2020. *Animal Feed Science and Technology*. 269: 114675 (11 p.).

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

Waly Ndianco Ndiaye[1,4], Marie-Hélène Deschamps[1], Yves Comeau[2], Kabir Chowdhury[3], Jean-Daniel Bunod[3], Marie-Pierre Letourneau-Montminy[1], Grant Vandenberg[1].

[1]Département des sciences animales, Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, 2425 Rue de l'Université Québec, QC, G1V0A6.
[2]Département des génies civil, géologique et des mines, Polytechnique Montréal, 2500 chemin de Polytechnique, Montréal, Québec, Canada H3T 1J4.
[3]Jefo Nutrition Inc, 5020 Avenue Jefo, Saint-Hyacinthe, Québec, Canada J2S 7B6.
[4]Institut Sénégalais de Recherches Agricoles (ISRA), Centre de Recheches Océanographiques de Dakar-Thiaroye (CRODT), Pôles de Recherches de Hann, Route du Front de Terre, BP : 2241, Dakar, Sénégal.

Running title: Intestinal binding of soluble phosphorus

*Corresponding Author: Grant W. Vandenberg,
Faculté des sciences de l'agriculture et de l'alimentation,
Département des sciences animales,
Université Laval,
2425 rue de l'Agriculture,
Québec, QC, Canada, G1V 0A6.
Tel: + 1(418) 656-2131 (ext. 6541).
E-mail address: grant.vandenberg@fsaa.ulaval.ca

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 encapulated 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 appraoch 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 ºC, 8 h). Then pellets (4.0 mm dia.) were dried in a forced-air oven (45 ºC, 24 h), sieved and stored at -20 ºC 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/m^3 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:

Weight gain = [(FBW - IBW) / IBW] × 100 %

FCR = [FI / (FBW - IBW)]

SGR = 100 × (lnFBW - lnIBW) / days

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

HSI = (liver weight/body weight) × 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 ºC. 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 ºC 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 ºC) 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 ºC) and room temperature (22 ºC) 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 filte*r*, 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 ± 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-PO_4 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 ± sd; dry basis). P-release was calculated using total P in feed taking in account ADC of total P (TP). The release of o-PO_4 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 ºC and 0.7 ± 0.5 and 70.4 ± 2.2 % of TP in feces at 22 ºC; the effect of chelating compounds was more pronounced at room temperature.After seven days, the o-PO_4 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 encapsualted Al released the lowest quantity of o-PO_4.

Experiment 2: increasing Al_2SO_4 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-PO_4 from the feces increased significantly during the incubation period (0-14 d). The level of encapsulated Al incorporated in diets influenced fecal o-PO_4 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-PO_4 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-PO_4 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-PO_4 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 beetwen 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 chonic 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-PO_4 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-PO_4 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-PO_4

