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Apparent availability of zinc, selenium and manganese as inorganic metal salts or organic forms in plant-based diets for Atlantic salmon (*Salmo salar*)

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ABSTRACT

The composition of salmonid diets has changed from the use of mainly marine ingredients to increased use of plant ingredients, and this has an impact on the mineral content and availability. Minerals, like zinc (Zn), selenium (Se) and manganese (Mn), are supplemented to diets as inorganic or organic forms to cover the nutritional requirements of fish. This study compared the apparent availability (AA) of Zn, Se and Mn from inorganic metal salts and their organic forms in Atlantic salmon. Sixteen diets were prepared based on a two-level factorial design (2^4). The tested factors were Zn additive source (A), Se additive source (B), Mn additive source (C) and phytic acid level (D). The diets were fed to Atlantic salmon for 11 days, faeces were collected by stripping, and the total content of mineral and yttrium in diets and faeces were determined by inductively coupled plasma mass spectrometry. Data obtained were used to estimate the AA for the minerals. Zinc additive source had no significant effect on the AA of Zn. However, the Se and Mn additive source had significant effects on the AA of Se and Mn, respectively. Higher AA of Se was achieved with selenomethionine than with selenite, and Mn sulphate was more available than Mn chelate of glycine. The phytic acid level did not significantly affect the AA of Zn, Se or Mn. However, several interactions between mineral additive sources and the phytic acid level significantly affected the AA of Zn, Se and Mn.

1. Introduction

For many years in aquaculture industry, fish meal and fish oil were used as main ingredients in diet formulation. Over the last 10–15 years, access to fish meal and fish oil has become more difficult due to their price and aquaculture industry growth. As a consequence, the formulation of salmonid diets has changed and nowadays most commercial salmonid diets contain > 70% of plant ingredients (Ytrestoyl et al., 2015). Minerals such as zinc (Zn), selenium (Se) and manganese (Mn) are naturally present in fish meal and in plant ingredients. In fish meal, Zn, Se and Mn are present in concentrations ranging from 64 to 74 mg kg^{-1}, 1.5 to 3.1 mg kg^{-1} and 3.6 to 12 mg kg^{-1}, respectively (Sanden et al., 2013). Zinc, Se and Mn are present in plant ingredients in concentrations ranging from 35 to 48 mg kg^{-1}, < 0.01 to 0.16 mg kg^{-1} and 26 to 46 mg kg^{-1}, respectively (Sanden et al., 2017). In addition to the native sources found in ingredients, Zn, Se and Mn are supplemented to salmon diets as inorganic metal salts or their organic forms to meet the nutritional requirements of the fish (NRC, 2011; Schlegel et al., 2008). In the European Union, the current upper limit for total Zn in complete feed of all fish except salmonids is 150 mg kg^{-1} and for salmonids feeds it is 180 mg kg^{-1} feed (European Commission, 2003, 2016). The current upper limit for total Se in fish feed is 0.5 mg kg^{-1} (European Commission, 2003) and the supplementation of organic Se must not exceed 0.2 mg kg^{-1} in complete feed (European Commission, 2013a, b, 2015, 2017a, b). The upper limit in feed for Mn is 100 mg kg^{-1} (European Commission, 2017c, 2003).

Phytic acid is commonly found in cereal grains. Thus, the use of plant-based ingredients will add phytic acid in fish diet (Kumar et al., 2012; Lall, 2003). The phytic acid molecule is a very reactive molecule due to the presence of phosphate groups which are highly negatively charged. Hence, the molecules tend to bind divalent cations (e.g. Ca^{+2}, Fe^{+2}, Zn^{+2}) rendering them poorly available to the fish (Cao et al., 2007; Kumar et al., 2012). In fish, the availability of minerals from a diet is dependent on the diet composition, the chemical form of the mineral, and possible interactions with other diet components and nutrients coexisting in the gastrointestinal tract (Lall, 2003; Watanabe

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et al., 1997). Finding mineral sources with higher availability can reduce the amount of minerals one needs to add to the diets. Consequently, there is an increasing interest of comparing the availability of inorganic metal salts and their organic forms. A systematic review, where a large number of studies were included, discussed the availability of organic mineral sources over the respective inorganic forms in fish (Prabhu et al., 2016). Herein it was concluded that Se organic forms (selenomethionine (SeMet) and selenoyeast (Se yeast)) have higher availability when compared with the inorganic form of Se (selenite). However, for Zn and Mn sources, the data available in literature was found to be highly variable and inconsistent across studies (Prabhu et al., 2016).

Mineral availability studies have been performed in salmonids species, such as Atlantic salmon (*Salmo salar*) (Bell and Cowey, 1989; Maage et al. 2001), coho salmon (*Oncorhynchus kisutch*) (Sugiura et al., 1998) and rainbow trout (*Oncorhynchus mykiss*) (Apines-Amar et al., 2004; Fontagne-Dicharry et al., 2015; Rider et al., 2010; Sugiura et al., 1998). Bell and Cowey studied the digestibility and bioavailability of dietary Se from fish meal, selenite, SeMet and selenocystine (SeCys) in Atlantic salmon. Selenomethionine was found to be the most available Se source (91.6 ± 1.0%) followed by selenite (63.9 ± 4.26%) (Bell and Cowey, 1989). Maage and co-workers compared the availability of an organic Zn form (Zn gluconate) with the availability of an inorganic Zn form (Zn sulphate) in Atlantic salmon. Herein, the results obtained showed no differences in Zn status between groups given different Zn forms (Maage et al., 2001). Sugiura and colleagues studied mineral availability of different ingredients (e.g. animal and plant-based ingredients) for salmonid diets. The apparent availability (AA) of calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), sodium (Na), phosphorous (P), strontium (Sr), Mn and Zn in ingredients were determined in coho salmon and rainbow trout, and the AA of the minerals studied varied among the ingredients (Sugiura et al., 1998). Rider and co-workers compared the digestibility of inorganic and organic forms of Zn and Se in rainbow trout. Three marine-based diets (diet without supplementation, diet supplemented with selenite and Zn sulphate, and diet supplemented with Se yeast + Zn proteinate) were tested. The outcome of this study was that the diet supplemented with Se yeast had higher digestibility than the diet without supplementation and the diet supplemented with selenite. The digestibility of Zn was similar in the three treatments (Rider et al., 2010). Fontagné-Dicharry and colleagues studied the influence of Se chemical forms and levels on antioxidant status in rainbow trout fry. They found that plant-based diets need to be supplemented with Se to ensure adequate antioxidant status. In the same study, the Se availability was higher in a diet supplemented with Se yeast than in a diet supplemented with selenite or a non-supplemented diet (Fontagne-Dicharry et al., 2015). Apines-Amar and co-workers compared the absorption of Zn, Mn and Cu in rainbow trout, using one diet supplemented with inorganic salts and two diets supplemented with amino acid chelate. Higher absorption of Zn and Mn was obtained using diets supplemented with amino acid chelate, while higher absorption of Cu was obtained using the diet supplemented with inorganic salt (Apines-Amar et al., 2004). In the current study, we will provide data on the effect of the inorganic and organic forms of Zn, Se and Mn on Zn, Se and Mn availability in Atlantic salmon.

