Development of novel methods to evaluate availability of zinc, selenium and manganese in Atlantic salmon (*Salmo salar*)

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Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

This thesis was submitted by Marta Silva to the Institute of Biology at the University of Bergen, Norway, as one of the requirements for the Degree of Philosophiae Doctor (PhD). The work described was carried out between November 2015 and April 2019 in the Feed Safety group at the National Institute of Nutrition and Seafood Research, now the Institute of Marine Research. Part of this PhD work was carried out at the National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark. This work is part of the project "APREMIA - Apparent availability and requirements of micro minerals in salmon", which is funded by the Research Council of Norway (grant no. 244490).

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Contents

Abstract

During the recent years, the composition of salmonids diets has changed from the use of mainly marine-based ingredients (e.g. fish meal and fish oil) to an increased use of plant-based ingredients (e.g. soybean meal and vegetable oil). These changes in diet composition have an impact on the mineral concentration and mineral availability. For instance, zinc (Zn) is naturally present in both fish meal and plant-based ingredients, but in different concentrations. The Zn concentration is usually higher in fish meal than in plant-based ingredients so, with the increased use of plant-based ingredients the Zn concentration tends to decrease in the basal mixes. In addition, compounds from plant-based ingredients can reduce mineral availability. For instance, phytic acid, which is typically found in plant-based ingredients, can decrease mineral availability due to its high binding affinity for metal ions. Therefore, minerals such as Zn, selenium (Se) and manganese (Mn) are supplemented to diets to cover the nutritional requirement of farmed fish. These minerals may be added as organic or inorganic forms. Taken all of this information into consideration, there is a need to study mineral availability in the current salmon feed composition which is formulated mainly using plant-based ingredients. Further knowledge in this area will provide a better understanding regarding mineral availability and necessary strategies to increase mineral availability in Atlantic salmon. Increasing mineral availability will promote fish health and robustness, but also decrease the environmental load via faeces.

Considering that several chemical species of minerals can be present in a fish feed, it was hypothesised that availability is affected by the chemical species. For that purpose, analytical methods were optimized for extraction, quantification and identification of Zn chemical species in fish feed. This included method optimization by fractional factorial design and evaluation of sample extracts by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry (SEC-ICP-MS) (**Paper I**). The impact of freshwater or seawater media ion composition and methionine chelation on Zn uptake was evaluated using a rainbow trout intestinal epithelial cell line (RTgutGC) (**Paper II**). This PhD work also compared the availability of Zn, Se and

Mn from inorganic metal salts and their organic forms in Atlantic salmon diets. Sixteen experimental diets were prepared based on a two-level full factorial design for four factors. The tested factors were Zn additive source, Se additive source, Mn additive source and phytic acid level. The Zn, Se, Mn and yttrium concentration in diets and faeces were determined using inductively coupled plasma mass spectrometry (ICP-MS) and the apparent availability of Zn, Se and Mn were estimated (**Paper III**). The availability of a nutrient depends on several factors, including solubility. An *in vitro* digestion method was developed to evaluate solubility of dietary Zn, Se and Mn in two diets for Atlantic salmon. The soluble fractions obtained were then evaluated as a measure to predict availability of Zn, Se and Mn (**Paper IV**). A summary of the work done in the PhD is described by the graphical abstract shown in Figure 1.

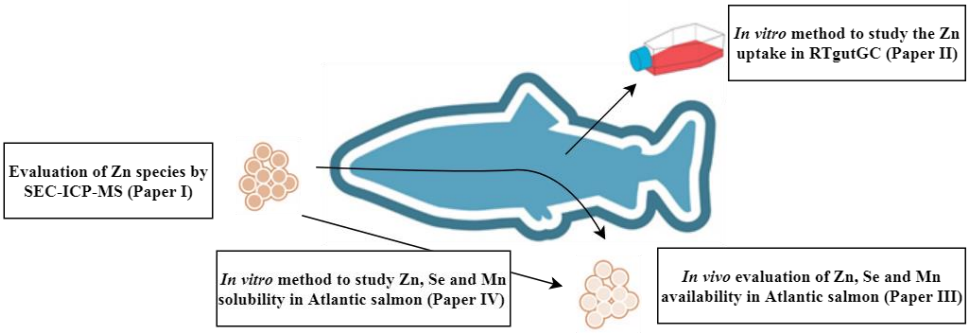

Figure 1 – Graphical abstract summarizing the PhD work.

The procedure to extract the Zn species from the diet included extraction conditions to keep the Zn species intact. The highest recovery of Zn (9.9±0.2%) was obtained using 100 mM Tris-HCl, pH 8.5 at a temperature of 4°C for 24 h. The same soluble fraction was further evaluated for Zn species by SEC-ICP-MS. Four Zn containing peaks were found, each peak with different molecular weights: peak 1 (high molecular weight), peak 2 and peak 3 (medium molecular weight) were the least abundant peaks (1-6%), while peak 4 (low molecular weight) was the most abundant peaks (84-95%) (**Paper I**). In RTgutGC, Zn uptake was not different between freshwater and seawater media ion composition. Conversely, in the presence of methionine, Zn uptake in seawater

media ion composition was lower compared to freshwater media ion composition, but only at high Zn concentrations (12 and 25 μM) (**Paper II**). The apparent availability of Zn was not affected by the Zn additive source. However, the Se and Mn additive sources affected their apparent availability. The apparent availability of Se was higher for selenomethionine than for selenite, and Mn sulphate was more available than Mn chelate of glycine. Several interactions between mineral additive sources and the phytic acid level affected the apparent availability of Zn, Se and Mn (**Paper III**). The solubility of Zn was similar in both diets tested. The amount of soluble Zn was low in the acidic hydrolysis (3-8%) and lower in the alkaline hydrolysis (0.4-2%). The solubility of Se was higher in the diet supplemented with organic mineral sources (7-34%) when compared with diet supplemented with inorganic mineral sources (3-12%). Regarding Mn, during the acidic hydrolysis the solubility was higher in the diet supplemented with inorganic mineral sources (6-25%) than in the diet supplemented with organic mineral sources (4-17%) (**Paper IV**).

Several Zn species were found in the soluble fraction of the Atlantic salmon diet studied but further work is needed to evaluate the effect of the different Zn species on availability (**Paper I**). Zinc uptake in RTgutGC cell line was influenced by the ionic concentration in the media, indicating that the intestinal ionic composition in a freshwater or in a seawater environment can influence Zn availability (**Paper II**). Regarding the apparent availability of Zn, Se and Mn in Atlantic salmon, it was demonstrated that the availability of Zn, Se and Mn depended on both the chemical form of the mineral supplemented to diets and on several interactions between Zn, Se and Mn and phytic acid level (**Paper III**). The solubility of Zn, Se and Mn was influenced both by the mineral chemical form supplemented in diet and by the gastrointestinal environment (**Paper IV**). Moreover, solubility and apparent availability of Mn showed a strong positive correlation, but a week positive correlation was seen for Zn and Se (**Paper IV**). Consequently, more work needs to be done for improving the *in vitro* digestion method.

List of publications

Paper I

Marta S. Silva, Veronika Sele, Jens J. Sloth, Pedro Araujo and Heidi Amlund (2019): "Speciation of zinc in fish feed by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry – using fractional factorial design for method optimization and mild extraction conditions", Journal of Chromatography B, Vol. 1104: 262-268, ISSN 1570-0232, https://doi.org/10.1016/j.jchromb.2018.11.010.

Paper II

P. Antony Jesu Prabhu, Thea Stewart, Marta Silva, Heidi Amlund, Robin Ørnsrud, Erik-Jan Lock, Rune Waagbø and Christer Hogstrand (2018): "Zinc uptake in fish intestinal epithelial model RTgutGC: Impact of media ion composition and methionine chelation", Journal of Trace Elements in Medicine and Biology, Vol. 50: 377-383, ISSN 0946-672X, https://doi.org/10.1016/j.jtemb.2018.07.025.

Paper III

Marta S. Silva, Saskia Kröckel, P. Antony Jesu Prabhu, Wolfgang Koppe, Robin Ørnsrud, Rune Waagbø, Pedro Araujo and Heidi Amlund (2019): "Apparent availability of zinc, selenium and manganese as inorganic metal salts or organic forms in plant-based diets for Atlantic salmon (*Salmo salar*)", Aquaculture, Vol. 503: 562-570, ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2019.01.005.

Paper IV

Marta S. Silva, P. Antony Jesu Prabhu, Robin Ørnsrud, Veronika Sele, Saskia Kröckel, Jens J. Sloth and Heidi Amlund (2019): "*In vitro* digestion method to evaluate solubility of dietary zinc, selenium and manganese in Atlantic salmon (*Salmo salar*) diets", submitted to Aquaculture.

List of abbreviations

CE	Capillary electrophoresis
Cu	Copper
EC	European Commission
EFSA	European Food Safety Authority
ESI	Electrospray ionisation
EU	European Union
Fe	Iron
FFF	Field-flow fractionation
GC	Gas chromatography
HPLC	High performance liquid chromatography
ICP	Inductively coupled plasma
ICP-MS	Inductively coupled plasma mass spectrometry
IEC	Ion exchange chromatography
InsP6	Myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate
IUPAC	International Union of Pure and Applied Chemistry
LA	Laser ablation
LC	Liquid chromatography
Mn	Manganese
MS	Mass spectrometry
P	Phosphorous
RPC	Reversed phase chromatography
RTgutGC	Rainbow trout intestinal epithelial cell line
Se	Selenium
SEC	Size exclusion chromatography
SeCys	Selenocystine
SeMet	Selenomethionine
TOF-MS	Time-of-flight mass spectrometry
Zn	Zinc

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1. Introduction

The continuous growth of the world population leads to an increasing demand for food, including fish and other seafood. The natural fish stocks of some of the most important commercial species are decreasing, thus, aquaculture is a promising approach to meet the demand for fish and other seafood [1]. In 2017, global aquaculture production included around 79.2 million tonnes of fish and seafood (i.e. fish, molluscs and crustaceans) [2]. China is by far the major producer followed by other major producers such as India, Indonesia, Vietnam, Bangladesh, Egypt and Norway [1]. In Norway, the most important species is the Atlantic salmon (*Salmo salar*), which, accounted for 94.5% of the total Norwegian aquaculture production in 2017 [3]. Other species like rainbow trout (*Oncorhynchus mykiss*), Atlantic halibut (*Hippoglossus hippoglossus*), Arctic char (*Salvelinus alpinus*), blue mussel (*Mytilus edulis*), great Atlantic scallop (*Pecten maximus*) and flat oyster (*Ostrea edulis*) are also being farmed in Norway [3].

For many years, fish meal was used as the main protein source in aquaculture diet formulation. However, high demand for and high prices of fish meal led the industry to explore and to increase the use of other protein sources [4]. There are several protein sources that have the potential of replacing fish meal in diets for many fish species without compromising their health or performance. Research has shown that it is possible to partially or fully replace fish meal with proteins from other sources [5,6]. These alternative protein sources include animal proteins from rendering or slaughter (e.g. poultry by-product meal, feather meal, blood meal from non-ruminants), plant proteins (e.g. soybean meal, maize gluten meal, wheat gluten) and novel proteins (e.g. algae, yeast, insect meal) [7].

Over the past decades, the composition of salmonid feeds has changed from the use of mainly marine-based ingredients to an increased use of plant-based ingredients. Nowadays, most commercial salmonid feeds contain around 70% of plant-based ingredients and around 30% of marine-based ingredients [8]. This change has an impact on nutrient composition of and mineral concentration in feeds. Minerals are naturally present in fish meal and in plant-based ingredients [9,10]. However, the amount of

mineral present is not always enough to cover requirements, the minerals chemical forms in plant-based ingredients may have low availability and compounds in plant-based ingredients can reduce mineral availability [11]. Thus, minerals are usually supplemented to feeds as inorganic or organic mineral sources to ensure that the nutritional requirements of fish are met. Therefore, choosing mineral sources with higher availability can reduce the needed amount of minerals supplemented to feeds and subsequently decrease environmental mineral load arising from salmon farming. Consequently, there is an increasing interest of investigating the availability of inorganic mineral sources and their respective organic forms [11].

In fish, the availability of minerals in a diet is dependent on dietary source and chemical form, and on possible interactions with other dietary components and nutrients present in the gastrointestinal tract [12,13]. Hence, to gain knowledge of concentration and chemical forms of a mineral present in ingredients and fish feeds is very important for fish nutrition purposes. Usually, the physicochemical properties of minerals are used to distinguish between them, with the most effective method for mineral determination being atomic spectroscopic techniques [14].

Overall, the use of plant-based ingredients led to a change in the mineral concentration and dietary components. Thus, there is a need for more knowledge to determine optimal mineral levels, avoiding deficiency or excess of minerals in salmon feeds. Understanding mineral availability is central to achieve this goal. At the Institute of Marine Research, there is a project with the goal of studying the apparent availability and requirement of minerals in Atlantic salmon (APREMIA). The project aims to expand the knowledge on the availability and requirement of minerals such as zinc (Zn), selenium (Se) and manganese (Mn) in plant-based diets for Atlantic salmon (*Salmo salar*). As part of this project, this PhD work evaluated the availability of Zn, Se and Mn in Atlantic salmon.