406 released from feces after 14 days were reduced by 15, 38 and 50% for the 3, 6, and 15 g/
407 kg feed of encapsulated alum, respectively.
408
409 The form of phosphorus has a significant effect on the solubilization of P from feces. Lall
410 and Lewis-McCrea (2007) noted that calcium-bound phosphorus (mainly hydroxyapatite)
411 fractions were insoluble, whereas fractions of organic P (OP; 60-80% of total P) were
412 dissolved over time (Foy and Rosell, 1991; Dosdat, 1992; Ackefors and Enell, 1994).
413 There is a consensus on this level of fecal organic P (Ouellet, 1999) despite great
414 variability from one experience to another (Dosdat, 1992; Lall, 1991). It seems that fecal
415 OP is largely mineralized to o-PO_4 within a few days. Garcia-Ruiz and Hall (1996) showed
416 with laboratory tests that 40% of TP in feces could be dissolved in 5 hours, which
417 corresponds to a proportion of 50-70% of the o-PO4 fraction. Dosdat (1992) reported 48%
418 TP mineralization after 15 days (at 17ºC). In our first study, 50-60% of fecal TP
419 mineralization (18-20 mg of P/g of feces, dry basis) was observed after one week from
420 feces of fish fed control diets (Ctrl and Ctrl+) devoid of chelating compounds and incubated
421 at 22 ºC. In the second experiment, 69% of the TP was mineralized at the end of the 14th
422 day (at 22ºC) for feces from control feed-derived feces.
423
424 These results clearly demonstrate that it is possible to reduce by about half the OP
425 solubilization from trout feces using the encapsulated chelating compounds, that are
426 already approved and widely employed as approaches for wastewater treatment (Morse
427 et al., 1998; Metcalf, 2003;). Several nutritional strategies have been developed to
428 minimize the P loads in fish farm effluent. Low-P diets (Ketola and Harland, 1993; Bureau
429 et al., 2000, Sarker et al., 2011), high nutrient-dense diets (Cho et Bureau, 1997) and
430 inclusion of plant-derived proteins (Médale et al., 1998) have been described as
431 approaches to address the problem. Considering that the requirements of nutrients in
432 most animals are known to decrease with age because the growth rate decreases and the
433 dietary nutrients including P are used mainly for maintaining metabolic functions.
434 According to this Sarker et al. (2011) reported that the feeding phase of P in diets
435 (alternating P-sufficient and Low-P diets) for larger fish is a clear opportunity to significantly
436 reduce P output from trout farm facilities.
437
438 This P output from aquaculture operations is predominantly represented by fish feces.
439 Mechanical filtration and settling suspend solids allowed to reduce 20 to 55% of total P
440 release into effluents (D'Orcastel, 2006). Using the technique described in this study we
441 obtained similar reductions to using both mechanical filtration and settling suspended
442 solids (34 to 54%) and higher P retention versus those found with sludge filtration in steel
443 slag filter beds (36%, e.g., Puigagut et al. (2011) and Kõiv et al., 2016). Contrary to steel
444 slag methods and liming using CaO, micro-encapsulating Al and Fe included in the diet
445 does not increase effluent pH. The combination of inclusion of encapsulated P-chelating
446 agents in early growing (50-250 g) phase and the use of low- P diets in the post-juvenile
447 growing phases (> 250 g) combined with sludge treatment (Kõiv et al., 2016) methods,
448 could allow fish farms to reduce P loading to the environment, improving the environmental
449 performance of rainbow trout diets.
450
451 **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 feeing 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.

Acknowledgments

We greatly thank the technical assistance of the staff at the *Laboratoire de Recherche des Sciences Aquatiques* (LARSA) and *Groupe de Recherche Intégré en Physiologie et sciences Animales* (GRIPHA) at the Université Laval, particularly to Yolaine Lebeuf and Nancy Bolduc. This project was supported by *Ministère de l'Agriculture, des Pêcheries et de l'Alimentation-INNOVAMER (MAPAQ)*, the network *Ressources Aquatiques Québec* (RAQ) and the *Canadian Francophonie Scholaship Program* (PCBF in french).

Declaration of Competing Interest

None

References

Ackefors, H., Enell, M., 1994. The release of nutrients and organic matter from aquaculture systems in Nordic countries. Journal of Applied Ichthyology 10, 225-241. https://doi.org/10.1111/j.1439-0426.1994.tb00163.x.

AOAC (Association of Official Analytical Chemists) 1990. Method numbers 927.05 and 930.30. In: Official methods of analysis, 15th ed. AOAC, Washington, DC, USA.

APHA (1992). Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC, USA.

Atkinson, J.L., Hilton, J.W., Slinger, S.J., 1984. Evaluation of acid-insoluble ash as an indicator of feed digestibility in rainbow trout (*Salmo gairdneri*). Canadian Journal of Aquatic Sciences 41, 1384-1386. https://doi.org/10.1139/f84-170.

Avila, E.M., Tu, H., Basantes, S., Ferraris, R.P., 2000. Dietary phosphorus regulates intestinal transport and plasma concentrations of phosphate in rainbow trout. Journal of Comparative Physiology B 170, 201-209.

Baker, R., Martin, P., Davies, S., 1997. Ingestion of sub-lethal levels of iron sulphate by African catfish affects growth and tissue lipid peroxidation. Aquatic Toxicology 40, 51-61. https://doi.org/10.1016/S0166-445X(97)00047-7.

Bureau, D., Harris, A., Bevan, D., Simmons, L., Azevedo, P., Cho, C., 2000. Feather meals and meat and bone meals from different origins as protein sources in rainbow trout (*Oncorhynchus mykiss*) diets. Aquaculture 181, 281-291. https://doi.org/10.1016/S0044-8486(99)00232-X.

Bury, N.R., Walker, P.A., Glover, C.N., 2003. Nutritive metal uptake in teleost fish. Journal of Experimental Biology 206, 11-23. https://doi.org/10.1242/jeb.00068.