Design of experiments (DOE), a multivariate experimental design approach, offers a large number of advantages over the one-factor-at-a-time approach. Two of the most important advantages of DOE are the ability to estimate the effect of each factor individually and to study interaction effects simultaneously (Miller, 2010; Montgomery, 2008). The DOE includes a wide range of designs such as Box-Behnken, latin square, randomized complete block design, central composite, fractional factorial design, and full factorial design. The full factorial design (FFD) is the most commonly used design due to the intuitive strategy of this experimental design (Hicks and Turner, 1999). The popularity of FFD has grown in the last years in different research fields, including aquaculture. Some successful applications of the FFD within aquaculture research have been reported (Hu et al., 2011; Nicolaisen et al., 2014; Søfteland et al., 2016). For instance, Nicolaisen and co-workers used FFD as a tool to optimize rearing conditions of fish larvae (Nicolaisen et al., 2014). Moreover, FFD was applied in a study examining how nutrients can modulate the toxicological outcome of contaminants in novel diets for Atlantic salmon (Søfteland et al., 2016) and in a study of CO_2 removal method in recirculating aquaculture waters (Hu et al., 2011). Until now, the FFD have not been used in mineral availability studies in fish. This is the first study using FFD to study the chemical forms of the supplemented minerals as well as the interactions among minerals.

The aim of the present study was to compare the AA of inorganic and organic forms of Zn, Se and Mn in Atlantic salmon (*Salmo salar*) diets. The research hypotheses are (i) the AA of Zn, Se and Mn are dependent on their chemical form, (ii) the interactions between Zn, Se and Mn sources in the diet have an influence on their AA, and (iii) the AA of Zn, Se and Mn are affected by dietary phytic acid.

2. Materials and methods

2.1. Experimental design

The experiment design was a two-level FFD with four factors ($2^4 = 16$ diets). The tested factors were Zn additive source (A), Se additive source (B), Mn additive source (C) and phytic acid level (D). Two factorial levels were used and coded as "−1" and "+1" for inorganic and organic mineral additive source (factors A, B and C) or low and high phytic acid level (factor D), respectively. The factors Zn additive source (A), Se additive source (B), Mn additive source (C) are qualitative variables and the factor phytic acid level (D) is a quantitative variable. Table 1 shows the variables and levels used for each factor, and Table 2 describes the 16 experimental diets.

2.2. Experimental diets

The 16 experimental diets were produced at Skretting Aquaculture Research Centre (Stavanger, Norway). All diets contained the same type of ingredients but the proportions were adjusted to have two basal mixtures with a low and a high phytic acid level, as described in Table 3. Yttrium oxide (Y_2O_3) was added as an inert marker. Zinc sulphate monohydrate ($ZnSO_4.H_2O$, Zn 35%, Vilomix, Hønefoss, Norway), zinc chelate of glycine hydrate ($Zn(x)_{1-3}.nH_2O$, x = anion of glycine ($C_2H_4NO_2^-$), Zn 26%, Phytobiotics, Eltville, Germany), sodium selenite (Na_2SeO_3, Se 4.5% BMP, DSM nutritional products, Basel, Switzerland), L-selenomethionine ($C_5H_{11}NO_2Se$, Se 0.16%, Orffa Additives, Werkendam, The Netherlands), manganous sulphate monohydrate ($MnSO_4.H_2O$, Mn 32%, Vilomix, Hønefoss, Norway), manganese chelate of glycine hydrate ($Mn(x)_{1-3}.nH_2O$, x = anion of glycine ($C_2H_4NO_2^-$), Mn 22%, Phytobiotics, Eltville, Germany) were used as mineral additive sources. The nominal concentration of Zn, Se and Mn

Table 1
Experimental design factors (A-D) and respective levels; two factorial levels coded as "−1" and "+1" for inorganic and organic mineral additive source (factors A, B and C) or low and high phytic acid level (factor D), respectively; factors A – zinc additive source, B – selenium additive source and C – manganese additive source are qualitative variables and factor D – phytic acid level is a quantitative variable.

Factor	Level -1	Level +1
A – zinc additive source	zinc sulphate	zinc chelate of glycine
B – selenium additive source	selenite	selenomethionine
C – manganese additive source	manganous sulphate	manganese chelate of glycine
D – phytic acid level	low	high

Table 2
Full factorial design, number of experimental diets, factors and experimental responses as apparent availability (AA, %) for zinc (Zn), selenium (Se) and manganese (Mn) in Atlantic salmon fed the 16 experimental diets for 11 days; the factors Zn additive source (A), Se additive source (B), Mn additive source (C) were coded as "−1" and "+1" for inorganic and organic mineral additive source, respectively; the factor phytic acid level (D) was coded as "−1" and "+1" for low and high phytic acid level, respectively; Factor level codes are shown as "-1" or "+1" followed by the real factor level (shown between parenthesis); ZnSul = Zn sulphate, ZnCheGly = Zn chelate of glycine, SeMet = selenomethionine, MnSul = Mn sulphate, MnCheGly = Mn chelate of glycine. The AA (%) was determined by AA = 100 – [100*(yttrium in diet/yttrium in faeces)*(Zn or Se or Mn in faeces/Zn or Se or Mn in diet)]; The AA (%) values are presented as average ± standard deviation (n = 3).