2. Background

2.1 Digestive system in Atlantic salmon

In Atlantic salmon, the digestive system can be divided in three parts: the pre-gastric, gastric, and post-gastric sections. The pre-gastric section comprises the mouth, pharynx and oesophagus. The gastric section comprises the stomach (Figure 2). The stomach mechanically digests the feed with the help of muscle contractions and relaxation movements. Also, the stomach chemically digests the feed by secreting hydrochloric acid (HCl) and pepsinogen. The pH of the stomach becomes acid due to secretion of HCl. The HCl denatures proteins present in the feed and converts the pepsinogen into its active form, pepsin [15]. The post-gastric section includes the pyloric caeca, the mid intestine, the distal intestine and anus (Figure 2).

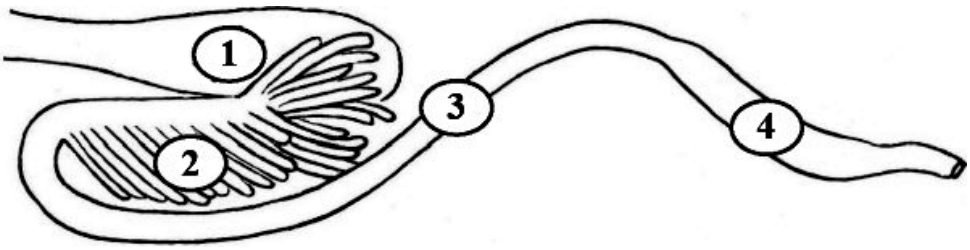

Figure 2 – Scheme of the gastrointestinal tract in Atlantic salmon; after being mechanically and chemically digested in the stomach (1), the feed enters the pyloric caeca (2) and then the mid intestine (3) and distal intestine (4) for absorption; adapted from Moldal et al., 2014 [16].

After passing the stomach, the semi-digested feed comes into the pyloric caeca. The pyloric caeca are blind extensions of the intestine that both secrete digestive enzymes and absorb nutrients [17]. In the intestine, nutrients are further digested with the help of digestive enzymes and bile salts secreted by the pancreas (e.g. trypsin, chymotrypsin, and elastase) [17]. The pH in the intestine becomes alkaline, mainly due

to secretion of bicarbonate from the pancreas, and this makes the pH optimal for the digestive enzymes. In the intestine, a layer of enterocytes line the intestinal walls forming a brush border. The enterocytes are key cells to the function of the digestive system as they have both digestive and absorptive functions [17]. Overall, digestion and absorption of nutrients may take place along most of the gastrointestinal tract (Figure 2). The main purpose of the gastrointestinal tract is to make the nutrients available for absorption [15]. Moreover, the fish gastrointestinal tract also has a number of other functions, including osmoregulation and regulation of the immune system [18].

2.2 Factors affecting nutrient bioavailability

The term bioavailability or biological availability has been defined several times over the last years. An overview of definitions for nutrient bioavailability is presented in Table 1.

Table 1 – An overview of proposed definitions for nutrient bioavailability.

Definition	Reference
A quantitative measure of utilisation of a nutrient under specific conditions to support the organism's normal structure and physiological processes.	Fox et al., 1981 [19]
The proportion of a nutrient in food which is absorbed and utilised.	O`Dell, 1984 [20]
The fraction of the dietary element which becomes biologically active.	Mutanen, 1986 [21]
A measure of the proportion of the total amount of a nutrient that is utilised for normal body functions.	Fairweather-Tait, 1992 [22]
The degree to which an ingested nutrient in a particular source is absorbed in a form that can be utilized in the metabolism by the animal.	Ammerman et al., 1995 [23]
The fraction of the ingested nutrient that is utilised for normal functions and storage.	Jackson, 1997 [24]

According to Fairweather-Tait, bioavailability is a measure of the proportion of the total amount of a nutrient that is utilised for normal body functions, and it involves various factors, each of which is affected by different dietary and physiological factors (Figure 3) [22]. In this work, the definition from Fairweather-Tait was used as it considers several factors affecting nutrient bioavailability.

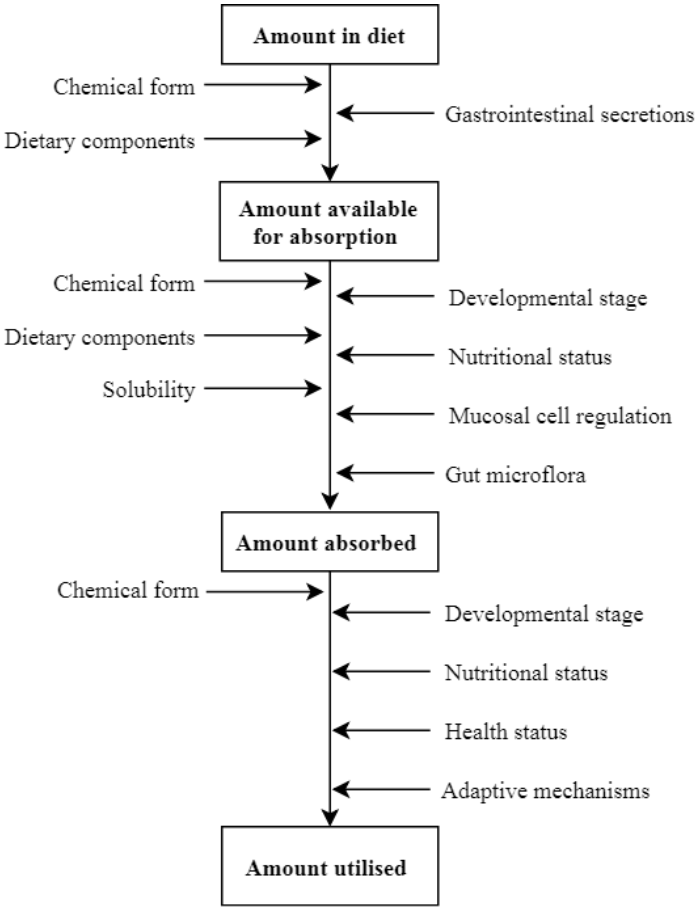

Figure 3 – Flowchart showing the different factors affecting nutrient bioavailability; adapted from Fairweather-Tait, 1992 [22]. Bioavailability is the amount of a nutrient that is utilised for normal body functions, and it involves various factors, each of which is affected by different dietary and physiological factors. The amount of a nutrient in diet which become available for absorption and the amount of a nutrient in diet which is absorbed are factors influencing bioavailability.

2.3 Mineral availability

Mineral availability is generally considered as the proportion of mineral that is absorbed from the diet [22]. As shown in Figure 3, the proportion of mineral that is absorbed from the diet can depend on dietary components, chemical form of the mineral, gastrointestinal secretions, solubility, developmental stage of the fish, nutritional status, mucosal cell regulation and gut microflora [22]. Moreover, mineral availability can be influenced by interactions with other nutrients and dietary components coexisting in the gastrointestinal tract [12,13]. Several factors can influence mineral availability simultaneously, which makes the evaluation of mineral availability challenging. In addition, it is important to take into account that fish have a close interaction with the aquatic environment. Indeed, fish take up minerals from the diet and from the water [25]. Moreover, Atlantic salmon is an anadromous fish, spending parts of their lives in both freshwater and seawater. Therefore, the osmoregulation in freshwater or seawater environment is one more factor to consider in mineral availability in Atlantic salmon [26].

The minerals need to be released from the dietary matrix and dissolved in the gastrointestinal fluids before becoming available for absorption [22,27]. The International Union of Pure and Applied Chemistry (IUPAC) defined solubility as "the analytical composition of a saturated solution expressed as a proportion of a designated solute in a designated solvent" [28]. Regardless of in which chemical form the minerals are ingested, their absorption depends on their solubility and chemical form at the point of contact with the absorbing membranes. In general, the availability of minerals is positively related to the aqueous solubility of the mineral. This principle has been demonstrated by Weerasinghe and co-workers who showed a good correlation between soluble phosphorous (P) in different feed ingredients and *in vivo* availability of P from these ingredients in feed [26]. For a nutrient to reach the apical membrane domain of the enterocyte, it must pass an unstirred water layer, emphasizing the necessity of solubility in transport across biologic interfaces. Although solubility is important for

absorption, it is important not to equate solubility of a mineral with absorption of that mineral, which also depends on other factors (Figure 3).

The mineral solubility can be determined *in vitro* using a ratio between the concentration of mineral which is soluble (i.e. [M] in soluble fraction) and the mineral concentration in diet (i.e. [M] in diet) [29], as described in Equation 1:

Equation 1

$$\text{Solubility (\%)} = \frac{\text{[M] in soluble fraction}}{\text{[M] in diet}} * 100$$

where [M] is the concentration of the mineral.

The use of *in vitro* methods has been used to study iron (Fe), P and Zn solubility in fish [30-32]. Also, the use of *in vitro* methods can be applied to study mineral uptake. For instance, a fish intestinal epithelial model established from a rainbow trout cell line (RTgutGC) was successfully used to study intestinal uptake of silver and Zn [33,34]. These cells possess an intestinal epithelial-like morphology, providing an excellent tool for assessing intestinal mineral uptake. A more conventional assessment of mineral availability in fish is measuring it *in vivo* by collecting faeces from water, by stripping faeces from the fish, dissecting the fish gut after sacrificing the fish, or collecting faeces directly from fish using anal suctioning [35]. The apparent availability of a mineral can be determined using a ratio between the concentrations of the mineral in diet and in faeces and the concentrations of an inert marker (e.g. chromium oxide or yttrium oxide) in diet and faeces, as described in Equation 2:

Equation 2

$$\text{App. availability (\%)} = 100 - \left(100 \frac{\text{[IM] in diet}}{\text{[IM] in faeces}} * \frac{\text{[M] in faeces}}{\text{[M] in diet}}\right)$$

where [M] is the concentration of the mineral, and [IM] the concentration of the inert marker.

The term apparent availability acknowledges the fact that the measured concentrations are not only related to the unabsorbed minerals from the diet but also to gastrointestinal secretions. Faeces from the fish are composed of undigested material, but also endogenous secretions, such as digestive enzymes, bile secretions, sloughed epithelium and mucus [36].

As described above, dietary components (e.g. proteins, carbohydrates, vitamins, minerals, lipids) may influence mineral availability. There is a range of possible dietary interactions influencing mineral availability, as reviewed by Hilton (1989) [37]. These interactions are divided into three groups; i) vitamin-mineral interactions, ii) mineral-mineral interactions and iii) mineral-other dietary component interactions [37]. However, the effect of the different interactions is not fully understood as several interactions can act simultaneously. Modern commercial salmonid feeds contain mostly plant-based ingredients (~70%) and marine-based ingredients (~30%) [8]. The different ingredients contribute with different dietary components. In addition to naturally occurring dietary components, different dietary components can also be supplemented to the feeds. This increases the level of complexity on understanding the effect of dietary components on mineral availability.

2.4 Minerals in feed ingredients

The shift from the use of mainly marine-based ingredients to increased use of plant-based ingredients changed the proximate composition of feeds [5]. At the Institute of Marine Research, determination of mineral concentrations are performed routinely at the inorganic chemistry laboratory. Between 2015 and 2018, data were collected regarding the mineral concentrations in several commercially used ingredients as summarized in Table 2. Minerals such as Fe, Zn, copper (Cu), Mn, cobalt (Co) and Se are naturally present in fish meal and in plant-based ingredients in different concentrations [10,38,39]. The mineral concentrations present in each type of ingredient differ greatly. For instance, Zn in fish meal ranged from 30 to 1365 mg kg^{-1} and Zn in plant-based ingredients ranged from 18 to 93 mg kg^{-1}. In fish meal, the

mineral concentrations depend on the source of raw materials and the processing method used. In plant-based ingredients, the mineral concentrations depend on fertilisation, genetic differences in plant species, and soil concentration and soil conditions (e.g. pH, ion exchange capacity) which influence mineral uptake in plants [40,41]. Also, the processing method will affect the mineral concentration in plant-based ingredients. The concentration of Fe, Zn, Cu, Mn, Co and Se found in fish meal (n=40) were 205 ± 169 mg of Fe kg $^{-1}$, 181 ± 315 mg of Zn kg $^{-1}$, 14 ± 21 mg of Cu kg $^{-1}$, 12 ± 14 mg of Mn kg $^{-1}$, 0.02 ± 0.04 mg of Co kg $^{-1}$ and 1.9 ± 1.0 mg of Se kg $^{-1}$, respectively The concentration of Fe, Zn, Cu, Mn, Co and Se in plant-based ingredients (n=76) were 146 ± 101 mg of Fe kg $^{-1}$, 46 ± 21 mg of Zn kg $^{-1}$, 8 ± 6 mg of Cu kg $^{-1}$, 28 ± 13 mg of Mn kg $^{-1}$, 0.01 ± 0.04 mg of Co kg $^{-1}$ and 0.1 ± 0.4 mg of Se kg $^{-1}$, respectively. The average mineral concentration of Fe, Zn, Cu, Co and Se was higher in fish meal than in plant-based ingredients. Moreover, the average concentration of Mn was higher in plant-based ingredients when compared with the average mineral concentration in fish meal.