500 Champagne C.P., and Fustier P., 2007. Microencapsulation for the improved delivery of
501 bioactive compounds into foods. Current Opinion in Biotechnology 18, 184-190.
502 https://doi.org/10.1016/j.copbio.2007.03.001.
503 Cho, C.Y., Bureau, D.P., 1997. Reduction of waste output from salmonid aquaculture
504 through feeds and feeding. The Progressive Fish-Culturist 59(2):155-160.
505 https://doi.org/10.1577/1548-8640(1997)059<0155:ROWOFS>2.3.CO;2.
506 Cho, C., Slinger, S., Bayley, H., 1982. Bioenergetics of salmonid fishes: energy intake,
507 expenditure, and productivity. Comparative Biochemistry and Physiology Part B:
508 Comparative Biochemistry 73, 25-41.https://doi.org/10.1016/0305-
509 0491(82)90198-5.
510 Claveau-Mallet, D., Lida, F., Comeau, Y., 2015. Improving phosphorus removal of
511 conventional septic tanks by a recirculating steel slag filter. Water Quality
512 Research Journal 50, 211-218. https://doi.org/10.2166/wqrjc.2015.045.
513 Codling, E.E., Chaney, R.L., Mulchi, C.L., 2000. Use of aluminum-and iron-rich residues
514 to immobilize phosphorus in poultry litter and litter-amended soils. Journal of
515 Environmental Quality 29, 1924-1931.
516 https://doi.org/10.2134/jeq2000.00472425002900060027x.
517 Correll, D.L., 1998.The role of phosphorus in the eutrophication of receiving waters: A
518 review. Journal of Environmental Quality 27, 261-266.https://doi.org/
519 10.2134/jeq1998.00472425002700020004x.
520 Deschamps, M.H., Poirier Stewart, N., Demanche, A., Vandenberg, G.W., 2014.
521 Preliminary study for a phenotypic description of vertebral abnormalities in triploid
522 trout subjected to a prolonged deficiency in phosphorus. Journal of Applied
523 Ichthyology 30, 833-839. https://doi.org/10.1111/jai.12518.
524 Desjardins, L., Hicks, B., Hilton, J., 1987. Iron catalyzed the oxidation of trout diets and its
525 effect on the growth and physiological response of rainbow trout. Fish Physiology
526 and Biochemistry 3, 173-182.https://doi.org/10.1007/BF02180278.
527 Dosdat A.,1992. L'excrétion chez les poissons téléostéens I: L'azote. La pisciculture
528 Française 108, 25-40.
529 D'Orbcastel, E.R., and Blancheton, J.P., 2006. The wastes from marine fish production
530 systems: characterization, minimization, treatment, and valorization. World
531 Aquaculture, 37, 28-35, 70.
532 Elser, J.J., Bracken, M.E., Cleland, E.E., Gruner, D.S., Harpole, W.S., Hillebrand, H.,
533 Smith, J.E., 2007. Global analysis of nitrogen and phosphorus limitation of primary
534 producers in freshwater, marine, and terrestrial ecosystems. Ecology letters 10,
535 1135-1142. https://doi.org/10.1111/j.1461-0248.2007.01113.x.
536 Gibbs, F., Kermasha, S., Alli, I., Mulligan, C.N., 1999. Encapsulation in the food industry:
537 a review. International Journal of Food Sciences and Nutrition 50, 213-224.
538 https://doi.org/10.1080/096374899101256.
539 Fournier J., 2012. *Optimisation de la formulation d'un régime à teneur réduite en*
540 *phosphore chez la truite arc-en-ciel (Oncorhynchus mykiss) dans le but de réduire*
541 *les rejets en phosphore*. MSc thesis, Laval University, Québec, Canada.
542 http://hdl.handle.net/20.500.11794/23697.
543 Fournier, J., 2008. *Amélioration de la stabilité des fèces de truites arc-en-ciel par*
544 *intervention alimentaire*. BSc thesis, Université Laval, Québec, Canada, p. 70.
545 Foy, R., Rosell R., 1991. Loadings of nitrogen and phosphorus from a Northern Ireland
546 fish farm. Aquaculture 96, 17-30.https://doi.org/10.1016/0044-8486(91)90136-U.

