is consistent with Fournier (2008). The second experiment also confirmed this study and highlighted the dose-effect of encapsulated dietary alum on the insolubilization of fecal P. Indeed, the levels of o-PO₄

released from feces after 14 days were reduced by 15, 38 and 50% for the 3, 6, and 15 g/kg feed of encapsulated alum, respectively.

The form of phosphorus has a significant effect on the solubilization of P from feces. Lall and Lewis-McCrea (2007) noted that calcium-bound phosphorus (mainly hydroxyapatite) fractions were insoluble, whereas fractions of organic P (OP; 60-80% of total P) were dissolved over time (Foy and Rosell, 1991; Dosdat, 1992; Ackefors and Enell, 1994). There is a consensus on this level of fecal organic P (Ouellet, 1999) despite great variability from one experience to another (Dosdat, 1992; Lall, 1991). It seems that fecal OP is largely mineralized to o-PO_4 within a few days. Garcia-Ruiz and Hall (1996) showed with laboratory tests that 40% of TP in feces could be dissolved in 5 hours, which corresponds to a proportion of 50-70% of the o-PO_4 fraction. Dosdat (1992) reported 48% TP mineralization after 15 days (at 17°C). In our first study, 50-60% of fecal TP mineralization (18-20 mg of P/g of feces, dry basis) was observed after one week from feces of fish fed control diets (Ctrl and Ctrl+) devoid of chelating compounds and incubated at 22 °C. In the second experiment, 69% of the TP was mineralized at the end of the 14th day (at 22°C) for feces from control feed-derived feces.

These results clearly demonstrate that it is possible to reduce by about half the OP solubilization from trout feces using the encapsulated chelating compounds, that are already approved and widely employed as approaches for wastewater treatment (Morse et al., 1998; Metcalf, 2003;). Several nutritional strategies have been developed to minimize the P loads in fish farm effluent. Low-P diets (Ketola and Harland, 1993; Bureau et al., 2000, Sarker et al., 2011), high nutrient-dense diets (Cho et Bureau, 1997) and inclusion of plant-derived proteins (Médale et al., 1998) have been described as approaches to address the problem. Considering that the requirements of nutrients in most animals are known to decrease with age because the growth rate decreases and the dietary nutrients including P are used mainly for maintaining metabolic functions. According to this Sarker et al. (2011) reported that the feeding phase of P in diets (alternating P-sufficient and Low-P diets) for larger fish is a clear opportunity to significantly reduce P output from trout farm facilities.

This P output from aquaculture operations is predominantly represented by fish feces. Mechanical filtration and settling suspend solids allowed to reduce 20 to 55% of total P release into effluents (D'Orcastel, 2006). Using the technique described in this study we obtained similar reductions to using both mechanical filtration and settling suspended solids (34 to 54%) and higher P retention versus those found with sludge filtration in steel slag filter beds (36%, e.g., Puigagut et al. (2011) and Kõiv et al., 2016). Contrary to steel slag methods and liming using CaO, micro-encapsulating Al and Fe included in the diet does not increase effluent pH. The combination of inclusion of encapsulated P-chelating agents in early growing (50-250 g) phase and the use of low- P diets in the post-juvenile growing phases (> 250 g) combined with sludge treatment (Kõiv et al., 2016) methods, could allow fish farms to reduce P loading to the environment, improving the environmental performance of rainbow trout diets.

Conclusion

Minimizing P wastes is a critical factor for the environmental sustainability of freshwater aquaculture operations. The proposed technique offers a novel approach to capture soluble P from feces. This will have the effect of limiting the level of effluent phosphorus and ultimately, the level of this element being discharged into receiving aquatic ecosystems. In the context of Quebec's freshwater aquaculture sector, where there exists a mandatory threshold of 4.2 kg of P/ton of fish produced, the dietary incorporation of microencapsulated chelating compounds described herein may provide a practical tool to assist in managing effluent P emissions and allow the sector to pursue anticipated sustainable industry growth and development. A complementary long-term study is required ensure no toxic impacts of chronic feeding of micro-encapsulated Al and Fe in diets. Finally, large-scale experiments on conventional rainbow trout farms over an entire grow-out cycle should be carried out to validate the reduction of P output from these facilities.