Table 2 – Concentrations of some of the minerals found in fish meal (n=40) and plant-based ingredients (e.g. soybean concentrates, corn gluten meal, soybean meal, wheat gluten meal) (n=76); iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), cobalt (Co) and selenium (Se) are naturally present in fish meal and in plant-based ingredients; data shown were collected between 2015 and 2018 at the Institute of Marine Research; average concentration ± standard deviation are presented in the first line as mg kg $^{-1}$; minimum and maximum concentration are presented in brackets as mg kg $^{-1}$.

	Fe *mg kg $^{-1}$*	**Zn** *mg kg $^{-1}$*	**Cu** *mg kg $^{-1}$*	**Mn** *mg kg $^{-1}$*	**Co** *mg kg $^{-1}$*	**Se** *mg kg $^{-1}$*
Fish meal (n=40)	205 ± 169 (13-839)	181 ± 315 (30-1365)	14 ± 21 (2-77)	12 ± 14 (1-49)	0.02 ± 0.04 (0-0.10)	1.9 ± 1.0 (0-3.0)
Plant-based ingredients (n=76)	146 ± 101 (11-512)	46 ± 21 (18-93)	8 ± 6 (2-31)	28 ± 13 (2-47)	0.01 ± 0.04 (0-0.34)	0.1 ± 0.4 (0-3.1)

2.5 Compounds in plant-based ingredients influencing mineral availability

Compounds in plant-based ingredients influencing mineral availability are phytic acid, saponins, tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens and alkaloids [42]. For instance, non-starch polysaccharides (e.g. fibres) impair mineral availability by increasing cell (e.g. enterocytes) sloughing in the intestine or through the formation of insoluble chelates [43]. Phytic acid forms complexes with divalent cations (e.g. Zn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+}) rendering them poorly available to the fish [12,44].

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, InsP6) is naturally present in most cereals in concentrations ranging from 0.5 to 2.0% [45]. Its molecular formula is $C_6H_{18}O_{24}P_6$ and its molecular weight is 660.03 g mol $^{-1}$. The phytic acid molecule is very reactive due to the presence of phosphate groups that are highly negatively charged (Figure 4). At moderate acid conditions (pH ≥ 5.2), six of these phosphate groups will be negatively charged, while the remaining six phosphate groups will be charged in more acidic conditions (pH < 3.2) [46].

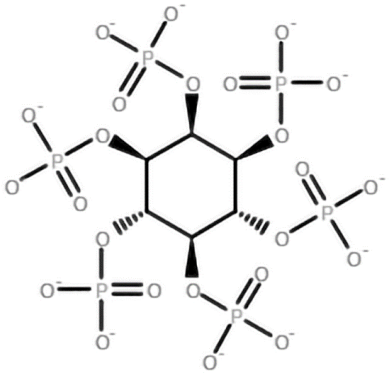

Figure 4 – A phytic acid molecule; the negatively charged phosphate groups can bind to divalent cations.

The hydrolysis of InsP6 occur by nonenzymatic or enzymatic processes. The nonenzymatic hydrolysis usually takes place when the feed is exposed to high temperatures and pressure (e.g. during feed production), or after treatment with strong acids. The enzymatic hydrolysis is mediated by phytases [47]. These enzymes catalyse the hydrolytic cleavage of InsP6 via several phosphorylated intermediary products (i.e. myo-inositol pentakis-, tetrakis-, tris-, bis- and monophosphate) down to myo-inositol [48]. Phytic acid and phosphorylated intermediary products are considered as anti-nutrients due to their high affinity for mineral polyvalent cations which hinders mineral absorption in the animal gastrointestinal tract [49].

2.6 Zinc, selenium and manganese additives in feed

In addition to the native sources found in feed ingredients, Zn, Se and Mn are often supplemented to feed as inorganic salts or as their organic forms to meet the nutritional requirements of fish [36,50], being categorised as feed additives. These feed additives must be authorised before being sold on the European market. The authorisations are valid for 10 years throughout the European Union (EU) and the European Economic Area. Applications for authorisation are submitted to the European Commission (EC). The applicant submits a dossier which includes: 1) name of the applicant; 2) identification of the additive; 3) method of production and method of analysis; 4) studies on safety and efficacy of the additive; 5) proposed conditions for use and animal species for which the additive is intended; 6) proposal for post market monitoring [51]. The European Food Safety Authority (EFSA) evaluates the safety and efficacy of the additive and assesses possible adverse effects on human and animal health and on the environment. Subsequently, the EC may approve feed additives, which are considered safe to use and establishes upper limits for the different minerals in complete diets. In the EU, the current upper limit for total Zn in complete feed of all fish, except salmonids, is 150 mg kg^{-1} and for salmonids feed it is 180 mg kg^{-1} [52,53]. The current upper limit for total Se in fish feed is 0.5 mg kg^{-1} [52], while supplementation of organic Se must not exceed 0.2 mg kg^{-1} in complete feed [54-57]. The upper limit for Mn in feed is 100 mg kg^{-1} [52,57]. Currently, the Zn additives approved are zinc acetate

dihydrate, zinc chloride anhydrous, zinc oxide, zinc sulphate heptahydrate, zinc sulphate monohydrate, zinc chelate of amino acids hydrate, zinc chelate of protein hydrolysates, zinc chelate of glycine hydrate (solid) and zinc chelate of glycine hydrate (liquid) [52,53]. The Se additives approved are sodium selenite, hydroxy-analogue of selenomethionine, L-selenomethionine, DL-selenomethionine and selenomethionine produced by *Saccharomyces cerevisiae* [52,54-57]. The Mn additives approved are manganous chloride tetrahydrate, manganese (II) oxide, manganous sulphate monohydrate, manganese chelate of amino acids hydrate, manganese chelate of protein hydrolysates, manganese chelate of glycine hydrate and dimanganese chloride trihydroxide [52,57]. Table 3 summarizes the list of approved feed additives by the EC and the respective current upper limit in EU for Zn, Se and Mn (information obtained in December 2018).

Table 3 – List of approved feed additives by the European Commission and the respective current upper limit in European Union for Zn, Se and Mn (information obtained in December 2018).

	List of approved feed additives	Upper limit in EU
Zn[(a)]	- zinc acetate dehydrate - zinc chloride anhydrous - zinc oxide - zinc sulphate heptahydrate - zinc sulphate monohydrate - zinc chelate of amino acids hydrate - zinc chelate of protein hydrolysates - zinc chelate of glycine hydrate (solid) - zinc chelate of glycine hydrate (liquid)	- all fish except salmonids: 150 mg kg^{-1} - salmonids: 180 mg kg^{-1}
Se[(b)]	- sodium selenite - hydroxy-analogue of selenomethionine - L-selenomethionine - DL-selenomethionine - selenomethionine produced by *Saccharomyces cerevisiae*	- 0.5 mg kg^{-1} - the organic Se must not exceed 0.2 mg kg^{-1} in complete feed

	List of approved feed additives	Upper limit in EU
Mn[(c)]	- manganous chloride tetrahydrate - manganese (II) oxide - manganous sulphate monohydrate - manganese chelate of amino acids hydrate - manganese chelate of protein hydrolysates - manganese chelate of glycine hydrate - dimanganese chloride trihydroxide	- 100 mg kg^{-1}

(a) Reg. (EC) No. 2003/1831 and amendments [52,53]

(b) Reg. (EC) No. 2003/1831 and amendments [52,54-57]

(c) Reg. (EC) No. 2003/1831 and amendments [52,57]

Currently, the Zn additives approved are zinc acetate dihydrate, zinc chloride anhydrous, zinc oxide, zinc sulphate heptahydrate, zinc sulphate monohydrate, zinc chelate of amino acids hydrate, zinc chelate of protein hydrolysates, zinc chelate of glycine hydrate (solid) and zinc chelate of glycine hydrate (liquid) [52,53]. The Se additives approved are sodium selenite, hydroxy-analogue of selenomethionine, L-selenomethionine, DL-selenomethionine and selenomethionine produced by *Saccharomyces cerevisiae* [52,54-57]. The Mn additives approved are manganous chloride tetrahydrate, manganese (II) oxide, manganous sulphate monohydrate, manganese chelate of amino acids hydrate, manganese chelate of protein hydrolysates, manganese chelate of glycine hydrate and dimanganese chloride trihydroxide [52,57].

2.7 Speciation analysis

Determination of total mineral concentration is commonly used in mineral availability studies. However, speciation analysis can provide valuable data, as specific information for each individual chemical species is provided [58]. As illustrated in Figure 3, the mineral availability is influenced by the chemical form of the mineral, which is again influenced by the ionic concentration, temperature and pH of the fluids in the gastrointestinal tract environment [22]. Information regarding speciation of a mineral is important since the biological role of any mineral depends on its chemical

form [59]. As defined by IUPAC, in analytical chemistry speciation analysis is “the analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample” [60]. In addition, IUPAC defined speciation of an element as “the distribution of an element amongst defined chemical species in a system” [60].

Speciation analysis comprises typically of three steps; 1) sample extraction, 2) separation and 3) detection of chemical species. Sample extraction is commonly achieved by hydrolysis procedures (e.g. acid, alkaline and enzymatic hydrolysis) or by using aqueous or organic solvents to solubilise the different compounds depending on their physicochemical properties [61]. In terms of separation, liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE) are the most commonly used techniques [59]. It is common to couple these techniques to element-specific detection techniques with high sensitivity, such as inductively coupled plasma mass spectrometry (ICP-MS) [59]. A challenge in speciation analysis is often the identification and characterization of the chemical structure of unknown species due to lack of analytical standards. To overcome this challenge, complementary techniques such as electrospray ionisation mass spectrometry (ESI-MS), time-of-flight mass spectrometry (TOF-MS) and other high resolution mass spectrometry (HR-MS) can be used [59,62]. As can be seen in Figure 5, between 1991 and 2019, a large number of peer-reviewed scientific publications (n=3308) reported the use of ICP-MS in speciation analysis (shown as grey bars). In addition, a large number of peer-reviewed scientific publications (n=1727) combined LC with ICP-MS (shown as white bars) in speciation analysis underlining that LC-ICP-MS is the most commonly applied methodology in speciation analysis.

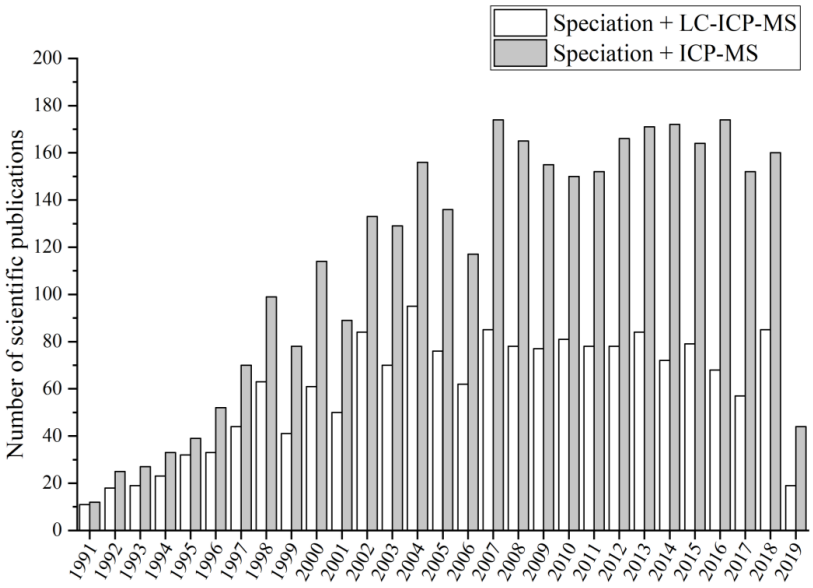

Figure 5 – Number of peer-reviewed scientific publications between 1991 and 2019 on speciation analysis and LC-ICP-MS (shown as white bars), and speciation analysis and ICP-MS (shown as grey bars) (data obtained in March 2019 using as keywords "speciation and liquid chromatography and inductively coupled plasma mass spectrometry" and "speciation and inductively coupled plasma mass spectrometry" in the Web of Science™ database).

Liquid chromatography is a separation technique in which the mobile phase is a liquid. The liquid mobile phase passes through the column and is used to elute the sample through the stationary phase. The components of the mobile phase and the sample can interact with the solid stationary phase, which is usually packed in a column support [63]. Currently, LC is mainly performed using high performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) columns [64]. In LC, some of the most common techniques used for speciation analysis are reversed phase chromatography (RPC), ion exchange chromatography (IEC) and size exclusion chromatography (SEC) [65]. An overview of column type, separation type, mobile phase and examples of applications in speciation analysis using RPC, IEC and SEC techniques is given in Table 4.