547 Furné, M., Hidalgo, M.C., Lopez, A., Garcia-Gallego, M., Morales, A. E., Domezain, A.,
548 Sanz, A., 2005. Digestive enzyme activities in Adriatic sturgeon Acipenser naccarii
549 and rainbow trout *Oncorhynchus mykiss.* A comparative study. Aquaculture 250,
550 391-398. https://doi.org/10.1016/j.aquaculture.2005.05.017.
551 Garcia-Ruiz, R., Hall, G.H., 1996. Phosphorus fractionation and mobility in the food and
552 faeces of hatchery reared rainbow trout (*Onchorhynchus mykiss*). Aquaculture 145
553 ,183-193. https://doi.org/10.1016/S0044-8486(96)01329-4.
554 Gu, i D., Liu, W., Shao, X., Xu, W., 2010. Effects of different dietary levels of cottonseed
555 meal protein hydrolysate on growth, digestibility, body composition and serum
556 biochemical indices in crucian carp (*Carassius auratus gibelio*). Animal Feed
557 Science and Technology 156, 112-120.
558 https://doi.org/10.1016/j.anifeedsci.2010.01.012.
559 Handy, R., Poxton, M., 1993. Nitrogen pollution in mariculture: toxicity and excretion of
560 nitrogenous compounds by marine fish. Reviews in Fish Biology and Fisheries 3,
561 205-241. https://doi.org/10.1007/BF00043929.
562 Hasnaoui, M., Kassila, J., Loudiki, M., Droussi, M., Balvay, G., Barrouin, G., 2001.
563 Relargage du phosphore à l'interface eau-sédiment dans des étangs de
564 pisciculture de la station Deroua (Béni Mellal, Maroc). Revue des sciences de
565 l'eau/Journal of Water Science 14, 307-322. https://doi.org/10.7202/705422ar.
566 Ketola, H.G., Harland, B.F., 1993. Influence of phosphorus in rainbow trout diets on
567 phosphorus discharges in effluent water. Transactions of the American Fisheries
568 Society. 122, 1120-1126. https://doi.org/10.1577/1548-
569 8659(1993)122<1120:IOPIRT>2.3.CO;2.
570 Khan, F.A., Ansari, A.A., 2005. Eutrophication: an ecological vision. The botanical review
571 71 4, 449-482. https://doi.org/10.1663/0006-
572 8101(2005)071[0449:EAEV]2.0.CO;2.
573 Kõiv, M., Mahadeo, K., Brient, S., Claveau-Mallet, D., Comeau, Y., 2016. Treatment of
574 fish farm sludge supernatant by aerated filter beds and steel slag filters—effect of
575 organic loading rate. Ecological Engineering 94, 190-199.
576 https://doi.org/10.1016/j.ecoleng.2016.05.060.
577 Koko K.D. G., 2007. *Une stratégie nutritionnelle de réduction du phosphore (P) dans les*
578 *effluents aquacoles: l'alimentation en phase des truites arc-en-ciel (Oncorhynchus*
579 *mykiss) avec alternances d'un régime carencé et d'un régime équilibré en*
580 *phosphore*. MSc thesis, Université Laval, Québec, Canada.
581 http://hdl.handle.net/20.500.11794/20376.
582 Lall, S. P., Lewis-McCrea, L.M., 2007. Role of nutrients in skeletal metabolism and
583 pathology in the fish-an overview. Aquaculture 267, 3-19.
584 https://doi.org/10.1016/j.aquaculture.2007.02.053.
585 Lall, S.P., 1991. Digestibility, metabolism, and excretion of dietary phosphorus in fish. In
586 Nutritional Strategies and Aquaculture Waste. Proceedings of the first international
587 symposium on nutritional strategies in management of aquaculture waste (Cowey
588 CB & Cho CY, eds). pp. 21–36. University of Guelph, Guelph, Ontario,
589 Canada,1991.
590 Le Luyer, J., Deschamps, M-H., Proulx, E., Poirier-Stewart, N., Robert, C., Vandenberg,
591 G., 2014. Responses of different body compartments to acute dietary phosphorus
592 deficiency in juvenile triploid rainbow trout (*Oncorhynchus mykiss*, Walbaum).
593 Journal of Applied Ichthyology 30, 825-832. https://doi.org/10.1111/jai.12519.

Maruo, M., Ishimaru, M., Azumi, Y., Kawasumi, Y., Nagafuchi, O., Obata, H., 2016. Comparison of soluble reactive phosphorus and orthophosphate concentrations in river waters. Limnology 17, 7-12. https://doi.org/10.1007/s10201-015-0463-6.