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Declaration of Competing Interest

None

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Figure and Tables

Table 1: Ingredients (g/kg) and proximate composition (g/kg dry-weight basis) of the test diets in experiment 1^a.

Ingredients (g/kg)	Diets (chelating inclusion) ^b			
	Ctrl	Ctrl+	Al	Fe
Herring meal ¹	300	300	300	300
Soybean meal ²	130	130	130	130
Corn gluten meal ²	167	147	147	147
Wheat grain ³	165	165	165	165
Dried whey ²	100	100	100	100
Fish oil ¹	115	115	115	115
Vitamin and mineral premix ^{4, e}	10	10	10	10
Sipernat 50 ^{5, c}	10	10	10	10
Guar gum ⁶	3	3	3	3
P-chelating microbeads ^d	0	20	20	20
<i>Lipid matrix</i>	<i>0</i>	<i>20</i>	<i>14</i>	<i>13.4</i>
<i>Chelating compound</i>	<i>0</i>	<i>0</i>	<i>6</i>	<i>6.6</i>
Chemical composition (g/kg, dry basis)				
Dry matter	940	934.2	937.6	941.9
Crude protein	422	427.8	419.2	403.4
Crude lipid	146.6	176.7	174.8	160.9
Ash	105.7	107.2	107.5	111.5
Energy (E. MJ/kg)	22.4	22.8	22.6	22.3
Total phosphorus (P)	11.4	12.9	12.7	12.7
Aluminium (Al, mg/kg)	57.1	56.8	960.2	50.5
Iron (Fe, mg/kg)	82.8	87.8	92.0	2490

^aValues were means of triplicate chemical analyses (n=3) per diet; ^bDiet designations: Ctrl, control diet without P-chelating microbeads; Ctrl+, positive control diet with 20g/kg of microbeads containing no chelating agent; Al, diet with microbeads containing Al₂SO₄; Fe, diet with microbeads containing FeSO₄; ^cSipernat 50: a source of insoluble acid ash comprised of 98.50% SiO₂ with an average particle size of 50 µm; ^dg/kg diet (italics) of lipid or chelating compound in microbeads.

^eSupplied the following: (to provide mg/kg except when noted): vitamin mix = thiamin HCl, 2; riboflavin, 3; pyridoxine HCl, 0.6; niacin, 1; calcium pantothenate, 4; folic acid, 0.2; biotin (1mg/g), 4; vitamin B12 (0.1%), 10; vitamin A palmitate, (250,000 IU/g) 2; vitamin D3 (400,000 IU/g), 1.5; vitamin E acetate (500 IU/g), 29.8; menadione sodium bisulfite (62.3% menadione), 3.2; t-BHQ, 0.03; dextrose, 938.66; mineral mix = potassium iodide (76%I), 2.63; ferrous sulfate 7H₂O (20%Fe), 50; manganese sulfate H₂O (32.5% Mn), 24.6; zinc sulfate H₂O (36.44%Zn), 37.48; cupric sulfate 5H₂O (25% Cu), 8; sodium selenite (45.6% Se), 0.35; cobalt chloride 6H₂O (24.77% Co), 0.085; dextrose, 876.855. Each mix was added at 5g/kg of diet.

North American supplier:

¹Comeau Seafood Ltd; ²Meunerie Gérard Soucy Inc. 926 route Laurier. Sainte-Croix. QC. G0S 2H0; ³Colabor. 820 rue St-Alphonse Desrochers. Lévis. Qc. G7A 5H9; ⁴Dyets. Inc. 2508 Easton Avenue. Bethlehem. PA 18017. Bethlehem. PA 18017; ⁵Evonik Corporation. 2 turner place Piscataway. NJ 08855-0365. USA; ⁶Laboratoire Mat Inc. Quebec. QC.