Table 4 – An overview of the some of the most common techniques used for mineral speciation analysis: reversed phase chromatography (RPC), ion exchange chromatography (IEC) and size exclusion chromatography (SEC); this table contains information concerning column type, separation type, mobile phase and examples of applications in speciation analysis; adapted from Pereira et al., 2012 [66].

	Chromatographic technique		
	RPC	**IEC**	**SEC**
Column type	- Hydrophobic stationary phase (e.g. C8 or C18)	- Stationary phase with charged groups binding ions with opposite charge	- Inert
Separation type	- Separates molecules based on hydrophobic interactions	- Separates charged molecules based on their ionic strength	- Separates molecules based on their molecular weight
Mobile phase	- Mixture of water/organic solvent (e.g. acetonitrile or methanol)	- Aqueous solution of a salt buffer	- Aqueous or organic or mixtures thereof
Applications in speciation analysis	- Metal porphyrins [67,68] - Species separation: platinum [69] iodine [70,71] chromium [72,73] mercury [74] thallium [75] lead [76] gold [77] selenium [78,79]	- Charged element--containing species: bromine [71] arsenic [80,81] iodine [71] antimony [82] chromium [83] zinc [84] selenium [79]	- Metalloproteins [85,86] - Metal-containing compounds [84,87]

Reversed phase chromatography is the most widely used mode of LC. In RPC, the separation of the molecules happens due to hydrophobic interactions. The stationary phase is often silica covalently bound to carbon chains of varying length (e.g. C8 and C18). The stationary phase is usually hydrophobic and consequently more hydrophobic molecules will have stronger interactions with the stationary phase resulting in longer retention times while, the less hydrophobic ones will have weaker interactions resulting in shorter retention times [64]. Several speciation studies used RPC and some examples are the separations of metal porphyrins [67,68], platinum [69], iodine [70,71], chromium [72,73], mercury [74], thallium [75], lead [76], gold [77] and Se [78,79] species.

In IEC, the separation of the molecules is based on interactions between charged functional groups in the stationary phase and ions in the sample. The stationary phase can be charged positively and interact with negatively charged molecules (anion exchange chromatography) or the stationary phase can be charged negatively and interact with positively charged molecules (cation exchange chromatography). The elution of molecules is controlled by adjusting the pH or ionic strength of the mobile phase. This type of chromatography is commonly used to separate proteins and peptides. However, it can be used to separate any kind of charged molecule [64]. As example, the IEC has been used to separate species of bromine [71], arsenic [80,81], iodine [71], antimony [82], chromium [83], Zn [84] and Se [79].

In SEC, the molecules are separated according to their sizes relative to the pores in the stationary phase. Porous silica beads are usually used as stationary phase in SEC. The separation of the molecules happens as the molecules travel through the stationary phase of the column. The larger the size of the molecule is, the less possibility they have to penetrate the pores of stationary phase beads and will elute earlier, while smaller molecules will travel slower and elute later [64]. Contrary to the chromatographic methods described above, in SEC the separation of molecules does not rely on any interaction between the sample molecule and the stationary phase or mobile phase [64]. The SEC has been used, for example, to study metalloproteins [85,86] and metal-containing compounds [84,87].

Inductively coupled plasma mass spectrometry is capable of detecting most of the elements in the periodic table. The use of ICP-MS has several advantages such as multielement detection capabilities, high sensitivity, use of small sample volumes, short time analysis and its possible hyphenation with other instruments such as HPLC, CE, GC, field-flow fractionation (FFF) or laser ablation (LA) systems [88]. As such, ICP-MS cannot be used in speciation analysis as all the molecules introduced will be broken down in the argon plasma of the ICP. Consequently, for speciation analysis, species of interest need to be separated before reaching the ICP-MS [88]. As can be seen in Figure 6, an ICP-MS includes several components such as a sample introduction port, plasma torch, radio frequency (RF) power supply, mass analyser and mass detector [89]. The detection by ICP-MS comprises several steps, briefly, a sample solution is converted in droplets by a nebuliser, droplets are desolvated, the sample molecules are broken down by a plasma flame into atoms and subsequently ionised, the ions are sorted based on their mass to charge and introduced into the mass detector.

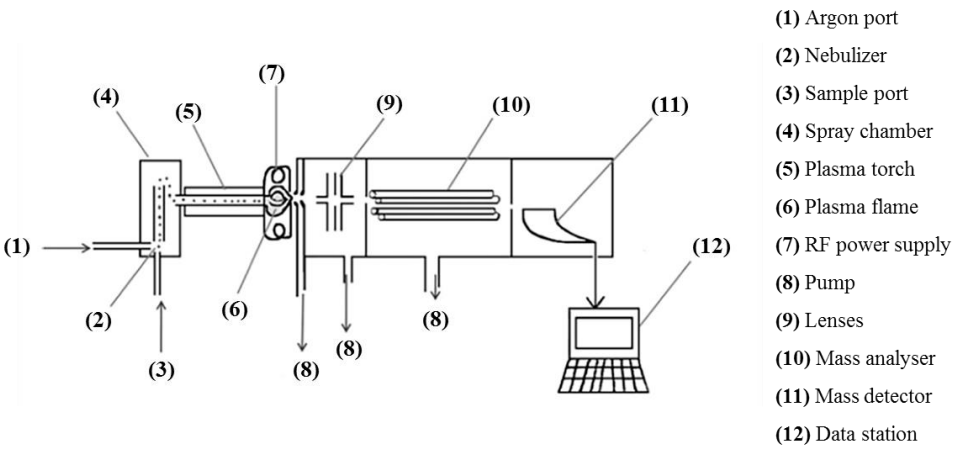

Figure 6 – An inductively coupled plasma mass spectrometry scheme (ICP-MS); the sample solution will travel from the sample port (3) to the mass detector (11); a sample solution is converted in droplets by a nebuliser (2), droplets are desolvated, the sample molecules are broken down by a plasma flame (6) into atoms and subsequently ionised, the ions are sorted based on their mass to charge and introduced into the mass detector (11); adapted from Ha et al. 2011 [89].

2.8 Multivariate statistical data analysis in chemometrics

Multivariate statistical data analysis may be used to solve problems involving large amounts of data. Large amounts of data are generated in fields such as method development, process monitoring control and laboratory routine analysis. In these fields, the use of single variables is often inadequate to describe or classify samples. Multivariate data analysis allows the simultaneous evaluation of several variables, which ensures that interactions, patterns and correlations are taken into consideration [90]. Chemometrics include the so-called Design of Experiments (DOE) and the analysis of the obtained data [91]. Traditionally, method development is performed using the one-factor-at-a-time (OFAT) strategy, which is a labour-intensive and material consuming approach when compared to the DOE approach [92]. One of the most important advantages of using DOE is the estimation of the effect of each factor individually and the study of interaction effects simultaneously. The DOE includes a wide range of designs such as Box-Behnken, Latin square, randomized complete block design, central composite and factorial design [92].

In a factorial design, the influence of all experimental variables, factors, and interaction effects on the response or responses are investigated. If the combinations of k factors are investigated at two levels, a factorial design will consist of 2^k experiments. For example, if the number of factors is 5, then the number of experiments is 32. The levels of the factors are denoted by "-" (minus) for low level and "+" (plus) for high level. Care should be taken when defining the low and high levels to ensure sufficient and reasonable variation in the response. Furthermore, replication allows for the estimation of variance. A factorial design can be either full or fractional design [92].

A full factorial designed experiment consists of all the possible combinations of levels for all factors. An example of a full factorial design is described in Figure 7. A 2^3 design can be used to study the effect of three factors at two levels by performing eight experiments (Figure 7). When the number of factors is equal to 5 or greater, a full factorial design requires a large number of experiments. For instance, if the number of

factors is 5, 6 or 7 then the number of experiments is 32, 64 and 128, respectively. Therefore, choosing a fractional factorial design can be a better choice.

Exp. No.	Factors		
	X_1	X_2	X_3
1	−	−	−
2	+	−	−
3	−	+	−
4	+	+	−
5	−	−	+
6	+	−	+
7	−	+	+
8	+	+	+

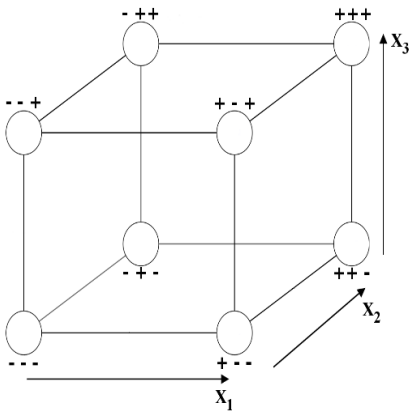

Figure 7 – An example of a two-level full factorial design scheme (2^3); this design can be used to study the effect of three factors at two levels by performing eight experiments; the factors are represented as X_1, X_2 and X_3; factor level codes are shown as "–" or "+".

A fractional factorial design is a design where the experiments conducted are only a subset of the experiments required in the full factorial design. A fractional factorial design is a good option for screening purposes. One drawback of this type of design is that some of the effects of the factors and interactions are confounded. This makes the interpretation of the results more difficult. However, one can always expand a fractional factorial design if needed, thereby increasing the resolution of the design [92]. Fractional factorial designs are defined according to their resolution (e.g resolution III, IV, and V), which states the effects of the factors which are confounded. An example of a fractional factorial design is shown in Table 5. A 2^{6-3} design can be used to study the effect of six factors at two levels by performing only eight experiments. This design has resolution III and the effects X_4, X_5 and X_6 are confounded as described in Table 5.

Table 5 – An example of a two-level full factorial design (2^{6-3}); this design can be used to study the effect of six factors at two levels by performing eight experiments; the factors are represented as X_1, X_2, X_3, X_4, X_5 and X_6; the effect of X_4 is confounded with the effect of X_1X_2 and similarly, X_5 is confounded with X_1X_3 and X_6 confounded with X_2X_3, respectively; factor level codes are shown as "–" or "+".

Exp. No.	**Factors**					
	$\mathbf{X_1}$	$\mathbf{X_2}$	$\mathbf{X_3}$	$\mathbf{X_4 = X_1X_2}$	$\mathbf{X_5 = X_1X_3}$	$\mathbf{X_6 = X_2X_3}$
1	−	−	−	+	+	+
2	+	−	−	−	−	+
3	−	+	−	−	+	−
4	+	+	−	+	−	−
5	−	−	+	+	−	−
6	+	−	+	−	+	−
7	−	+	+	−	−	+
8	+	+	+	+	+	+

3. Aims of the PhD work

The main aim of the PhD work was to evaluate the availability of Zn, Se and Mn in Atlantic salmon. The PhD work was divided in five tasks as follows:

1) Development of analytical methods for Zn speciation

The aim of this task was to develop analytical methods for extraction, quantification and identification of different chemical Zn forms in fish diets.

2) Evaluation of Zn intestinal uptake using the RTgutGC cell line

The aim of this task was to evaluate Zn intestinal uptake in different ionic media composition representing the intestine of freshwater and seawater salmonids using the RTgutGC cell line.

3) Evaluation of solubility of Zn, Se and Mn in Atlantic salmon

The aim of this task was to develop an *in vitro* digestion method to evaluate solubility of dietary Zn, Se and Mn in Atlantic salmon.

4) Evaluation of apparent availability of Zn, Se and Mn in Atlantic salmon

The aim of this task was to study apparent availability of Zn, Se and Mn in Atlantic salmon.

5) Evaluation of correlation between solubility and apparent availability of Zn, Se and Mn

The aim of this task was to evaluate if solubility and apparent availability of Zn, Se and Mn correlates. Therefore, solubility data obtained in task 3 were compared to apparent availability data in task 4.