Diet	Factors				Response (AA, %)								
	A Zn additive source	B Se additive source	C Mn additive source	D Phytic acid level	Zn			Se			Mn		
1	-1 (ZnSul)	-1 (Selenite)	-1 (MnSul)	-1 (low)	31	±	12	63	±	4	31	±	12
2	1 (ZnCheGly)	-1 (Selenite)	-1 (MnSul)	-1 (low)	31	±	3	66	±	2	21	±	2
3	-1 (ZnSul)	1 (SeMet)	-1 (MnSul)	-1 (low)	34	±	9	74	±	2	35	±	16
4	1 (ZnCheGly)	1 (SeMet)	-1 (MnSul)	-1 (low)	34	±	5	74	±	2	24	±	14
5	-1 (ZnSul)	-1 (Selenite)	1 (MnCheGly)	-1 (low)	24	±	1	64	±	4	4	±	10
6	1 (ZnCheGly)	-1 (Selenite)	1 (MnCheGly)	-1 (low)	35	±	2	61	±	3	27	±	12
7	-1 (ZnSul)	1 (SeMet)	1 (MnCheGly)	-1 (low)	44	±	6	76	±	4	14	±	10
8	1 (ZnCheGly)	1 (SeMet)	1 (MnCheGly)	-1 (low)	29	±	13	67	±	6	31	±	17
9	-1 (ZnSul)	-1 (Selenite)	-1 (MnSul)	1 (high)	27	±	8	58	±	5	20	±	13
10	1 (ZnCheGly)	-1 (Selenite)	-1 (MnSul)	1 (high)	34	±	5	68	±	4	38	±	7
11	-1 (ZnSul)	1 (SeMet)	-1 (MnSul)	1 (high)	27	±	6	69	±	4	28	±	15
12	1 (ZnCheGly)	1 (SeMet)	-1 (MnSul)	1 (high)	36	±	5	72	±	4	32	±	14
13	-1 (ZnSul)	-1 (Selenite)	1 (MnCheGly)	1 (high)	38	±	5	65	±	11	36	±	12
14	1 (ZnCheGly)	-1 (Selenite)	1 (MnCheGly)	1 (high)	45	±	16	64	±	10	25	±	32
15	-1 (ZnSul)	1 (SeMet)	1 (MnCheGly)	1 (high)	28	±	4	62	±	8	1	±	10
16	1 (ZnCheGly)	1 (SeMet)	1 (MnCheGly)	1 (high)	23	±	5	68	±	3	9	±	13

Table 3
Formulation and composition of the experimental diets (n = 16); all the diets were prepared using the same ingredients but the proportions were adjusted to have basal mixtures for low and high phytic acid; low phytic acid and high phytic acid refers to the concentration of phytic acid.

Ingredients (%)	Low phytic acid	High phytic acid
Wheat	8.3	8.1
Corn gluten	15.0	15.0
Hi-pro soya	14.4	10.0
Wheat gluten	20.0	14.3
Soya protein concentrate	10.0	20.0
Fish meal[a]	5.0	5.0
Fish oil[a]	9.9	10.1
Rapeseed oil[b]	12.3	12.6
Microingredients and premixes[c]	5.4	5.2
Experimental premixes (zinc, selenium and manganese)[d]	0.6	0.6
Proximate composition (analysed, n = 8)	Average ± SD	Average ± SD
Dry weight (%)	92.2 ± 0.6	92.6 ± 0.5
Lipid (%)	21.2 ± 0.6	22.0 ± 0.3
Protein, analysed as N × 6.25 (%)	48 ± 2	46 ± 2
Ash (%)	4.2 ± 0.2	4.3 ± 0.1
AD of protein (%) (n = 2)	93.1 ± 0.1	93.24 ± 0.05
AD of lipid (%) (n = 2)	97.8 ± 0.3	97.6 ± 0.2
Phytic acid (μmol g^{-1}) (n = 2)	11.3 ± 0.1	12.0 ± 0.1

[a] North-Atlantic.
[b] European, non-GM.
[c] Contains monoamonium phosphate, histidine HCl, yttrium oxide, L-lysine and DL-methionine and astaxanthin; standard vitamin and mineral mix, excluding the target minerals zinc, selenium and manganese.
[d] The experimental premixes were manually prepared and added to the diets following the full factorial matrix.

were 150 mg kg^{-1} diet, 0.5 mg kg^{-1} diet and 25 mg kg^{-1} diet, respectively. The concentration of Zn, Se and Mn in the ingredients was determined and this information was taken into consideration when preparing the diet formulation.

2.3. Fish and experimental conditions

The feeding trial took place at Lerang Research Station (Skretting Aquaculture Research Centre, Lerang, Norway) according to Norwegian (FOR-2015-06 − 18-761) and European legislation (Directive 2010/63/EU). Atlantic salmon (SalmoBreed strain) were reared in seawater with continuous light (24 h). The salmon used in this trial had a mean initial body weight of 294 ± 11 g (n = 1584). Each diet was tested in triplicate tanks, thus the salmon were randomly distributed to 48 tanks (450 L tanks) and each tank contained 33 fish. The fish were acclimated in their respective tank for 20 days while being fed a commercial diet (Spirit 3 mm, Skretting). The fish were fed the experimental diets for 11 days. The fish were fed using automatic feeders three times a day. Collection and weighing of uneaten diet were conducted 30 min after the end of each meal and based on these data, diet intake was calculated. The average diet intake was 0.55 ± 0.08% (n = 48) of body weight day^{-1}. During the trial, the average saturation of dissolved oxygen in the seawater was 101 ± 5% (n = 9) and the average temperature in the seawater was 11.9 ± 0.3 °C (n = 29).

2.4. Sampling

Fish were killed by overdose using 6 mL of tricaine methanesulphonate stock solution per L^{-1} of water (PharmaQ, Bergen, Norway). Subsequently, the fish was individually weighed and length measured. A pooled sample of faeces from fish (n = 20) from the same tank was collected into a plate by stripping from the ventral fin to anus. The sample was collected in a 50 mL falcon and immediately stored at −20 °C. The samples were kept at −20 °C until further analysis.

2.5. Chemical analysis

2.5.1. Chemicals and reagents

Analytical reagent grade chemicals and Milli-Q® water

(18.2 MΩ cm) (EMD Millipore Corporation, Billerica, MA, USA) were used throughout the study unless otherwise stated. Hydrogen peroxide (H_2O_2, Emsure® ACS, ISO, 30% *w*/w) was obtained from Merck (Darmstadt, Germany). Nitric acid (HNO_3, trace select, ≥ 69.0% w/w) was acquired from Sigma-Aldrich (St. Louis, MO, USA). High purity ethylenediaminetetraacetic acid (EDTA) was purchased from Leco Corporation (Saint Joseph, MI, USA). Sulphanilamide ($C_6H_8N_2O_2S$, 98% purity) was acquired from Alfa Aesar GmbH & Co (Karlsruhe, Germany).

2.5.2. Biochemical analysis of diets and faeces

Diets were homogenised for 10 s at 3000 rpm using a knife mill (GM 300, Retsch GmbH, Haan, Germany) and kept at 4 °C until further analysis. Faeces samples were freeze dried for 72 h at −80 °C, homogenised with a pestle and mortar into a fine powder and stored at room temperature until further analysis. The 16 diets were analysed for dry matter, ash content, lipid content, protein content, and faeces were analysed for lipid content and protein content following standard procedures. Dry matter content was measured gravimetrically after drying at 104 °C for 24 h, ash content was determined by combustion in a muffle furnace flame combustion at 550 °C for 16–18 h, and lipid content was determined after acid-extraction (Lie, 1991). Total nitrogen was measured with a nitrogen analyser (Vario Macro Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany) according to AOAC official methods of analysis (AOAC, 2002), and protein calculated as N × 6.25. The instrument was calibrated with EDTA (certified reference material). Sulphanilamide and a standard meat reference material (SMRD 2000, LGC Standards, Teddington, UK) were used as control samples. The phytic acid content was determined in the two basal diets (i.e. low phytic acid diets and high phytic acid diets) following the procedure described by Zeller and co-workers (Zeller et al., 2015). Briefly, samples were extracted with a solution containing 0.2 M EDTA and 0.1 M NaF (pH 10). For sample clean-up, the extracts were filtered through a 0.2 µm cellulose acetate filter (VWR, Darmstadt, Germany) into a Microcon® filter (cutoff 30 kDa) device (Millipore, Bedford, MA, USA) following manufacturer's instructions. Filtrates were analysed using ion chromatography (Carbo Pac 200 column) and UV detection at 290 nm after post-column derivatisation using an ICS-3000 system (Dionex, Idstein, Germany).