Pelleting machine: Model CPM CL-5, California Laboratory Pellet Mill, Crawfordsville, IN, USA

Table 2: Ingredients (g/kg) and proximate composition (g/kg dry-weight basis) of the test diets in experiment 2^a.

Ingredients (g/kg)	Diets ^b (alum inclusion)			
	0	3	6	15
Herring meal ¹	300	300	300	300
Soybean meal ²	130	130	130	130
Corn gluten ²	167	167	157	127
Wheat grain ³	155	155	155	155
Dried whey ²	100	100	100	100
Fish oil ¹	115	115	115	115
Vitamin and mineral premix ^{4, e}	10	10	10	10
Sipernat 50 ^c	10	10	10	10
Guar gum	3	3	3	3
P-chelating microbeads ^d	10	10	20	50
<i>Lipid matrix</i>	<i>10</i>	<i>7</i>	<i>14</i>	<i>35</i>
<i>Chelating compound (Alum)</i>	<i>0</i>	<i>3</i>	<i>6</i>	<i>15</i>
Proximate composition (g/kg, dry basis)				
Dry matter	964.3	961.5	974.6	971.0
Crude protein	416.8	409.9	406.9	380.7
Crude lipid	160.3	160.3	166.1	186.2
Ash	109.0	108.9	109.2	111.3
Energy (E, MJ/kg)	21.8	21.6	21.7	22.0
Total phosphorus (P)	13.6	13.7	13.2	13.0

^aValues were means of triplicate chemical analyses (n = 3) per diet; ^bDiet designations: Ctrl+ or 0, positive control diet with 10g/kg of microbeads containing without chelating compound; 3, diet with 3 g/kg of alum inclusion, 6, diet with 6 g/kg of alum inclusion; 15, diet with 15 g/kg of alum inclusion. Microbeads with Al₂SO₄ as chelating compound was used for inclusion. ^cSipernat 50: a source of insoluble acid ash comprised of 98.50% SiO₂ with an average particle size of 50 µm, ^dg/kg diet (italics) of lipid or chelating compound in microbeads.

^eSupplied the following: see table 1

¹⁻⁴North American supplier: see table 1

Table 3: Nutrient digestibility for diets used in the two experiments (g/kg or mg/kg dry basis).

Experiment	Experiment 1 (chelating inclusion)						Experiment 2 (alum inclusion)					
Diets	Ctrl	Ctrl+	Al	Fe	Pooled SEM	P-value	0	3	6	15	Pooled SEM	P-value
Digestible dry matter ¹	646	644	661	643	28.5	0.072	656	672	648	645	21.5	0.063
Digestible protein ¹	355	356	362	352	6.3	0.092	NA	NA	NA	NA	NA	NA
Digestible lipid ¹	97	121	120	107	18.2	0.045	NA	NA	NA	NA	NA	NA
Digestible ash ¹	43	48	48	47	1.5	0.052	47	57	51	48	2.4	0.031
Digestible E ¹	16.2	16.6	16.8	16.2	3.2	0.520	NA	NA	NA	NA	NA	NA
Digestible P ¹	0.53	0.82	0.72	0.62	0.141	0.022	0.72	0.54	0.63	0.54	0.130	0.041
Digestible Al (mg/kg) ¹	8.5	8.2	9.1	7.3	0.52	0.055	NA	NA	NA	NA	NA	NA
Digestible Fe (mg/kg) ¹	8.2	8.2	8.3	7.9	0.34	0.105	NA	NA	NA	NA	NA	NA

¹The digestibility study was conducted in triplicate tanks. Energy values as MJ/kg. Values are indicated as mean (n = 3 tanks) ± standard deviation (sd). NA: in Experiment 2, only the digestibility of dry matter, ash, and P content in the diet were evaluated.

Table 4: Growth performance indicators of fish at the end (5 w) of the experiment 1.