4. General discussion

4.1 Challenges in development of analytical methods for zinc speciation

A method using SEC-ICP-MS was developed for Zn speciation analysis and several challenges were encountered during method development; i) low solubility of Zn-containing compounds under mild extraction conditions; ii) possible loss of Zn species or species transformation during sample preparation and the chromatographic run; iii) lack of standards and certified reference materials making method validation, identification and quantification of Zn-containing compounds difficult (**Paper I**). These challenges are further discussed below.

i) Low solubility of Zn-containing compounds under mild extraction conditions. In speciation analysis, a common approach is to solubilise the sample before analysis [61]. Several mild extraction conditions were tested to extract Zn, but the Zn recovery was low (~10%) (**Paper I**). Mild extraction conditions were applied to keep the integrity of the Zn chemical species intact, which may have compromised the extraction efficiency (**Paper I**). Furthermore, Zn ions (Zn^{+2}) can easily bind to other compounds which are less soluble in water (i.e. phytic acid, sulphides). The lower solubility could be related to Zn binding to other compounds present in the fish feed and thereby forming water insoluble Zn species (**Paper I**). Indeed, the feed analysed was supplemented with 66.9 mg of Zn kg^{-1} as Zn oxide and the average Zn concentration was 110 ± 8 mg kg^{-1} of feed (n= 10). As Zn oxide was ~61% of the Zn concentration, it was expected to have higher Zn recovery than was actually found. However, Zn oxide has very low solubility in water but dissolves in most acids and form soluble Zn compounds in alkalis [93]. The narrow pH range tested (i.e. pH 6.5 and 8.5) may partly explain why the soluble fraction did not contain more than 10% of the total Zn (**Paper I**). A screening of the effect of six factors (extraction solution, molar concentration of the extraction solution, pH, addition of 4% sodium dodecyl sulphate solution, temperature, and extraction time) on Zn extraction was performed

(**Paper I**). However, several other extraction conditions could have been selected (i.e. the use of other buffers, temperature, pH) possibly leading to higher Zn extraction efficiency but then potentially at the cost of species integrity. In addition, there are a number of speciation protocols, which include the use of microwave and ultrasound-assisted extraction for enhancing the extraction efficiency [94,95]. The effect of the ultrasound-assisted extraction was examined in preliminary tests. However, the results obtained did not show any improvement of Zn recovery when using ultrasound-assisted extraction. Furthermore, microwave and ultrasound-assisted extraction could affect the species integrity [94,95]. Consequently, in the present study emphasis was put on the use of mild extraction conditions to keep the chemical species intact, and microwave and ultrasound-assisted extraction were not included (**Paper I**).

ii) Possible loss of Zn species or species transformation during sample preparation and chromatographic run. To obtain reliable speciation data, it is important to preserve species integrity during sample preparation and chromatographic run. Zinc standards could be useful tools to evaluate the possible loss of Zn species or species transformation during the sample preparation and/or the chromatographic run, however such standards are not available. Chemical synthesis and custom-made standards could overcome this, but this process is costly and it would require knowledge on the compounds to be synthesised.

Techniques like RPC, IEC and SEC are some of the most commonly used chromatographic methods for speciation studies [65]. In this work, IEC and RPC were disregarded for the analysis of Zn species as the chromatograms obtained by anion exchange and RPC showed poor resolution and severe peak broadening. The poor resolution and severe peak broadening can be indicators of loss of Zn species integrity during the chromatographic run. When applying SEC, the chromatograms obtained did not show poor resolution and severe peak broadening, which indicate higher stability of the Zn species. Therefore, SEC was chosen as the chromatographic method for Zn speciation (**Paper I**).

iii) Lack of standards and certified reference materials making method validation, and identification and quantification of Zn-containing compounds difficult. According to international guidelines for the validation of analytical methods, the confirmation of the identity of an analyte in a sample is achieved, for instance, by comparison of retention time of the analyte to retention time of a reference standard [96]. The SEC-ICP-MS method provided qualitative and semi-quantitative information regarding Zn species present in the soluble fractions of the feed (**Paper I**). A full validation of the SEC-ICP-MS method was not possible due to the lack of standards and certified reference materials. Four Zn-containing compounds with different molecular weights were found in the soluble fraction of the fish feed studied (**Paper I**). However, due to a lack of standards the identification and quantification of the unknown Zn-containing compounds were challenging. In SEC, the separation of molecules is based on the molecular sizes. Therefore, each Zn-containing peak might contain several compounds with similar molecular weight [97], which also contributes to the challenge of identifying Zn compounds. A quick method was used to investigate the nature of the Zn species in the soluble fraction. The different ingredients sources and the molecular weight range of the Zn peaks suggest that the observed Zn peaks could be metalloproteins. One of the most studied metalloproteins are metallothioneins (MTs). As MTs are thermally stable proteins, a heating step is a commonly used procedure to confirm MTs presence. The soluble fraction of the fish feed was heated and data obtained suggested that the observed Zn-containing compounds could be MTs.

4.2 Intestinal epithelium uptake: example of zinc

There is limited knowledge about the intestinal uptake of Zn in fish [25]. However, it is acknowledged that the uptake of Zn across the intestinal epithelium includes different stages [25]. First, Zn species released from a fish feed reach the absorption sites of the intestinal epithelium. Secondly, Zn is absorbed across the epithelial cells by means of diffusion (paracellular uptake) or by means of transporters (intracellular uptake). Transporters from the Zrt- and Irt-like protein (ZIP) family and amino acid

transporters (AAT) are examples of active transporters acting at the brush border membrane of the intestine [25]. A hypothetical representation of Zn intestinal uptake in Atlantic salmon is shown in Figure 8. This figure summarizes the uptake of Zn across the intestinal epithelium including the different possible stages and information obtained in this PhD work (**Paper I and II**). The uptake of Zn across the intestinal epithelium will be influenced by inhibitors and enhancers at the point of contact with the absorbing membranes.

In this PhD work, Zn-containing compounds were found in the soluble fraction of an Atlantic salmon feed and the data obtained suggested that the observed Zn-containing compounds were high, medium and low molecular weight compounds (**Paper I**). The molecular weight of the compounds will influence whether compounds will be absorbed or not and also their route of uptake [98]. One study regarding Zn uptake was performed in rainbow trout using an *in vivo* perfusion technique [99]. It was proposed that diffusive pathway was blocked as a consequence of increased secretion of epithelial mucus [99]. Results obtained using an *in vivo* perfusion technique indicated that epithelial mucus can hinder Zn intestinal uptake. In this PhD work, it was demonstrated that amino acid transporters are involved in Zn intestinal uptake (**Paper II**). The RTgutGC cells were exposed to amino acids along with the amino acid transporter blocker (BCH). A significant reduction in Zn intestinal uptake was seen in cells treated with BCH when compared to cells untreated with BCH (**Paper II**). Also, Zn intestinal uptake was positively influenced by the presence of methionine (**Paper II**).

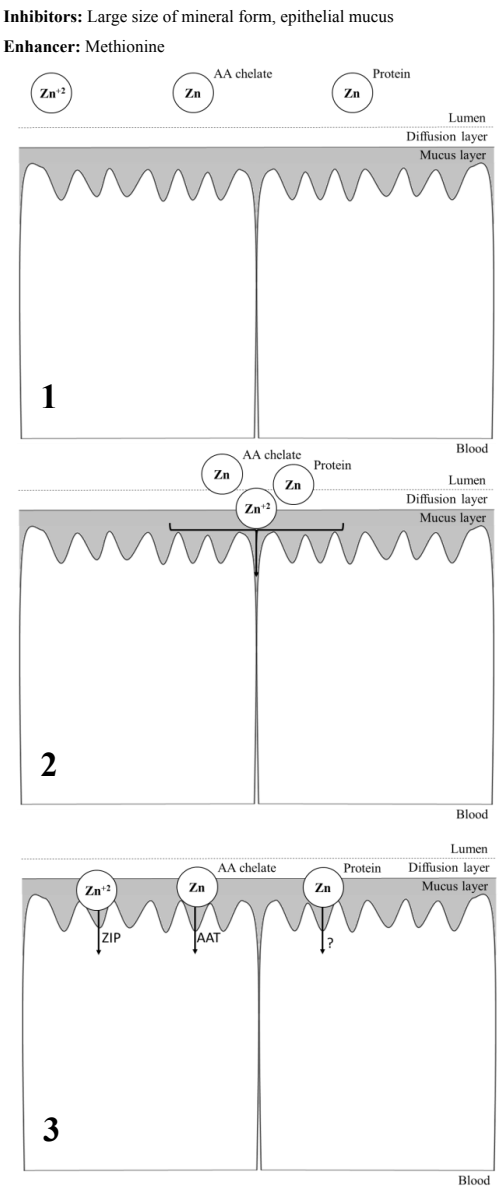

Figure 8 – A hypothetical representation of intestinal zinc (Zn) uptake in Atlantic salmon; 1) Zn-containing compounds reach the intestine, chemical species such as Zn^{+2}, Zn chelated with an amino acid (AA) and Zn bonded with proteins or peptides; 2) paracellular uptake; 3) intracellular uptake; Transporters from the Zrt- and Irt-like protein (ZIP) family and amino acid transporters (AAT) are examples of active transporters acting at the brush border membrane of the intestine; adapted from Bury et al. 2003 [25].

4.3 Mineral availability in Atlantic salmon

The apparent availability of inorganic and organic sources of Zn, Se and Mn in Atlantic salmon diets was studied (**Paper III**). This was done by preparing 16 experimental diets and these diets were fed to Atlantic salmon for 11 days. Table 6 summarizes the average concentration of Zn, Se and Mn in the basal mix and in the diets. In the same table, solubility, apparent availability, available level in diets (i.e. [(average of Zn or Se or Mn in diet)*(average of Zn or Se or Mn apparent availability)]/100), requirement in Atlantic salmon, and the upper limit in EU for Zn, Se and Mn.

Table 6 – Concentrations of Zn, Se and Mn in the basal ingredients mix (mg kg $^{-1}$, n=7) and in the experimental diets (mg kg $^{-1}$, n=16); apparent availability (%, n=16), available level in diets (mg kg $^{-1}$), requirement in Atlantic salmon and the upper limit in EU for Zn, Se and Mn (mg kg $^{-1}$). The values for basal mix, diets, solubility and apparent availability are presented as average ± standard deviation.

	Zn	**Se**	**Mn**
Basal mix, mg kg $^{-1}$ (n=7)	27.3 ± 0.1	0.20 ± 0.01	15.6 ± 0.8
Diets, mg kg $^{-1}$ (n=16)	142 ± 6	0.58 ± 0.03	25 ± 4
Solubility, % (n=2)	3 ± 3	16 ± 11	10 ± 7
App. availability, % (n=16)	33 ± 7	67 ± 5	24 ± 12
Available level in diet [(a)], mg kg $^{-1}$	47	0.39	6
Requirement, mg kg $^{-1}$	37 [(b)]	n.d.	10 [(b)]
Upper limit in EU, mg kg $^{-1}$	180 [(c)]	0.5 [(d)]	100 [(e)]

n.d. – not defined

(a) Available level in diets = [(average of Zn or Se or Mn in diet)*(average of Zn or Se or Mn apparent availability)]/100

(b) NRC (2011) [36]

(c) Reg. (EC) No. 2003/1831 and amendments [52,53]

(d) The supplemented organic Se must not exceed 0.2 mg kg $^{-1}$ in complete feed; Reg. (EC) No. 2003/1831 and amendments [52,54-57]

(e) Reg. (EC) No. 2003/1831 and amendments [52,57]

As reviewed by Prabhu and co-workers, the mineral requirement of fish can be affected by one or a combination of the following factors: biological factors (e.g. species, life stage, sex, trophic level, feeding habits and the nutritional status of the fish), dietary factors (e.g. diet composition, availability, nutrient and anti-nutrient interactions) and environmental factors (e.g. salinity, temperature and mineral concentration in water) [11]. The findings obtained in the PhD work regarding Zn, Se and Mn availability are compared to the mineral requirement in Atlantic salmon. This comparison was done separately for Zn, Se and Mn.

Zinc. Fish meal is known to have higher level of Zn (181 mg kg $^{-1}$) when compared with plant-based ingredients (46 mg kg $^{-1}$) (Table 2). The Zn concentration in the basal mix (27.3 ± 0.1 mg kg $^{-1}$) was lower than the Zn requirement (37 mg kg $^{-1}$) (Table 6) [36] and not all Zn in the basal mix is expected to be available. Consequently, it is recommended to supplement Zn (using inorganic or organic sources) to cover the requirement of the fish ensuring good performance in Atlantic salmon [100]. In the feeding trial (**Paper III**), average of Zn apparent availability was 33% and the available Zn level was 47 mg kg $^{-1}$ (Table 6). The available Zn concentration is higher than the concentration established as Zn requirement in Atlantic salmon (37 mg kg $^{-1}$) (Table 6) [36], meaning that the available Zn concentration was enough to cover the requirement of the fish. The low values obtained for apparent availability of Zn can be related to the high dietary level of Zn (**Paper III**). In Norway, the average Zn concentration in feeds in 2017 was on average 170 mg kg $^{-1}$ (n=40) [10]. In general, the higher the dietary level in comparison to the requirement, the lower the apparent availability will be. This was reported by Prabhu and co-workers who found that in rainbow trout, the apparently absorbed proportion of Zn decreased with increasing Zn dietary level above the requirement [101]. The Zn concentration in the basal mix was 27.3 ± 0.1 mg kg $^{-1}$ and the Zn concentration in the diets was 142 ± 6 mg kg $^{-1}$ (Table 6). Thus, it remains a question of whether Zn supplementation at such high level is needed. The EFSA proposed to decrease the upper limit to 150 mg Zn kg $^{-1}$ complete feed for salmonids [102]. According to EFSA, the decrease of the upper limit to 150 mg Zn kg $^{-1}$ complete feed for salmonids would ensure good health, welfare and productivity of the target

species, and would result in an overall reduction of Zn emissions from animal production of about 20% [102]. There is an environmental concern in terms of Zn in water and sediments around cages for farming fish [102]. Indeed, in the feeding trial (**Paper III**), average of Zn apparent availability was 33% (Table 6) meaning that around 67% of the Zn added in diets will be not be absorbed ending in the fish faecal matter. Therefore, it is important to expand the knowledge on Zn availability and how Zn availability can be increased. Higher Zn availability would promote good fish health and simultaneously decrease the environmental load via faeces.