2.5.3. Element determination in seawater, ingredients, diets and faeces by inductively coupled plasma mass spectrometry

The Zn, Se and Mn concentration in the seawater was measured. Seawater samples were collected from the water inlet ($n = 3$) and from tanks fed diets 1, 8, 9 and 16 ($n = 3$) using 50 mL plastic containers. The samples were kept at 4 °C and shipped on ice to an accredited laboratory where the analyses were performed (ALS, Oslo, Norway). The determination of Zn, Se and Mn was performed by inductively coupled plasma mass spectrometry (ICP-MS) according to the method EPA 200.8 (EMSL, 1996).

Ingredients were homogenised for 10 s at 10000 rpm using a knife mill (GM 300, Retsch GmbH, Haan, Germany) and kept at room temperature until further analysis. The samples were decomposed using microwave assisted acid digestion based on the procedure previously described (Julshamn et al., 2007). Briefly, approximately 0.2 g of diet was digested using 2 mL of HNO_3 (69% w/w) and 0.5 mL of H_2O_2 (30% w/w) in a Milestone-MLS − 1200 microwave oven (Milestone Inc., Shelton, CT, USA). The digested samples were subsequently diluted to 25 mL with Milli-Q® water. A similar procedure was applied to digest the ingredients and the faeces samples. Approximately 0.2 g of sample was digested using 2 mL of HNO_3 in an ultrawave digestion system (UltraWAVE, Milestone, Sorisole, Italy). The samples were capped and placed in the ultrawave system with a container of 130 mL Milli-Q® water and 5 mL H_2O_2. The extracts were then diluted to 25 mL with Milli-Q® water. The Zn, Se, Mn and yttrium concentrations were determined in the ingredients, diets and faeces by ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) equipped with an auto sampler (FAST SC-4Q DX, Elemental Scientific, Omaha, USA). The eluate was introduced directly into the nebulizer tube of the ICP-MS and Zn, Se, Mn and yttrium were detected at *m/z* 66, 78, 55, and 89, respectively, in the KED reaction mode. A solution of germanium and rhodium was added on-line for correction of instrumental drift during the analysis. As specified by the manufacturer, the tuning of the ICP-MS was performed using a tuning solution (1 ppb tuning solution B, Thermo Fisher, in 2% HNO_3 and 0.5% HCl) prior to analysis. Data were collected and processed using the Qtegra ICP-MS software (Thermo Scientific, version 2.1, 2013). For the quantitative determination of Zn, Se and Mn, an external calibration curve (10 to 500 ng mL^{-1}) was used. Two certified reference materials (CRM) were included to assess the accuracy of the method, i.e. lobster hepatopancreas (TORT-3; National Research Council Canada, Ottawa, Ontario, Canada) and oyster tissue (SMR 1566b; National Institute of Standards and Technology, Gaithersburg, USA). The obtained values for each CRM ($n = 5$) were in agreement with the certified values.

2.6. Calculations and statistical analysis

The experimental design matrix was drawn by using R Commander Plugin for DOE (Groemping, 2014; R Core Team, 2018). The apparent digestibility (AD) was determined for protein and lipid, and the apparent availability (AA) was determined for minerals. The formula used to determine AD (%) and AA (%) was previously described by Cho and Slinger (Cho and Slinger, 1979). The AD of protein and AD of lipid was determined according to Eq. 1:

$$\text{AD}\ (\%) = 100 - \left(100\frac{\text{yttrium in diet}}{\text{yttrium in faeces}} * \frac{\text{protein or lipid in faeces}}{\text{protein or lipid in diet}}\right) \quad (1)$$

The AA of Zn, AA of Mn and AA of Se was determined according to Eq. 2:

$$\text{AA}\ (\%) = 100 - \left(100\frac{\text{yttrium in diet}}{\text{yttrium in faeces}} * \frac{\text{Zn or Se or Mn in faeces}}{\text{Zn or Se or Mn in diet}}\right) \quad (2)$$

The AA of Zn, AA of Se and AA of Mn (%) were used as responses for statistical analysis. Data analysis was performed using the R commander plugin for DOE (Groemping, 2014; R Core Team, 2018). The Ryan-Joiner test was performed to evaluate normality of the data and the Grubb's test was performed to check for outliers at a confidence level of 95%. A ranking test to choose the diet with highest availability simultaneously for Zn, Se and Mn was performed at a confidence level of 95%. This ranking was performed comparing the median to the value of AA obtained in each replicate and only the values above the second quartile were considered. A two-tailed *t*-test was used to determine the magnitude of the effect of the main factors and interactions at a confidence level of 95%.

3. Results

3.1. Experimental diets

The proximate composition of the diets (i.e. dry matter, ash, crude lipid and crude protein) used in this work is presented in Table 3. The measured values for dry matter, ash, crude lipid and crude protein were similar to the expected values.

The phytic acid concentration in low level and high level phytic acid diets was 11.3 ± 0.1 µmol g^{-1} ($n = 2$) and 12.0 ± 0.1 µmol g^{-1} ($n = 2$), respectively. The total phytic acid content is a sum of myo-inositol hexakisphosphate (InsP6) and myo-inositol pentakisphosphate (InsP5) (i.e. Ins(12345)P5- and Ins(12456)P5) as these were the isomers quantified in the diets of this study.