Growth performance	Unit	Diets				Statistics	
		Ctrl	Ctrl+	Al	Fe	Pooled SEM	P-value
IBW ¹		179 ^{ab}	190 ^b	185 ^{ab}	175 ^a	3.4	0.033
FBW ¹	g	269 ^{ab}	286 ^b	260 ^{ab}	256 ^{ab}	6.2	0.005
FI		64.8 ^c	62.2 ^b	60.1 ^b	55.1 ^a	1.53	<0.001
Weight gain		46.3	49.2	38.0	43.2	3.54	0.112
FCR ¹	g/g	1.28	1.30	1.44	1.24	0.150	0.431
TGC ¹		0.21	0.20	0.15	0.19	0.034	0.177
HSI ^{1,2}	%	1.62	1.44	1.40	1.42	0.201	0.001

¹IBW, Initial body weight; FBW, Final body weight; FI, Feed Intake; FCR, feed conversion ratio; TGC, thermal-unit growth coefficient; HSI, hepatosomatic index. Values (IBW, FBW, FI, weight gain, FCR, TGC, and HSI) were means of 3 tanks by treatment (experimental unit). For parameter pooled standard error of means (SEM) were shown. Means were analyzed with one-way ANCOVA (effect of diet), as covariable IBW ($P=0.033$). Values not sharing identical letters were significantly different ($P<0.05$). ²For HSI at the beginning of the trial (0 w), measurements from 12 fish were taken. This mean was 1.03% and was significantly ($P=0.001$) different to 4 means from 4 treatments (diets, Ctrl = control, Ctrl+ = control positive, Al = diet with alum inclusion, Fe = diet with Iron inclusion) at the end of the experiment.

Table 5: Growth performance indicators of fish at the end (5 w) of the experiment 2.

Growth performance	Unit	Diets				Statistics	
		0	3	6	15	Pooled SEM	<i>P</i> -value
IBW ¹	g	113 ^b	109 ^a	114 ^b	112 ^b	2.2	0.007
FBW ¹		171	168	173	167	4.5	<0.001
FI		49.5	44.9	50.2	43.8	2.4	0.095
Weight gain	g/g	31.0 ^{ab}	30.8 ^{ab}	31.6 ^b	29.3 ^a	1.15	0.032
FCR ¹		1.61	1.44	1.61	1.45	0.057	0.539
TGC ¹		0.17	0.176	0.174	0.162	0.010	0.075

¹IBW, initial body weight; FBW, final body weight; FI, feed intake; FCR, feed conversion ratio; TGC, thermal-unit growth coefficient. Values were means of 3 tanks by treatment (experimental unit). For parameter pooled Standard Error of Means (SEM) are shown. Means were analyzed with one-way ANCOVA (effect of diet). Values not sharing identical letters were significantly different ($P<0.05$).

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708 Table 6: Ash and P level (% , dry basis) in carcasses and scales at the beginning (0 w) and the end (5 w) of the first
 709 experiment¹.

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P statut indicator	0 w	5 w				Statistics	
		Ctrl	Ctrl+	Al	Fe	Poled SEM	P-value
Scale ash	31.4	31.3	28.3	31.5	30.9	2.35	0.303
Carcass ash	9.4 ^b	8.3 ^a	7.7 ^a	7.8 ^a	7.5 ^a	0.70	0.012
Scale P	2.9 ^a	3.9 ^{ab}	3.2 ^{ab}	3.0 ^a	4.4 ^b	0.08	0.026
Carcass P	1.51 ^b	1.36 ^a	1.26 ^a	1.29 ^a	1.22 ^a	0.81	<0.001

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714 ¹Values were indicated as means. The different letters indicate significantly different means ($P<0.05$). Ash (or P) (%) = ash (or P) content/dry sample
 715 weight (g). For parameter pooled Standard Error of Means (SEM) were shown. At the beginning of the trial (0w), one sample of 12 fish was taken.
 716 This mean is compared to 4 means from 4 treatments (diets, Ctrl = control, Ctrl+ = control positive, Al = Diet with alum inclusion, Fe = diet with Iron
 717 inclusion) at the end. One way ANCOVA follows by Tuckey pairwise comparison.