Médale, F., Boujard, T., Vallée, F., Blanc, D., Mambrini, M., Roem, A., Kaushik, S.J., 1998. Voluntary feed intake, nitrogen and phosphorus losses in rainbow trout (*Oncorhynchus mykiss*) fed increasing dietary levels of soy protein concentrate. Aquatic Living Resources 11, 239-246.https://doi.org/10.1016/S0990-7440(98)89006-2.

Metcalf, E.E., Eddy, H., 2003. Wastewater engineering: treatment and reuse. Wastewater Engineering, Treatment, Disposal and Reuse. In: Tchobanoglous G., Burton F.L., Stensel, H.D., 2003. Wastewater engineering treatment and reuse (No. 628.3 T252s). Boston, US: McGraw-Hill Higher Education.

Moore, P., Daniel, T., Edwards, D., 1999. Reducing phosphorus runoff and improving poultry production with alum. Poultry Science 78, 692-698. https://doi.org/10.1093/ps/78.5.692.

Morse, G., Brett, S., Guy, J., Lester, J., 1998. Review: phosphorus removal and recovery technologies. Science of the Total Environment 212, 69-81. https://doi.org/10.1016/S0048-9697(97)00332-X.

Naumann, K., Bassler, R., 1976. Die chemische Untersuchung von Futtermitteln. In: The Chemical Analysis of Feeds. Methodenbuch Bd. III. Verlag J. Neumann-Neudamm, Germany, 24 chapters.

NRC 2011. Nutrient requirements of fish and shrimp. National Academy Press, Washington, DC: The national Academies of Sciences.

Olfert, E.D., Cross BM, McWilliam, A.A., 1993. Guide to the care and use of experimental animals, vol 1. Canadian Council on Animal Care Ottawa, Canada. URL: http://www2.psych.utoronto.ca/users/psy3001/files/Experimental_Animals_vol1.pdf

Ouellet, G.,1999. Les rejets des stations piscicoles et leurs impacts environnementaux., MAPAQ, Québec, Canada. 42 p.

Shreve, B., Moore, P., Daniel, T., Edwards, D., Miller, D.M., 1995. Reduction of phosphorus in runoff from field-applied poultry litter using chemical amendments. Journal of Environmental Quality 24, 106-111.https://doi.org/ 10.2134/jeq1995.00472425002400010015x.

Sims, J., and Luka-McCafferty, N., 2002. On-farm evaluation of aluminum sulfate (alum) as a poultry litter amendment: Effects on litter properties. Journal of Environmental Quality 31, 2066. https://doi.org/ 10.2134/jeq2002.2066.

Sugiura, S.H., McDaniel, N.K., Ferraris, R.P., 2003. In vivo, fractional Pi absorption and NaPi-II mRNA expression in rainbow trout are upregulated by dietary P restriction. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 285 (4): R770-R781.https://doi.org/10.1152/ajpregu.00127.2003.

Vandenberg, G.W., 2001. Encapsulation de phytase microbienne: L'influence sur la disponibilité de nutriments chez la truite arc-en-ciel. PhD Thesis Université Laval, Québec, Canada.

Zar, J.H., 1999. Biostatistical Analysis, 4 ed. Upper Saddle River, Prentice Hall, London, UK. ISN: 9780130815422.

Figure and Tables

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

	Diets (chelating inclusion)[b]			
Ingredients (g/kg)	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
Lipid matrix	*0*	*20*	*14*	*13.4*
Chelating compound	*0*	*0*	*6*	*6.6*
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

[a]Values were means of triplicate chemical analyses (n=3) per diet; [b]Diet 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 Al_2SO_4; Fe, diet with microbeads containing $FeSO_4$; [c]Sipernat 50: a source of insoluble acid ash comprised of 98.50% SiO_2 with an average particle size of 50 µm; [d]g/kg diet (italics) of lipid or chelating compound in microbeads. [e]Supplied 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 $7H_2O$ (20%Fe), 50; manganese sulfate H_2O (32.5% Mn), 24.6; zinc sulfate H2O (36.44%Zn), 37.48; cupric sulfate $5H_2O$ (25% Cu), 8; sodium selenite (45.6% Se), 0.35; cobalt chloride $6H_2O$ (24.77% Co), 0.085; dextrose, 876.855. Each mix was added at 5g/kg of diet.