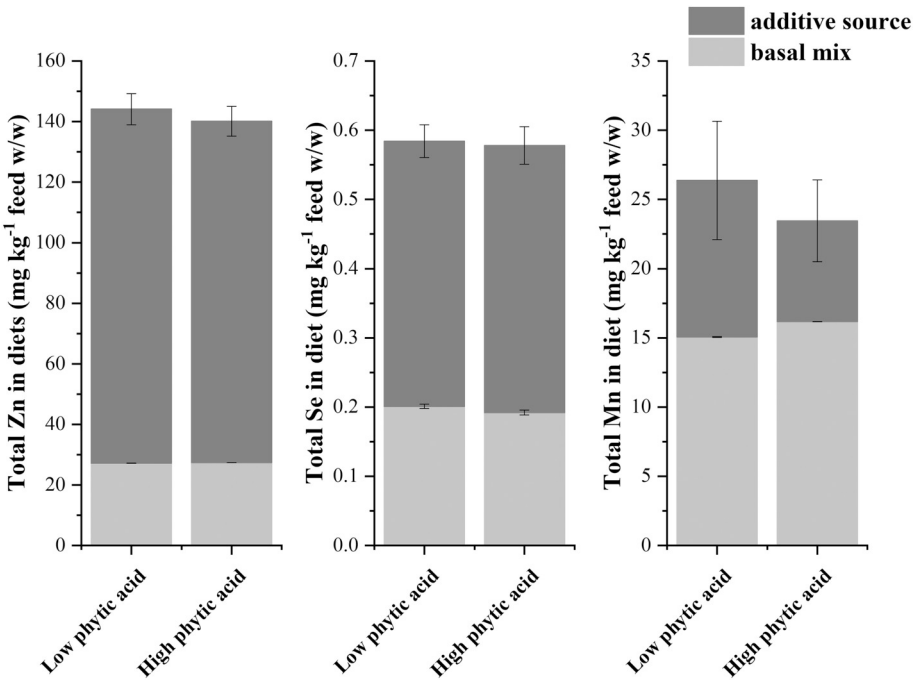

Fig. 1. Total concentration (mg kg^{-1} feed w/w) of zinc (Zn), selenium (Se) and manganese (Mn) in the low phytic acid diets ($n = 8$) and in the high phytic acid diets ($n = 8$); total concentration values are presented as average ± standard deviation; the light grey represents the concentration of Zn, Se and Mn from ingredients and the dark grey represents the concentration of Zn, Se and Mn added as additive source.

The total concentrations of Zn, Se and Mn in the low phytic acid diets were 144 ± 5 mg of kg^{-1} feed w/w ($n = 8$), 0.58 ± 0.02 mg kg^{-1} feed w/w ($n = 8$) and 26 ± 4 mg kg^{-1} feed w/w ($n = 8$), respectively. The total concentrations of Zn, Se and Mn in the high phytic acid diets were 140 ± 5 mg of kg^{-1} feed w/w ($n = 8$), 0.58 ± 0.03 mg kg^{-1} feed w/w ($n = 8$) and 24 ± 3 mg kg^{-1} feed w/w ($n = 8$), respectively. The total concentration of Se was slightly above the current upper limit (0.5 mg kg^{-1}) and the total concentration of Mn and Zn were below the current upper limit (current upper limit for total Mn is 100 mg kg^{-1} and for total Zn it is 180 mg kg^{-1} in salmonids feeds). There was little variation between the analysed mineral concentrations and the nominal concentrations (i.e. 150 mg Zn kg^{-1} diet, 0.5 mg Se kg^{-1} diet and 25 mg Mn kg^{-1} diet), and there was also little variation between the total concentration of Zn, Se and Mn in the low phytic acid and high phytic acid diets. As can be seen in Fig. 1, the total concentration of Zn, Se and Mn in the low phytic acid diets and in the high phytic acid diets is a sum of the concentration of Zn, Se and Mn from the basal mix and the concentration of Zn, Se and Mn supplemented as additive source.

3.2. Zinc, selenium, and manganese concentrations in the seawater

The Zn, Se, and Mn concentrations in the seawater inlet were 3.5 ± 0.3 µg L^{-1} ($n = 3$), 9 ± 3 µg L^{-1} ($n = 3$) and 4 ± 1 µg L^{-1} ($n = 3$), respectively. In the seawater samples collected from the tanks the Zn, Se, and Mn concentrations were 4 ± 1 µg L^{-1} ($n = 12$), 11 ± 2 µg L^{-1} ($n = 12$) and 3 ± 2 µg L^{-1} ($n = 12$), respectively. The concentrations between the analysed minerals in the seawater at inlet and the outlet of the tanks were not statistically different.

3.3. Apparent availability of zinc, selenium and manganese

Faeces are composed of undigested material but also endogenous secretions (e.g. digestive enzymes, bile secretions, sloughed epithelium and mucus). The term apparent availability is used to acknowledge the fact that the values obtained are not only related to the unabsorbed minerals from the diet but, also digestive secretions (NRC, 2011). Table 2 shows the factors chosen, the different factor level settings and the estimated values for AA of Zn, Se and Mn (%) in Atlantic salmon. The two highest AA of Zn (44 ± 6% and 45 ± 16%, $n = 3$) were obtained using diet 7 (i.e. low phytic acid diet, supplemented with Zn sulphate, SeMet and Mn chelate of glycine) and diet 14 (i.e. high phytic acid diet, supplemented with Zn chelate of glycine, selenite and Mn chelate of glycine), respectively. The two lowest AA of Zn (24 ± 1% and 23 ± 5%, $n = 3$) were obtained using diet 5 (i.e. low phytic acid diet, supplemented with Zn sulphate, selenite and Mn chelate of glycine) and diet 16 (i.e. high phytic acid diet, supplemented with Zn chelate of glycine, SeMet and Mn chelate of glycine). The highest AA of Se (76 ± 4%, $n = 3$) was obtained using diet 7 (i.e. low phytic acid diet, supplemented with Zn sulphate, SeMet and Mn chelate of glycine), while, the lowest AA of Se (58 ± 5%, $n = 3$) was obtained using diet 9 (i.e. high phytic acid diet, supplemented with Zn sulphate, selenite and Mn sulphate). The highest AA of Mn (38 ± 7%, $n = 3$) was obtained using diet 10 (i.e. high phytic acid diet, supplemented with Zn chelate of glycine, selenite and Mn sulphate). The lowest AA of Mn (1 ± 10%, $n = 3$) was obtained using diet 15 (i.e. high phytic acid diet, supplemented with Zn sulphate, SeMet and Mn chelate of glycine).