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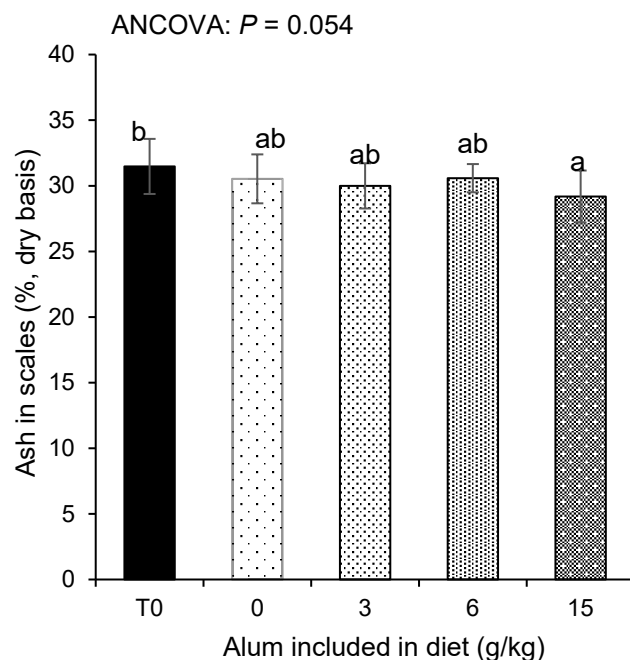
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731 Figure 1: Scale mineralization of graded levels of alum included at the beginning
 732 (T0) and the end of experiment 2 (0, 3, 6, 15 g/kg). The values represent mean $\pm$
 733 sd ($n = 3$). Values were analyzed with one-way ANCOVA (effect of treatment), as
 734 covariable IBW ($P=0.007$). Tucky test was used to identify significant differences
 735 between treatments. Values not sharing identical letters were significantly different
 736 ($P<0.1$).