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

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

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

	Diets[b] (alum inclusion)			
Ingredients (g/kg)	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
Lipid matrix	*10*	*7*	*14*	*35*
Chelating compound (Alum)	*0*	*3*	*6*	*15*
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

666
667 [a]Values were means of triplicate chemical analyses (n = 3) per diet; [b]Diet designations: Ctrl+ or 0,
668 positive control diet with 10g/kg of microbeads containing withour chelating compound; 3, diet with
669 3 g/kg of aum inclusion, 6, diet with 6 g/kg of alum inclusion; 15, diet with15 g/kg of alum inclusion.
670 Microbeads with Al_2SO_4 as chelating coumpound was was used for inclusion. [c]Sipernat 50: a
671 source of insoluble acid ash comprised of 98.50% SiO_2 with an average particle size of 50 µm,
672 [d]g/kg diet (italics) of lipid or chelating compound in microbeads.
673
674 [e]Supplied the following: see table 1
675 [1-4]North 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

[1]The 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.

		Diets				Statistics	
Growth performance	Unit	Ctrl	Ctrl+	Al	Fe	Pooled SEM	*P*-value
IBW[1]	g	179[ab]	190[b]	185[ab]	175[a]	3.4	0.033
FBW[1]		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

[1]IBW, 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). [2]For 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 experiement.

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

		Diets				Statistics	
Growth performance	Unit	0	3	6	15	Pooled SEM	*P*-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		31.0[ab]	30.8[ab]	31.6[b]	29.3[a]	1.15	0.032
FCR[1]	g/g	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

[1]IBW, 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 ($P<0.05$).

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 experiment[1].

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

[1]Values were indicated as means. The different letters indicate significantly different means ($P<0.05$). Ash (or P) (%) = ash (or P) content/dry sample 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. 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 inclusion) at the end. One way ANCOVA follows by Tuckey pairwise comparison.

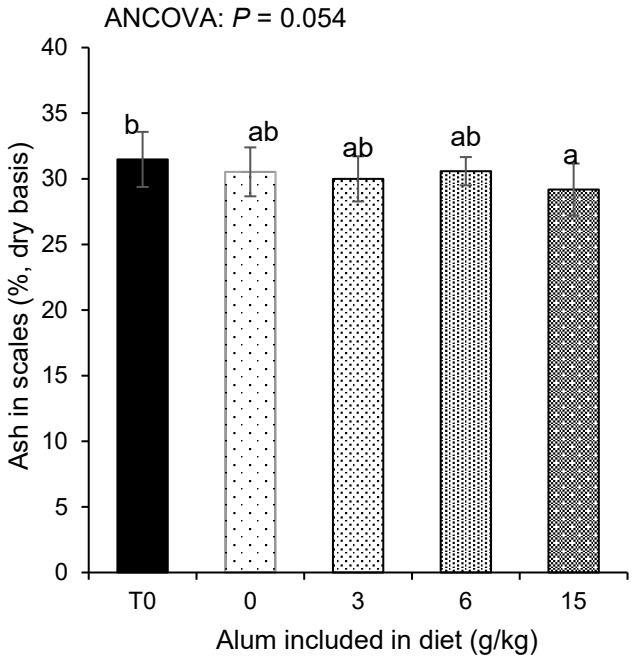
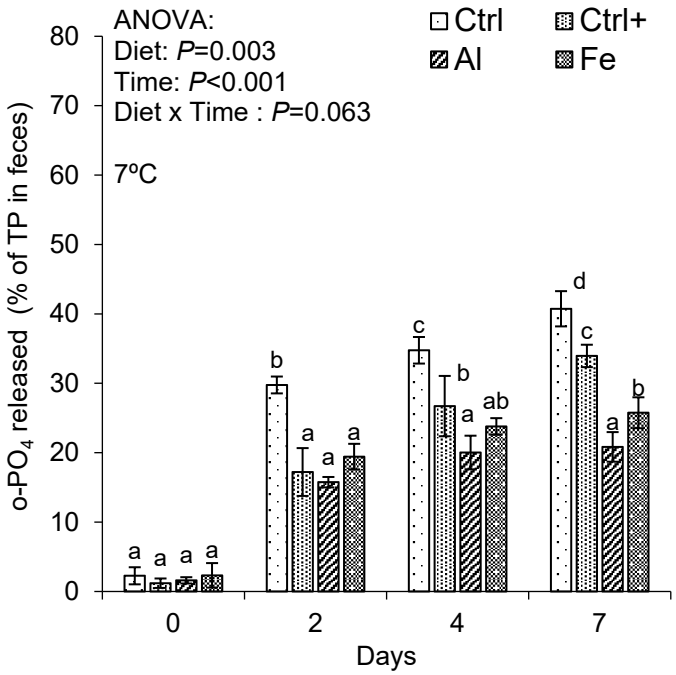
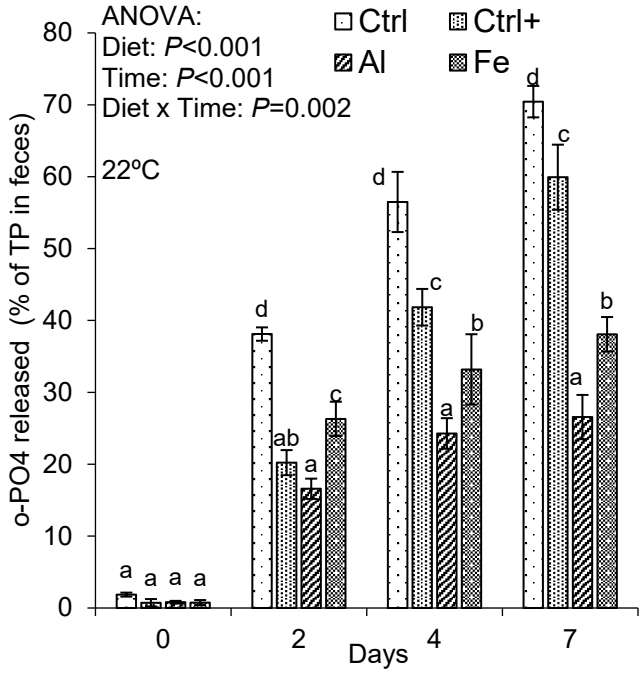