3.4. Main factors and interactions effects

The effect of the main factors and their interactions on the AA of Zn, Mn and Se are presented graphically in three Pareto charts (Fig. 2). The Pareto chart for Zn shows that the factor B (Zn additive source) did not significantly affect the AA of Zn ($p > .05$) (see Fig. 2(a)). However, the interaction between Zn additive source and Se additive source (A × B), the interaction between Se additive source and the phytic acid level (B × D), the interaction between Zn, Se and Mn additive sources (A × B × C) and the interaction between Se additive source, Mn additive source and phytic acid level (B × C × D) significantly affected the AA of Zn ($p < .05$) (See Fig. 2(a)). All these interactions showed a negative interactive effect which lowered the AA of Zn. The effect of B × D had the highest interaction effect on the AA of Zn, followed by B × C × D, A × B × C, and A × B, respectively. The higher the t-value, the higher the effect on the AA of Zn. As can be seen in Fig. 2(a), the Se additive source (factor B) had an effect in all the interactions with a significant effect for AA of Zn. The effect of interactions between Se additive source and phytic acid level (i.e. B × D, interaction between Se additive source and the phytic acid level, and B × C × D, interaction

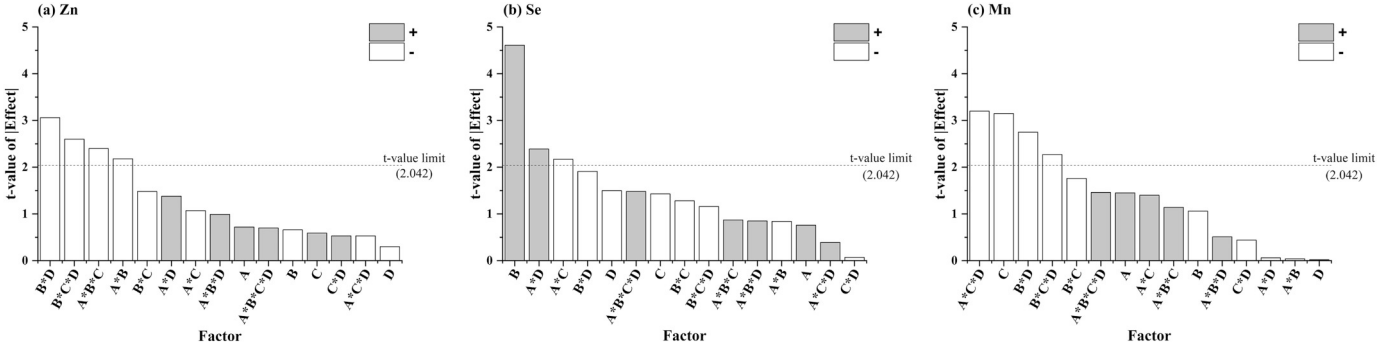

Fig. 2. Pareto chart showing the t-value of the effect using individually apparent availability (AA, %) of zinc (Zn) (a), selenium (Se) (b) and manganese (Mn) (c); the horizontal axis shows the factors and interactions ordered according to their magnitude, the factors are Zn additive source (A), Se additive source (B), Mn additive source (C) and phytic acid level (D); the vertical axis shows the t-value of the absolute effect; in grey, the effects with positive t-value and, in white, the effects with negative t-value; the reference line on the chart is the t-value limit ($\alpha = 0.05$; d.f. = 30); any effect that is over this reference line is statistically significant ($p < .05$).

between Se additive source, Mn additive source and phytic acid level) had significant effects ($p < .05$) on the AA of Zn. The Pareto chart for Se shows that Se additive source (factor B) significantly affected the AA of Se ($p < .05$) (Fig. 2(b)). Factor B had a positive t-value suggesting that this factor should be kept at the level "+1" and that the organic Se (SeMet) had higher availability than inorganic Se (selenite). The effect of factor B had the highest t-value of absolute effect on the AA of Se followed by A × D and A × C, respectively. The interactions between Zn additive source and phytic acid level (A × D) and between Zn and Mn additive sources (A × C) significantly affected the AA of Se ($p < .05$). The interaction A × D showed a positive effect and the interaction A × C showed a negative effect on the AA of Se (Fig. 2(b)). The Pareto chart for Mn shows that the effect of factor C (Mn additive source) significantly affected the AA of Mn ($p < .05$) and this factor had a negative t-value effect (Fig. 2(c)). This suggests that to have a higher AA of Mn, factor C should be kept at the level "-1", which implies using an inorganic source of Mn (Mn sulphate). Moreover, the interactions between Se additive source and phytic acid level (B × D), between Zn additive source, Mn additive source and phytic acid level (A × C × D), and between Se additive source, Mn additive source and phytic acid level (B × C × D) significantly affected the AA of Mn ($p < .05$). These interactions had a negative effect on the AA of Mn. The Mn additive source (C) and the interaction between Zn additive source, Mn additive source and phytic acid level (A × C × D) had the highest t-value of the absolute effect on the AA of Mn followed by the interaction between Se additive source and phytic acid level (B × D) and the interaction between Se additive source, Mn additive source and phytic acid level (B × C × D). This indicates that Mn additive source and the interaction between Zn additive source, Mn additive source and phytic acid level had the highest effect on AA of Mn.

4. Discussion

The average AD of protein and the average AD of lipid was approximately 93% and 98%, respectively. Our results are in line with other studies in Atlantic salmon also fed plant-based diets (i.e. lower fish meal inclusion) (Espe et al., 2012; Pratoomyot et al., 2010; Storebakken et al., 2000). Espe and co-workers (Espe et al., 2012) as well as Storebakken and co-workers (Storebakken et al., 2000) have reported AD of protein values between 89 to 94% and 88.6 to 93.6%, respectively. Moreover, the AD of lipid obtained in this study (~ 98%) was comparable to the values obtained by Storebakken and co-workers (90.9–93.1%) (Storebakken et al., 2000) and Pratoomyot and co-workers (90.5%) (Pratoomyot et al., 2010). Taken together, this indicates that the experimental diets had a good protein and lipid digestibility.

4.1. Apparent availability of zinc, selenium and manganese

The total concentration of Zn, Se and Mn in the experimental diets is a sum of the concentration of Zn, Se and Mn from the basal mix and the concentration of Zn, Se and Mn supplemented as additive source (Fig. 1). This study demonstrated that in Atlantic salmon, the availability of Zn, Se and Mn from a diet is dependent on the diet composition, the chemical form of the Zn, Se and Mn, the interactions between Zn, Se, Mn, and the interactions between Zn, Se, Mn and dietary phytic acid. The obtained values for AA of Zn (23 to 45%) reported in this study are similar to the AA of Zn found in rainbow trout fed plant-based diets supplemented with Zn sulphate (34.5 to 40.4%) (Prabhu et al., 2018b). However, the values for AA of Zn in our study are lower when compared with the Zn availability values found in coho salmon fed diets supplemented with Zn sulphate (60.0 to 89.3%) (Sugiura et al., 1998). The difference previously reported regarding the AA of Zn might be related to the fact that, purified diets were used in the study performed in coho salmon by Sugiura and co-workers (Sugiura et al., 1998). It is well known that AA of minerals is higher in purified diets than in practical diets (NRC, 2011). Moreover, the lower values obtained for AA of Zn can be related to the high dietary level of Zn. The Zn concentration in diets was ~150 mg kg^{-1} and the requirement of Zn for Atlantic salmon is between 37 and 67 mg kg^{-1} (Maage and Julshamn, 1993). In general, the higher the dietary level in comparison to the requirement, the lower the AA will be. This was reported by Rodehutscord and co-workers (Rodehutscord et al., 2000), who found that the apparently absorbed proportion of P became lower with increasing P dietary levels above the requirement (Rodehutscord et al., 2000). The AA of Se obtained in our study (58 to 74%) was similar to the Se availability found in a study performed in Atlantic salmon post-smolts fed a diet supplemented with selenite (63.9 ± 4.26%) (Bell and Cowey, 1989). However, in the same study of Atlantic salmon post-smolts fed a diet supplemented with SeMet, the value obtained for AA of Se (91.6 ± 1.0%) was higher (Bell and Cowey, 1989) than the values of AA of Se obtained in current study (58 to 74%). The values obtained for AA of Se in our study are slightly lower than the ones obtained in a study in rainbow trout fed plant-based diets supplemented with selenite (79.8 to 81.9%) (Prabhu et al., 2018b). Regarding the AA of Mn, the values obtained in our study (1 to 38%) were similar to the ones obtained for Mn availability in rainbow trout (4.2 to 53.7%, except wheat gluten diet (67.7%) (Sugiura et al., 1998) and 6.6 to 31% (Prabhu et al., 2018b) and coho salmon (5.1 to 52.6%). The low AA obtained for Mn (1 to 38%) can be related to the high dietary level of Mn. The Mn concentration in diets was ~25 mg kg^{-1} and the requirement of Mn for Atlantic salmon is between 7.5 and 15 mg kg^{-1} (Lorentzen et al., 1996).The AA determination was based on the ratio between Mn in diet and Mn in faeces. As a result of a higher level of Mn analysed in faeces than Mn in diet (i.e. diets 5, 14, 15, 16;