Selenium. Fish meal is known to have higher level (1.9 mg kg $^{-1}$) compared with Se level in plant-based ingredients (0.1 mg kg $^{-1}$) (Table 2). The requirement for Se in Atlantic salmon is not known. However, Se requirement in rainbow trout was estimated as 0.15 mg kg $^{-1}$ [36]. The concentration of Se in the basal mix was 0.20 ± 0.01 mg kg $^{-1}$ (Table 6) thus, enough to cover Se requirement assuming that rainbow trout and Atlantic salmon have a similar requirement of Se. However, it is important to consider partial dietary availability thus, organic or inorganic sources of Se are often supplemented to cover Se requirement. In the feeding trial (**Paper III**), average of Se apparent availability was 67% and available Se was 0.39 mg kg $^{-1}$ (Table 6). There is a small difference between the concentration of Se in basal mix (0.2 mg kg $^{-1}$) and the Se upper limit (0.5 mg kg $^{-1}$) which leaves a narrow window for supplementation (Table 6). In Norway, the Se concentration in feeds in 2017 was on average 0.8 mg kg $^{-1}$ (n=40) [10] and this concentration is higher than the current upper limit (0.5 mg kg $^{-1}$). Moreover, the addition of organic Se must not exceed 0.2 mg kg $^{-1}$ in complete feed [52,54-57]. Selenium speciation methods as the ones reported by Sele and co-workers [79] are important to differentiate between several forms of Se in feed (i.e. selenite, selenate, Se-methyl-seleno-cysteine, selenomethionine (SeMet) and selenocystine (SeCys)) but there is no method available to differentiate between supplemented Se forms and Se forms naturally present in feed ingredients and feed.

Manganese. Fish meal is known to contain a lower level of Mn (12 mg kg $^{-1}$) compared with plant-based ingredients (28 mg kg $^{-1}$) (Table 2). The Mn concentration in the basal mix (15.6 ± 0.8 mg kg $^{-1}$) was higher than the Mn requirement (10 mg kg $^{-1}$) of Atlantic

salmon (Table 6). However, Mn from basal mix might not be all available, and as a precautionary measure Mn is supplemented to Atlantic salmon feeds (inorganic or organic sources). In the feeding trial (**Paper III**), average Mn apparent availability was 24% and available Mn was 6 mg kg $^{-1}$ (Table 6). The available Mn concentration was slightly lower (6 mg kg $^{-1}$) than the level of Mn requirement in Atlantic salmon (10 mg kg $^{-1}$), thus, not enough to cover Mn requirement. However, it is important to consider that the Mn apparent availability varied greatly (**Paper III**). Manganese is secreted via bile into the gut. Thus, faecal Mn includes a portion of endogenous Mn in addition to unabsorbed Mn [103]. The high variability of Mn apparent availability can be related with having more Mn in the faeces (i.e. endogenous and unabsorbed Mn) than in the diet (**Paper III**). In Norway, the Mn concentration in feeds in 2017 was on average 47 mg kg $^{-1}$ (n=40) [10] and this concentration is approximately half of the current upper limit (100 mg kg $^{-1}$) (Table 6). In rainbow trout, the apparent availability Mn decreased with increasing Mn dietary level above the requirement [101]. In this feeding trial (**Paper III**), Mn concentration in diets was 25 ± 4 mg kg $^{-1}$ (n=16) and this is higher than the level of Mn requirement in Atlantic salmon (10 mg kg $^{-1}$). Thus, the lower values obtained for apparent availability of Mn can also be related to the high dietary level of Mn (**Paper III**).

4.3.1 Mineral availability is influenced by the chemical form

There is little known about mineral requirements for fish when compared to what is known for land animals [11]. Mineral supplementation is recommended in salmonid feeds to maintain whole body and tissue levels of important trace minerals, such as Zn, Mn and Se [26]. Whether supplementation should be performed using inorganic or organic mineral sources is unclear. In this PhD work, the apparent availability of Zn, Se and Mn was evaluated in Atlantic salmon (**Paper III**). The results from this study suggested that the apparent availability of Se and Mn depends on the chemical form of the minerals (**Paper III**). Selenomethionine was more available than selenite, while manganese sulphate was more available than manganese chelate of glycine (**Paper III**). The apparent availability of Zn was not affected by the chemical form of the additive source (**Paper II**). There are studies demonstrating the benefits of organic

minerals over inorganic forms in salmonids diets [104-109]. Bell and Cowey studied the digestibility and bioavailability of dietary Se from fish meal, selenite, SeMet and SeCys in Atlantic salmon. Selenomethionine was found to be the most available Se source followed by selenite [104]. Apines-Amar and co-workers compared the availability of inorganic sources of Zn, Mn and Cu (sulphates) with the organic source of Zn, Mn and Cu (amino acid chelates) in experimental diets [107], and in experimental diets containing tricalcium phosphate and phytate [106]. Both studies were performed in rainbow trout, and the organic sources of Zn, Cu and Mn (amino acid chelates) were more available than the Zn, Cu and Mn inorganic sources (sulphates) [106,107]. Rider and co-workers studied the bioavailability of inorganic and organic Se and Zn sources in rainbow trout fed a fish meal based diet [108]. Three diets were tested; control diet (no added Se or Zn); one diet supplemented with inorganic sources of Se and Zn (sodium selenite and Zn sulphate); and one diet supplemented with organic sources of Se and Zn (Se yeast and Zn proteinate). The bioavailability of Se was influenced by the chemical form, the diet supplemented with Se yeast had higher bioavailability than the diet supplemented with selenite [108]. However, the influence of the Zn chemical form on Zn bioavailability was not clear [108]. Fontagne-Dicharry and co-workers studied the influence of chemical forms and levels of dietary Se on antioxidant status and oxidative stress-related parameters in rainbow trout [109]. The results obtained demonstrated that the Se availability was higher in a diet supplemented with Se yeast than in a diet supplemented with selenite or a non-supplemented diet [109]. In salmonids, organic Se supplementation has higher availability when compared with inorganic Se supplementation as shown in this work (**Paper III**) and elsewhere [104,108,109]. However, the effect of the chemical form on mineral availability is not so clear for other minerals such as Zn and Mn [11]. Data showed that the Mn chemical form influenced Mn availability, but did not influenced Zn availability (**Paper III**). Mineral uptake is influenced by interactions with other dietary components leading to the formation of insoluble complexes or of large compounds which cannot be absorbed [110]. Inorganic mineral forms (e.g. sulphates and chlorides) are very weakly bound and for that reason chemical bounds can be easily destroyed, leaving the mineral ions free to interact [110,111]. Theoretically, organic

mineral sources are chemically more stable, which prevents the mineral sources from interacting [112,110]. In addition, the influence of gastrointestinal environment on mineral chelation needs to be considered. The evaluation of metal chelation at different pH will give more information. Brown and Zeringue performed an evaluation of solubility and structural integrity of chelated minerals at pH 2 and 5 [113]. In this work, the effect of gastrointestinal environment on Zn speciation was also considered (**Paper II**). Two media representing the intestine environment of freshwater or seawater salmonids were tested. The Zn ion uptake was not affected by the ionic concentration in the media. However, Zn uptake was influenced by the ionic concentration in the media when methionine was present (**Paper II**). In addition, the influence of gastrointestinal environment tested *in vitro* affected the solubility of Zn and Mn (**Paper IV**). There was a clear drop in solubility of these two minerals when the acidic hydrolysis ended and the alkaline hydrolysis started. The amount of soluble Zn was low in the acidic hydrolysis (3-8%) and in the alkaline hydrolysis (0.4-2%). For Mn, during the acidic hydrolysis the Mn solubility was higher in the diet supplemented with an inorganic mineral source (6-25%) than the diet supplemented with an organic mineral source (4-17%) but the amount of soluble Mn in the alkaline hydrolysis was similar for both diets (4-8%) (**Paper IV**). Overall, these results suggested that the gastrointestinal environment influenced Zn and Mn chemical forms.

4.3.2 Mineral availability is influenced by interactions

As reviewed by Hilton (1989) a range of dietary interactions can influence mineral availability [37]. The mineral-mineral interactions and mineral-other dietary component interactions will be discussed below as these are interactions relevant for this PhD work.

i) Mineral-mineral interactions. Previous studies in Atlantic salmon showed that Zn requirement was higher in the seawater phase than in the freshwater phase [26]. This led to investigate whether Zn availability is influenced by the variation in the gastrointestinal ionic concentration after shifting from freshwater to seawater (**Paper II**). The data obtained in **Paper II** indicated an interaction effect between Zn intestinal

uptake and the gastrointestinal ionic concentration media ionic composition but only when methionine was present in the media. The Zn uptake across the epithelial cells exposed to seawater media ion composition was significantly lower compared to the Zn uptake across the epithelial cells exposed to freshwater media ion composition, but only at Zn concentrations of 12 and 25 μM. The study gave some suggestions regarding the differences seen in Zn requirement of Atlantic salmon in the freshwater and seawater phase. However, in order to have a broader understanding, the influence of factors such as physiological conditions and diet composition are important to be considered and should be studied. Mineral-mineral interactions between Zn, Se and Mn were found to influence Zn, Se and Mn apparent availability (**Paper III**). The data obtained in **Paper III** suggested that these interactions had a negative effect which lowered the apparent availability. For instance, the interaction between Zn additive source and Se additive source, and the interaction between Zn, Se and Mn additive sources, decreased the apparent availability of Zn (Figure 9) (**Paper III**). In addition, the interaction between Zn and Mn additive sources decreased the apparent availability of Se (**Paper III**). This encourages further research to understand the interaction mechanisms. Interactions involving Zn, Se and Mn have been studied in rainbow trout [114-116] and in Atlantic salmon [105,117,118]. Knox and co-workers studied the effects of dietary Cu and Cu:Zn ratio [114] and the effects of dietary Zn intake on Cu metabolism in rainbow trout [115]. Results from the first study suggested that the dietary Cu:Zn ratio caused small changes in the plasma and hepatic levels of Mg, Na, Ca and K, but interaction effects between Zn and Cu were not seen [114]. Moreover, results from the second study showed that increased dietary Zn reduced the activity of Mn superoxide dismutase and increased the activity of Cu-Zn superoxide dismutase [115]. Ojo and co-workers examined *in vitro* interactions between Cu and Zn in rainbow trout [116]. The study was performed by increasing one at the time the concentration of Cu or Zn. A higher concentration of Zn reduced the availability of Cu and a higher concentration of Cu reduced the availability of Cu [116]. The interactions between Zn and Cu are antagonistic, as Zn and Cu interfere on each other absorption by upregulating synthesis of MTs within enterocytes [110].

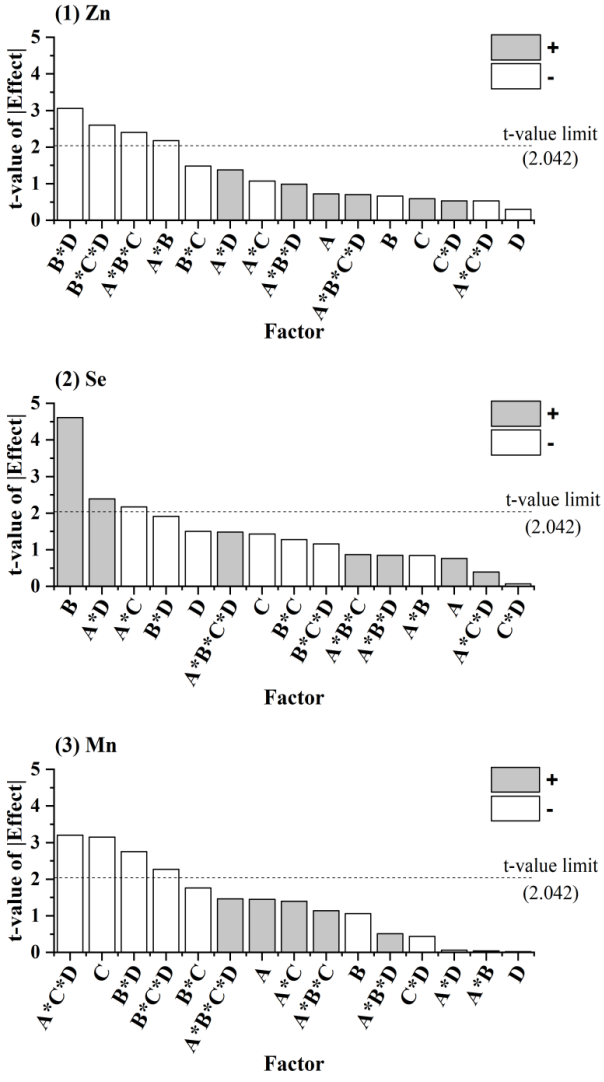

Figure 9 – Pareto charts showing the t-value of the effect using separately apparent availability (%) of zinc (Zn) (1), selenium (Se) (2) and manganese (Mn) (3); the factors are Zn additive source (A), Se additive source (B), Mn additive source (C) and phytic acid level (D); the horizontal axis shows the factors and interactions ordered according to their magnitude; 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 ($p < .05$); adapted from Paper III.