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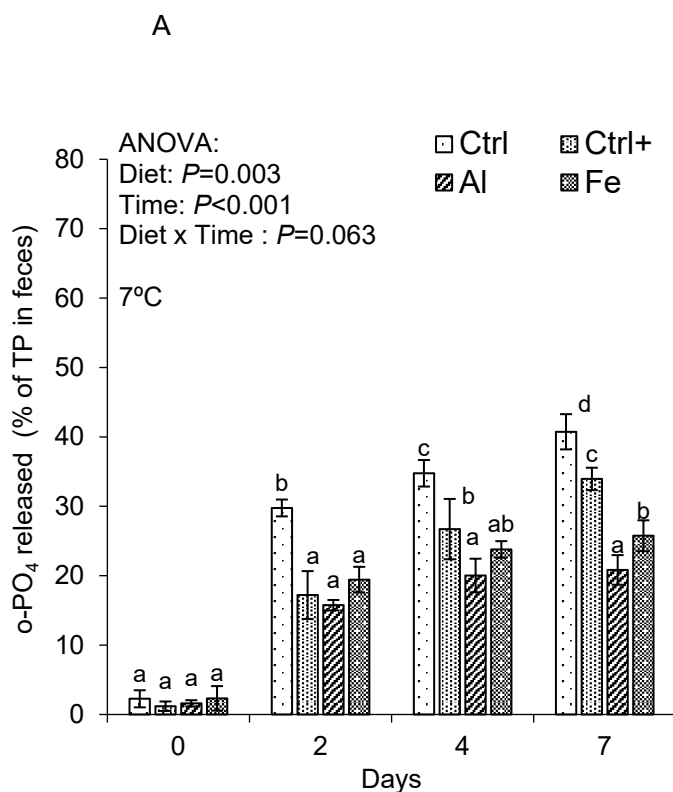
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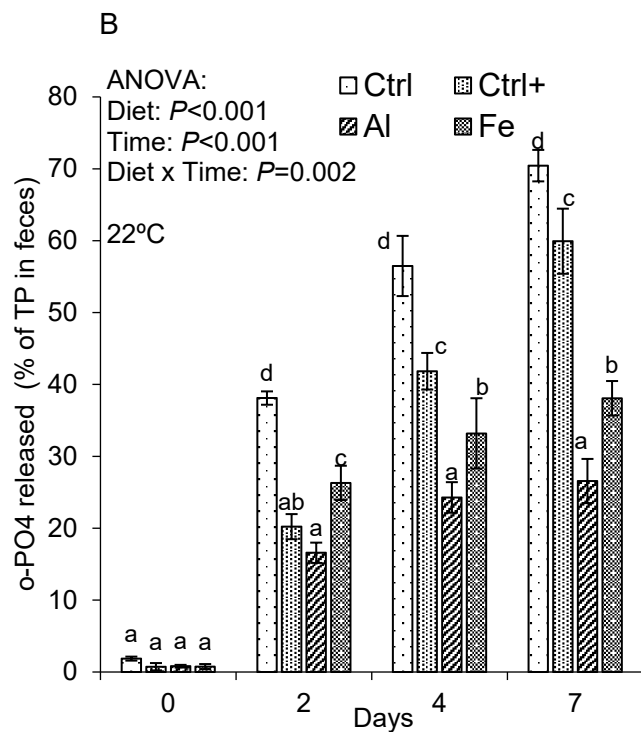
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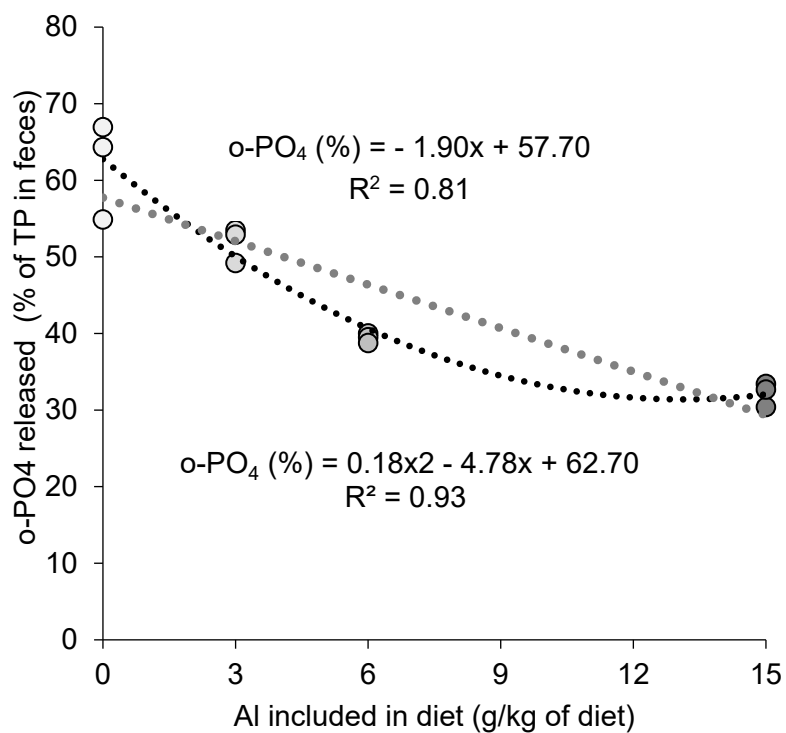


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759 Figure 2: o-PO₄ release from feces over 7-day incubation (expressed as % of total P in
760 feces) from fish fed with experimental diets and incubated at 7 °C (A) and 22 °C (B). The
761 value represents the o-PO₄ release (mean ± sd, n = 3). Two-way ANOVA followed by

762 Tukey pairwise comparison was used to identify the differences treatment in each time.
 763 The different letters indicate significantly different means ($P < 0.05$).
 764



765 Figure 3: Relation between o-PO₄ feces (0, 3, 6, 15 g/kg of alum) released after
 766 14 days and the level of encapsulated alum included in diets.