Figure 1: Scale mineralization of graded levels of alum included at the beginning (T0) and the end of experiment 2 (0, 3, 6, 15 g/kg). The values represent mean ± sd (n = 3). Values were analyzed with one-way ANCOVA (effect of treatment), as covariable IBW (P=0.007). Tucky test was used to identify significant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

754
755 A
756
757



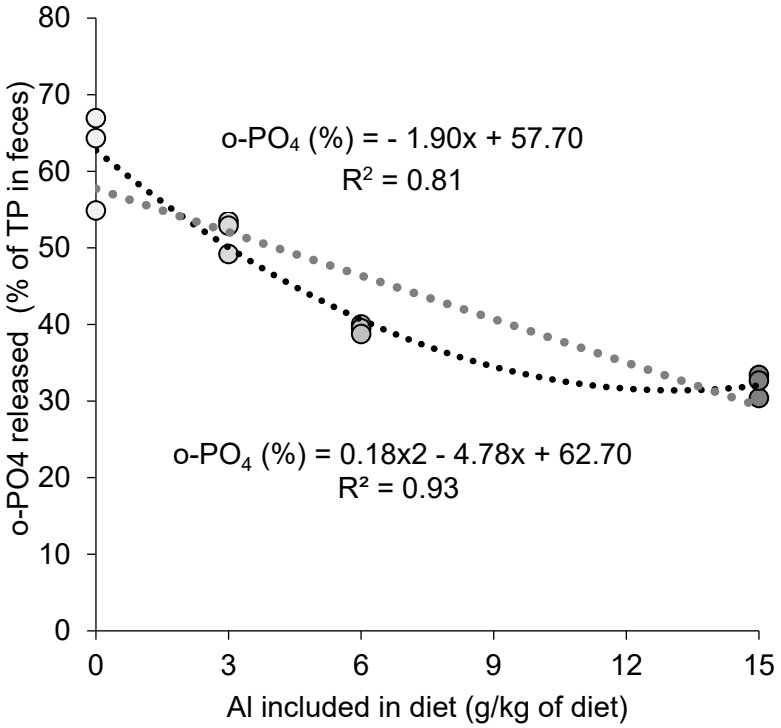
758 B



759 Figure 2: o-PO_4 release from feces over 7-day incubation (expressed as % of total P in
760 feces) from fish fed with experimentals diets and incubated at 7 ºC (A) and 22 ºC (B). The
761 value represents the o-PO_4 release (mean ± sd, n = 3). Two-way ANOVA followed by

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

764



765 Figure 3: Relation between o-PO_4 feces (0, 3, 6, 15 g/kg of alum) released after
766 14 days and the level of encapsulated alum included in diets.