One study in Atlantic salmon reported a synergistic interaction between Zn and Fe [105]. Andersen and co-workers studied the bioavailability and interactions with other micronutrients of three dietary Fe sources in Atlantic salmon [105]. The study found a positive correlation between whole body Fe concentrations and whole body Zn concentrations [105]. One study demonstrated an antagonist interaction effect between Se and Cu in Atlantic salmon [118]. Berntssen and co-workers found a reduction in tissue Se levels in the high dietary Cu group [118]. In rainbow trout, the Se and Cu interaction lead to the formation of a Se-Cu complex which reduced the availability of Se and Cu [119]. In this PhD work, the effect of minerals, such as Cu and Fe, on the availability of Zn, Se and Mn were not evaluated. However, it is likely that Cu and Fe can be involved in interactions involving Zn, Se and Mn, influencing mineral availability in Atlantic salmon.

ii) Mineral-other dietary component interactions. Several interactions between minerals and other dietary components have been pointed out as important in animal nutrition [110,120]. For the past decades, with the introduction of plant-based ingredients, the main focus has been to study the effect of plant-based ingredients and related compounds on fish performance. Consequently, several studies were performed in salmonids [121-139]. In this PhD work, experimental diets with low and high levels of phytic acid were fed to Atlantic salmon (**Paper III**). The phytic acid level as a factor did not affect apparent availability of Zn, Se and Mn (Figure 9) but the difference in phytic acid concentrations between the low (11.3 ± 0.1 μmol g^{-1}, $n = 2$) and high (12.0 ± 0.1 μmol g^{-1}, $n = 2$) phytic acid level diets was not large (**Paper III**). Conversely, several interactions between the phytic acid level and Zn, Se and Mn affected the apparent availability of Zn, Se and Mn (**Paper III**). For instance, the interaction between Se additive source, Mn additive source and phytic acid level ($B \times C \times D$) affected the apparent availability of Zn and Mn, and the interaction between Zn additive source and phytic acid level ($A \times D$) affected the apparent availability of Se (Figure 9). Spinelli and co-workers evaluated the effect of phytic acid on mineral availability in rainbow trout [140]. They did not observe effects of the dietary phytic acid level (0.5%) on Zn availability [140]. These results support the

findings in this study. Conversely, Richardson and co-workers reported that the concentration of Zn in plasma of Chinook salmon was directly related to the concentration of dietary Zn and inversely related to the dietary level of phytic acid, suggesting an interaction effect between Zn and phytic acid in juvenile Chinook salmon [141]. The studies performed by Spinelli and co-workers, and Richardson and co-workers used purified diets supplemented with sodium phytate, thus the difference in results between the two studies might be related to the use of different salmonids species, fish age or interactions with other dietary components. The effect of dietary components on mineral availability was shown by Prabhu and co-workers [101]. They studied the availability of a range of minerals including Mn, Zn and Se in rainbow trout [101]. Two basal diets, a fish meal diet (62.8 mg of Zn kg^{-1} feed and 9.4 mg of Mn kg^{-1} feed) and a plant-based diet (42.9 mg of Zn kg^{-1} feed and 88.6 mg of Mn kg^{-1} feed), were fed to rainbow trout. The results demonstrated that the ingredients used to formulate the diet influenced the apparent availability of Zn and Mn, but did not influenced Se apparent availability [101]. The Zn apparent availability was higher in fish fed the fish meal diet (56.1 ± 7.4%) when compared with fish fed plant-based diet (40.2 ± 3%) [101]. Similar results were seen for Mn. The Mn apparent availability was higher in fish fed the fish meal diet (31 ± 4.8%) than in fish fed plant-based diet (7.3 ± 0.4%) [101].

4.4 Correlation between solubility and apparent availability of zinc, selenium and manganese

Mineral availability is usually studied *in vivo* but having a fast and reliable *in vitro* method for the estimation of dietary mineral availability would greatly facilitate screening of new feed ingredients and feeds. The correlation between *in vitro* solubility and *in vivo* apparent availability of Zn, Se and Mn was evaluated (**Paper IV**). In general, the values obtained for *in vitro* solubility were lower than the values obtained for *in vivo* apparent availability of Zn, Se and Mn (**Paper IV**). The results shown a strong significant positive correlation between solubility and apparent availability of Mn and a weak non-significant positive correlation between solubility and apparent

availability of Zn and Se (**Paper IV**). Despite the weak correlation, the effect of the chemical form of the minerals were similar for Zn, Se and Mn solubility and Zn, Se and Mn apparent availability. The chemical form (Zn sulphate or Zn chelate of glycine) did not affect the Zn solubility (**Paper IV**) and the Zn apparent availability (**Paper III**). For Se, the organic form (SeMet) was more soluble (**Paper IV**) and also had higher availability than the inorganic form (selenite) (**Paper III**). Moreover, the Mn inorganic form (Mn sulphate) was more soluble (**Paper IV**) and more available (**Paper III**) than the Mn organic form (Mn chelate of glycine). This shows that *in vitro* evaluation of the mineral solubility gives promising insights on mineral availability. Thus, *in vitro* methods can be considered as a screening method, replacing some of the *in vivo* feeding trials. However, more work needs to be done to improve the *in vitro* digestion method to better estimate mineral availability. The low solubility of Zn, Se and Mn can be related with several factors. Mineral solubility is influenced by chemical structure, oxidation state of the mineral, mineral concentration, and changes in pH and temperature [111,142,143]. In addition, minerals can bind to other compounds forming complexes which are less soluble in water [144]. The influence of changes in pH and temperature on the solubility of Zn was evaluated (**Paper I and IV**). The solubility of Zn was low (3.5-10%) at a temperature of 4 or 20 °C and pH values of 6.5 or 8.5 (**Paper I**). Also, low Zn solubility (0.4-8%) was obtained at a temperature of 15 ºC, and pH values of 2.1 or 8.0 (**Paper IV**). The influence of changes in pH on the solubility of Mn and Se were evaluated (**Paper IV**). The change in pH did not affect Se solubility. However, low Mn solubility (4-25%) was obtained at a temperature of 15 ºC, and pH values of 2.1 or 8.0 (**Paper IV**). These results show that mineral solubility was influenced by changes in pH and temperature. An Atlantic salmon feed contains both marine and plant-based ingredients, and it is a lipid rich sample (~20-35% fat). The different ingredients can have an effect on the mineral solubility. A recent review described the ability of divalent minerals to bind free fatty acids and these chemical reactions can limit mineral solubility due to formation of poorly soluble soaps and salts [145]. Furthermore, divalent ions can easily bind to other compounds which are less soluble in water (i.e. phytic acid, sulphides) [111,146] and this might be also a reason for the lower solubility of Zn and Mn (**Paper I and IV**).

5. Conclusions

1) Development of analytical methods for Zn speciation

The present work developed an analytical method to study Zn speciation. The fish feed contained several Zn species originating from different sources as the feed contains both marine-based ingredients, plant-based ingredients, and supplemented forms. The developed SEC-ICP-MS method provided qualitative and semi-quantitative information on Zn chemical species present in the soluble fractions of feed. Four Zn-containing peaks were found, each with different molecular weights. In this work, the SEC-ICP-MS method was applied to the study of Zn species in fish feed but could potentially be applied to other types of feed and feed ingredients. Overall, the developed analytical methods provided complementary information for understanding the effect of speciation on mineral availability.

2) Evaluation of Zn intestinal uptake using RTgutGC cell line

The *in vitro* uptake of Zn was studied using RTgutGC cell line under media compositions mimicking the intestinal ionic concentration of freshwater or seawater acclimatised salmonids. The Zn intestinal uptake was not affected by the ionic concentration in the media. However, the Zn intestinal uptake was influenced by the ionic concentration in the media when methionine was present. The results obtained in this task demonstrated that RTgutGC cell line can be used to identify mechanisms involved in Zn intestinal uptake such as the effect of chemical forms and the effect of interactions occurring over the intestinal tract of salmonids.

3) Evaluation of solubility of Zn, Se and Mn in Atlantic salmon

The present work developed an *in vitro* digestion method to evaluate solubility of Zn, Se and Mn in Atlantic salmon diets. Data obtained demonstrated that solubility of Zn, Se and Mn was influenced both by the mineral chemical form supplemented to the diet and by the gastrointestinal environment. Regarding the mineral form supplemented to the diets, SeMet was more soluble than selenite, and Mn sulphate was more soluble

than Mn chelate of glycine. Conversely, the Zn additive source did not influence the solubility of Zn. The gastrointestinal environment did not affect the solubility of Se but affected the solubility of Zn and Mn. For both Zn and Mn there was a clear drop in solubility when shifting from acidic to alkaline hydrolysis, suggesting that the solubility of the Zn and Mn compounds was affected by the increased pH.

4) Evaluation of apparent availability of Zn, Se and Mn in Atlantic salmon

This work compared apparent availability of Zn, Se and Mn from inorganic metal salts and their organic forms in Atlantic salmon. Results demonstrated that in practical diets with low inclusion of fish meal, the availability of the three minerals depended on their chemical form. Selenomethionine was more available than selenite, and Mn sulphate was more available than Mn chelate of glycine. Conversely, the Zn additive source did not affect Zn availability. A number of mineral-mineral interactions were found to have a significant negative effect on the apparent availability of Zn, Se and Mn. In addition, phytic acid was involved in several interactions. The results regarding the interactions between the different factors were obtained using a full factorial design. This type of experimental design should be considered more often when studying mineral availability and mineral interactions in fish. Finding mineral chemical forms with higher availability supports health and robustness of Atlantic salmon and simultaneously decreases the environmental load via faeces.

5) Correlation between solubility and apparent availability of Zn, Se and Mn

The correlation between solubility and apparent availability of Zn, Se and Mn was evaluated. The results obtained suggested that there is a significant positive correlation between Mn solubility and Mn apparent availability and non-significant positive correlations between Zn solubility and Zn apparent availability Zn and Se solubility and Se apparent availability. Even though significant correlations were not found for Zn and Se, the effect of the chemical form was similar for Zn, Se and Mn solubility and Zn, Se and Mn apparent availability. This demonstrated that *in vitro* evaluation of the mineral solubility gives promising insights on mineral availability and can potentially be used as screening method, replacing some of the *in vivo* feeding trials.

6. Future perspectives

6.1 Development of analytical methods for zinc speciation

One of the tasks of the PhD work was the development of analytical methods to study Zn speciation. However, one of the challenges encountered during the method development for Zn speciation analysis was the identification of the Zn-containing peaks. Considering that Zn species will influence Zn uptake, information about the chemical structure of the Zn-containing species is important. Since the ICP-MS detector only provides information about the atomic mass of elements, alternative techniques are needed to get complementary molecular information. In order to pursue this approach a parallel coupling of HPLC (SEC) to ICP-MS and ESI-MS was set up (Figure 10).

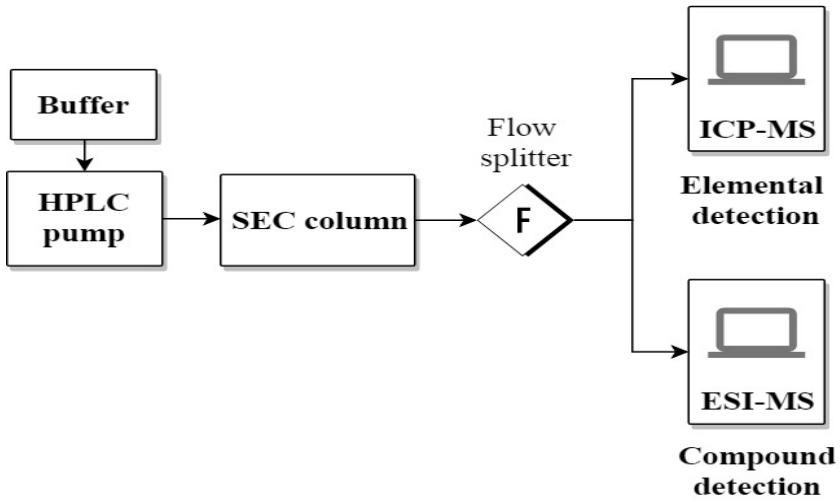

Figure 10 – Flowchart showing the set-up for characterization of unknown Zn-containing compounds by size exclusion chromatography (SEC) simultaneously coupled to inductively coupled plasma mass spectrometry (ICP-MS) and electrospray ionization mass spectrometer (ESI-MS); the sample is pumped into the SEC column by the high-performance liquid chromatography (HPLC) pump; the compounds present in the sample are separated by the SEC column; the flow splitter will split the flow of the sample between both detectors, ICP-MS and ESI-MS.