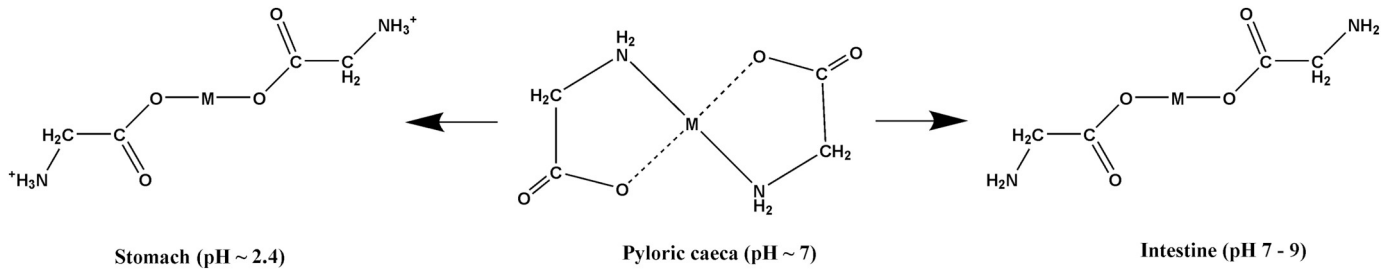

Fig. 3. – Changes in chemical conformation of glycine chelate during the fish intestinal tract: stomach (pH ~ 2.4), pyloric caeca (pH ~ 7) and intestine (pH 7–9).

supplemented with Mn chelate of glycine), negative values for AA of Mn for some of the replicates (within the same diet) were obtained. A similar finding was reported by Sugiura and co-workers in coho salmon fed diets supplemented with Mn sulphate (Sugiura et al., 1998). Manganese is secreted via bile into the gut, thus faecal Mn includes a portion of endogenous Mn in addition to the unabsorbed Mn. This could be the reason for having more Mn in the faeces than in the diet, explaining the higher standard deviation values for AA of Mn in diets 5, 14, 15, 16 (see Table 2).

4.2. Apparent availability is affected by the chemical form of zinc, selenium and manganese

One of the aims of this study was to evaluate the effect of the chemical form of Zn, Se and Mn on the AA of Zn, Se and Mn. Selenium is the only element for which there is evidence of a higher availability of organic sources over inorganic forms in fish (Prabhu et al., 2016). The result of this study shown that the organic Se (SeMet) had higher availability than inorganic Se (selenite). This is in agreement with other studies, which have demonstrated that organic sources of Se (e.g. SeMet) are more available than selenite to fish (Bennett et al., 1986; Dominguez et al., 2017; Lorentzen et al., 1994; Wang and Lovell, 1997; Wang et al., 2007). Regarding salmonid studies, SeMet was found to be the most available form of dietary Se to Atlantic salmon when compared with SeCys and selenite (Bell and Cowey, 1989), and in rainbow trout when compared with selenite (Fontagne-Dicharry et al., 2015; Rider et al., 2010). This study demonstrated that Zn organic and inorganic sources had similar AA. A similar finding was reported by Maage and colleagues. In their study, similar Zn availability was obtained in Atlantic salmon fed diets supplemented with organic Zn source (Zn gluconate) and inorganic Zn source (Zn sulphate) (Maage et al., 2001). Regarding Mn, inorganic source (Mn sulphate) had higher availability than organic source (Mn chelate of glycine). Conversely, a study in rainbow trout reported higher Mn availability when supplemented as organic source (Mn amino acid chelate) than inorganic source (Mn sulphate) (Apines-Amar et al., 2004).

Several studies have discussed the availability of organic versus inorganic forms of minerals in fish (Apines-Amar et al., 2004; Bell and Cowey, 1989; Dominguez et al., 2017; Fontagne-Dicharry et al., 2015; Lorentzen et al., 1994; Rider et al., 2010; Wang and Lovell, 1997; Wang et al., 2007). In this study, three organic minerals were used; Zn chelate of glycine, SeMet and Mn chelate of glycine. These compounds are grouped as organic minerals but they differ in their chemical properties. Selenomethionine is a biological synthesised molecule where Se is covalently bound to two carbon atoms creating an amino acid containing Se (replacing sulphur) (Shils and Shike, 2006). Zinc chelate of glycine and Mn chelate of glycine are products of a chemical reaction, mixing the inorganic mineral with the glycine amino acid. The glycine establishes two chemical bonds with the metal forming a ring structure; one covalent bond between the metal and the nitrogen in the NH_2 group from the glycine and one ionic chemical bond between the metal ion and the oxygen from the –COOH group (Ashmead, 2012b). Glycine is a small ligand and, as metal ion can form octahedral transition metal complexes, it is possible to find a metal atom such Zn or Mn attached to one, two or three glycine anions. The abundance of each form (i.e. ZnGly, $ZnGly_2$, $ZnGly_3$ or MnGly, $MnGly_2$, $MnGly_3$) is dependent on the molar ratio between the metal ion and glycine (Murphy and Martell, 1957). This creates some complexity in terms of understanding the availability of Zn and Mn chelate of glycine. Mineral absorption occurs mainly in the intestine and there is a concern regarding the stability of the chelate compounds until they reach the intestine (Goff, 2018). One assumption considers that the digestive tract fluids and chyme may contain molecules that can act as ligands. Thus, if any of these ligands have higher stability constants then they could pull the metal ion from the glycine chelate. The temperature, concentration and pH in the luminal environment have also influence on the stability constants, hence the affinity of the metal to the ligand (Brown and Zeringue, 1994). For example, glycine has a pKa of 2.35 and a pKb of 9.78 (Owen, 1934) and the InsP6, a possible ligand, has 12 ionizable protons, six of them have a pKa $\geq$ 5.2 and the remaining pKa values are $<$ 3.2 (Turner et al., 2006). This means that the metal ion will bind glycine or phytic acid depending on the intestinal conditions (temperature, pH, ligand concentration). Another assumption is that glycine chelate changes its chemical nature with changes in pH throughout the gastrointestinal tract (Fig.3). In the stomach (pH ~ 2.4), the bond between the metal and the nitrogen in the NH_2 group break; but, the ion continues to be attached to the glycine via carboxyl bonds. In the pyloric caeca (pH ~ 7), the molecule is a chelated configuration with the amino acid ligands forming heterocyclic rings with the metal ion. In the intestine (pH 7–9), the amine bond from the nitrogen to the metal is once again broken as occurred earlier in the acid pH environment. These changes in pH result in a molecule in which the metal continues to be bound to the amino acid via the carboxyl bond but it is not a chelate as such (Ashmead, 2012a). The availability of Zn and Mn chelate of glycine throughout the intestinal tract is complex, as the changes in Zn and Mn chelate of glycine chemical conformation due to pH can influence the route of uptake. Our previous research performed *in vitro* using rainbow trout derived intestinal cell line (RTgutGC) demonstrated that, in the presence of methionine, Zn uptake increased; whereas it decreased when an amino acid transport blocker was used, suggesting that Zn chelate of methionine is transported through an amino acid mediated uptake pathway (Prabhu et al., 2018a), in line with the reports for histidine facilitated uptake of Zn and Cu (Glover and Wood, 2008; Glover et al., 2003). In primary rat intestinal epithelial cells, Mn chelate of lysine has been suggested to be transported by amino acid uptake pathways (Zhang et al., 2015).

4.3. Minerals interact in the fish intestinal tract

This study is the first focused on quantifying the interactions among minerals and the effect of these interactions on mineral AA. Mineral interactions are known to occur in the fish gastrointestinal tract (Watanabe et al., 1997). The exact location of these interactions is not fully understood, but evidence suggests that Se and Mn may share the Zn transporters systems (Cousins, 2012). Moreover, Mn^{+2} and Zn^{+2} ions may compete for common ligands as the positively charged metal

ions become stable in the presence of anions (Crichton, 2012). This could explain the interaction seen between factors Zn, Se and Mn additive sources (A, B and C, respectively in Table 1). The interaction between Zn and Se additive sources (A × B) had a significant effect on AA of Zn suggesting that there is an interaction between Zn and Se additive sources that impacts Zn availability. A Zn-Se antagonist effect where a Zn-Se complex was formed in the intestinal tract of rats fed wheat grain was previously described (House and Welch, 1989). Moreover, an antagonist effect of Mn-Se was also previously reported in pigs (Burch et al., 1975) which can corroborate our results of interaction effect between Mn and Se.

In mineral nutrition, it would be appropriate to have a diet with the highest availability possible for all the minerals. In practice, this could mean that instead of choosing one diet that has higher availability of a single mineral the diet of choice takes in consideration the availability of several minerals simultaneously. In this work, it was studied which diet would have the highest mineral availability for Zn, Se and Mn simultaneously. It was found that diet 4 and diet 12 fulfilled this condition for Zn, Se and Mn. Diets 4 and 12 were supplemented with organic forms of Zn and Se (i.e. Zn chelate of glycine and SeMet) and inorganic form of Mn (i.e. Mn sulphate) in two levels of phytic acid. The phytic acid concentrations in low level and high level phytic acid diets were $11.3 \pm 0.1\,\mu mol\,g^{-1}$ ($n = 2$) and $12.0 \pm 0.1\,\mu mol\,g^{-1}$ ($n = 2$), respectively. The difference in phytic acid concentrations between the low and high phytic acid level diets was not large. However, fish diets with a higher amount of phytic acid can be produced if phytic acid is supplemented in diets as a salt (Sajjadi and Carter, 2004). In this study, it was decided to change the ingredient ratios to obtain diets with low and high levels of phytic acid instead of supplementing phytic acid as a salt. This decision means to have a shorter range between the low and high phytic acid level diets (i.e. ranging from $11.3 \pm 0.1\,\mu mol\,g^{-1}$ to $12.0 \pm 0.1\,\mu mol\,g^{-1}$) but meaningful in terms of Atlantic salmon farming industry.

The diets with higher AA of Zn were diet 7 (i.e. low phytic acid diet and supplemented with Zn sulphate) and diet 14 (i.e. high phytic acid diet and supplemented with Zn chelate of glycine). This suggested that depending on the level of phytic acid present in the diet, supplementation with inorganic source or organic source of Zn should be considered. Similar evidence was found in channel catfish, where the beneficial effects of adding Zn as an amino acid chelate were greater in diets that contained high levels of phytic acid (Paripatananont and Lovell, 1995). In the fish gut, the phytic acid may bind cations such as Mn^{+2} and Zn^{+2} thus reducing the mineral availability of Zn and Mn (Kumar et al., 2012). This can explain the significant effect on AA of Mn of the interaction of Zn additive source (A), Mn additive source (C) and phytic acid level (D). Moreover, the interaction of Se additive source (B) and phytic acid level (D) had a significant effect on AA of Mn and AA of Zn. The influence of phytic acid on minerals, such as Zn, Cu, Mn, has been investigated in fish (Kumar et al., 2012). However, data regarding the influence of phytic acid on Se is lacking in fish. This is the first study reporting an interaction between Se and phytic acid. The data obtained regarding the interactions encourages further research to understand the interaction mechanisms.

5. Conclusions

The present study compared the AA of inorganic and organic forms of Zn, Se and Mn in Atlantic salmon diets using a FFD. This study demonstrated that in low fish meal practical diets for Atlantic salmon, the availability of the minerals depends on the chemical form. Inorganic source of Mn ($MnSO_4$) and organic source of Se (SeMet) had better AA; there were no significant difference in Zn availability between inorganic source and organic source of Zn. In addition, this study reports several interactions between the Zn, Se and Mn additive sources. A number of these interactions were found to have a significant impact on AA of Zn, Se and Mn. The phytic acid level did not significantly affect AA of Zn, Se and Mn. However, several interactions with phytic acid level had a significant effect on AA of Zn, Se and Mn. The knowledge obtained regarding the interactions between the different factors was achieved using the FFD as a multivariate experimental design approach. This type of design should be considered more often when studying the mineral availability in fish, as it is known that mineral availability is influenced by other minerals.

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None of the authors has any conflicts of interest to declare.

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