The preliminary results obtained from the analysis of an extract of an Atlantic salmon feed suggested that this approach could be used as a promising set-up for the characterisation of unknown Zn species (Figure 11), providing simultaneous elemental and molecular information of Zn species. However, these are only preliminary results and further work needs to be done. For instance, the method needs further optimization and mass spectrometric techniques with high mass resolution could be used in the set-up. High mass resolution tools could be employed to achieve accurate mass information of the unknown Zn species (e.g. quadrupole time-of-flight mass spectrometry or orbitrap mass spectrometer). In addition to the identification of the organic-bound Zn species, it is also relevant to develop other Zn speciation methods to determine ionic Zn species in the soluble fraction of a fish feed. For instance, determination of free ionic Zn, as it is known that ionic Zn has a physiological relevance in Zn absorption.

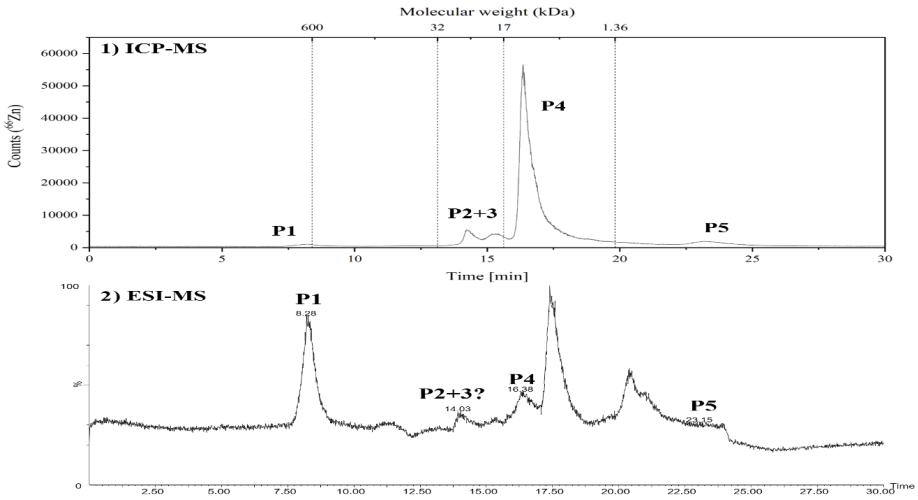

Figure 11 – Chromatograms of Zn species in the soluble fraction of an Atlantic salmon feed analysed by (a) SEC-ICP-MS and (b) SEC-ESI-MS; a molecular weight calibration was performed using thyroglobulin (660 kDa, monitoring ^{127}I), Zn/Cu superoxide dismutase (32 kDa, monitoring ^{66}Zn), myoglobin (17 kDa, monitoring ^{57}Fe), vitamin B12 (1.36 kDa, monitoring ^{59}Co); Peak 1 (P1): ~ 600 kDa, Rt 8.2 min; Peak 2+3 (P2+3): from 32 to 17 kDa, Rt 14.2 + 15.3 min; Peak 4 (P4): from 17 to 1.36 kDa, Rt 16.3 min; Peak 5 (P5): > 1.36 kDa, Rt 23.2 min.

6.2 Mineral availability

This work studied Zn, Se and Mn availability in plant-based diets and the data suggested that in practical diets with low inclusion of fish meal for Atlantic salmon, the availability of Zn, Se and Mn depend on their chemical form. In addition, several interactions had an effect on mineral availability. This encourages further research to understand the interaction mechanisms. However, the complexity of understanding the multiple dietary interactions and their effect on mineral availability can be challenging as several factors play a role. In this regard, the use of experimental factorial design allows to simultaneously study the influence of several factors and their respective interaction effects on the response. This approach should be considered more often in mineral availability studies. In this study, the effect of the chemical form of Zn, Se and Mn was evaluated. However, there are many other relevant minerals. For instance, in further feeding trials, Fe and Cu could be added to the study as it is likely that Cu and Fe are involved in interactions influencing mineral availability in Atlantic salmon.

During this PhD work, emphasis was given to the study of solubility of Zn, Se and Mn. However, there are several other minerals relevant in salmonids nutrition. Thus, the *in vitro* digestion method could be used to study solubility of other minerals in salmonid feeds. Moreover, a strong positive correlation between solubility and apparent availability was obtained for Mn but not for Zn or Se. This indicates that the mineral solubility estimation performed can be used to evaluate Mn availability, while for Zn and Se, the *in vitro* method needs to be optimised, for instance with longer digestion times and wider pH ranges. In addition, more data points could lead to stronger correlation for Zn and Se, thus more feeds should be evaluated.

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Paper I

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Speciation of zinc in fish feed by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry – using fractional factorial design for method optimization and mild extraction conditions

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Speciation of zinc in fish feed by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry – using fractional factorial design for method optimisation and mild extraction conditions

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ABSTRACT

Zinc (Zn) is an element essential to all living organisms and it has an important role as a cofactor of several enzymes. In fish, Zn deficiency has been associated with impaired growth, cataracts, skeletal abnormalities and reduced activity of various Zn metalloenzymes. Fish meal and fish oil traditionally used in salmon feed preparation are being replaced by plant-based ingredients. Zinc additives are supplemented to salmon feed to ensure adequate Zn levels, promoting good health and welfare in Atlantic salmon (*Salmo salar*). The main objective of the present study was to evaluate Zn species found in an Atlantic salmon feed. This work describes a Zn extraction method that was optimized using a fractional factorial design (FFD), whereby the effect of six factors could be studied by performing only eight experiments. The effects of the type of extraction solution and its molar concentration, pH, presence of sodium dodecyl sulphate, temperature and extraction time on Zn extraction were investigated. Mild extraction conditions were chosen in order to keep the Zn species intact. Total Zn (soluble fractions and non-soluble fractions) was determined by inductively coupled plasma mass spectrometry (ICP-MS). The highest Zn recovery was obtained using 100 mM Tris-HCl, pH 8.5 at a temperature of 4 °C for 24 h where the total Zn in soluble fraction and non-soluble fraction was 9.9 ± 0.2% and 98 ± 6%, respectively. Zinc speciation analysis (on the soluble fractions) was further conducted by size exclusion inductively coupled plasma mass spectroscopy (SEC-ICP-MS). The SEC-ICP-MS method provided qualitative and semi-quantitative information regarding Zn species present in the soluble fractions of the feed. Four Zn-containing peaks were found, each with different molecular weights: Peak 1 (high molecular weight - ≥600 kDa), peak 2 and peak 3 (medium molecular weight – 32 to 17 kDa) were the least abundant (1–6%), while peak 4 (low molecular weight – 17 to 1.36 kDa) was the most abundant (84–95%).

1. Introduction

Zinc (Zn) is an element that occurs naturally in water, air and soil and it is essential to all living organisms [1]. Zinc plays an essential role as a cofactor of several enzymes and it has also paracellular and intracellular signalling functions [2]. In farmed fish, Zn deficiency has been associated with impaired growth, cataracts, skeletal abnormalities and reduced activity of various Zn metalloenzymes [3,4]. Feed consumption and waterborne mineral uptake are the main sources of Zn in Atlantic salmon (*Salmo salar*) [5]. The composition of salmon feed have during recent years changed from the use of mainly marine feed ingredients, such as fish meal and fish oil, to an increasing replacement with plant-based ingredients, e.g. soybean meal, maize gluten meal, wheat gluten and rapeseed oil [6]. Zinc is naturally present in fish meal and in plant-based ingredients, with typical concentrations ranging from 64 to 74 mg kg^{-1} (data for 2008) [7] and from 35 to 48 mg kg^{-1} (data for 2016) [8], respectively. Zinc is added to feeds to prevent diseases and ensure animal welfare [9].

The European Union regulation EC No. 2003/1831 and amendments set the rules for the use of Zn additives in animal nutrition [10]. Examples of these additives are zinc acetate dihydrate, zinc chloride anhydrous, zinc oxide, zinc sulphate heptahydrate, zinc sulphate monohydrate, zinc chelate of amino acids hydrate, zinc chelate of protein hydrolysates, zinc chelate of glycine hydrate (solid) and zinc chelate of glycine hydrate (liquid). In the European Union, the current upper limit for total Zn in complete feed of all fish except salmonids is

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150 mg kg^{-1} and for salmonids feeds it is 180 mg kg^{-1} feed [11].

Elemental speciation analysis is the quantification or/and the identification of different chemical compounds, or element species [12]. There is limited knowledge on the chemical species of Zn in fish feeds. As the bioavailability of an element depends on its chemical form (i.e. its species) [13], Zn speciation analysis can provide valuable information with regards to fish nutrition studies. Zinc may be present in organic or inorganic forms. However, it is not so clear which forms have enhanced bioavailability [2,14]. Hence, development of proper analytical methods is needed to characterize Zn species present in feeds.

For separation of element species, high performance liquid chromatography (HPLC) is the traditional separation technique [15]. Other separation techniques, such as gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE) have also been used for the separation of element species [15,16]. Inductively coupled plasma mass spectrometry (ICP-MS) is the preferred detection method for elemental analysis as it gives high sensitivity and selectivity, provides isotope information and has multi-element capability [17]. For speciation analysis of Zn in plant-based matrices, samples are generally extracted using buffers, and subsequent analysis for Zn species is performed by size exclusion chromatography (SEC) coupled to ICP-MS [18–20]. Also, ion-exchange chromatography (IEC) coupled to ICP-MS [19] and CE-ICP-MS [21] have been used for Zn speciation in plant tissue and horse feed, respectively. So far, however, there is no reported study on Zn speciation in fish feed.

Traditionally, method development is performed using the one-factor-at-a-time (OFAT) strategy, which is a labour-intensive and material consuming approach. However, the use of design of experiments (DOE) is a much more efficient way to evaluate not only individual but also joint effects of the variables compared to the OFAT approach [22,23]. A DOE is selected based on experimental objectives, number of factors to be studied and on the amount of resources available. For screening purposes and a large number of factors to be studied, there are typically two types of design that are recommended, the Plackett-Burman and the fractional factorial design (FFD). A FFD is a design where the experiments conducted are only a subset of the runs in the full factorial design. The design can be expanded if needed [23,24].

The DOE has been applied in speciation studies of elements such as copper [25], selenium [26], mercury [27], chromium [28–30], arsenic [31,32] and antimony [32]. In speciation analysis, one of the most critical points is to keep the native structure of each chemical species intact along the extraction process and during the chromatographic separation [33,34]. For the extraction of Zn from a horse feed and tissues of barley grains, the use of ammonium acetate, Tris-HCl and NaCl in a range of concentrations from 10 to 100 mM as extraction solutions were reported [20,21]. In addition, different temperatures and extraction times were evaluated. Considering the lack of methods for Zn speciation in feed, the aim of the present study was to develop an extraction method for Zn in Atlantic salmon feed. The approach included (i) a FFD experimental setup, (ii) mild extraction conditions to keep chemical species intact, and (iii) a chromatographic method to characterize Zn species in Atlantic salmon feed.

2. Experimental

2.1. 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 stated otherwise. Methanol (MeOH, LiChrosolv®, HPLC grade), acetic acid (CH_3COOH, Emsure® ACS, ISO, 96% w/w), hydrochloric acid (HCl, Emsure® ACS, ISO, 37% w/w), hydrogen peroxide (H_2O_2, Emsure® ACS, ISO, 30% w/w) were obtained from Merck (Darmstadt, Germany). Nitric acid (HNO_3, trace select, ≥ 69.0% w/w) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Multielement (product number SS60835) and germanium (product number SS1230) standard solutions were obtained from Spectrascan TeknoLab (Drøbak, Norway). Tris(hydroxymethyl)aminomethane [Tris-HCl, $NH_2C(CH_2OH)_3$], ammonium acetate ($NH_4CH_3CO_2$), sodium dodecyl sulphate (SDS), thyroglobulin (T1001), glutathione peroxidase (G6137), superoxide dismutase (S7446), myoglobin (M1882) and vitamin B12 (V2876) were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Sample

The feed sample used (L1, 3 mm) is described elsewhere [35]. The feed was formulated based on commercial feed for Atlantic salmon, containing protein sources mainly from plant-based ingredients (i.e. 15% marine protein, 8% fish oil, 65% plant proteins and 10% plant oils). Zinc oxide was added to the feed. The feed was grinded by hand with a pestle and a mortar, and sieved to ensure a feed fraction with similar particle size (from 850 µm to 1.12 mm). To establish a target value for total Zn, the feed sample was analysed at the laboratory and two other accredited laboratories.

2.3. Experimental design

Based on previous Zn speciation studies [20,21], the factors included in the experimental design were type of extraction solution (A), molar concentration of the extraction solution, mM (B), pH (C), addition of 4% sodium dodecyl sulphate (SDS) solution (D), temperature, °C (E), and extraction time, hour/s (F) (Table 1). Factors were set at low (−1) and high (+1) levels. The experimental procedure was performed according to a 2^{6-3} fractional factorial design (resolution III). Eight experiments in triplicate (n = 3) and a blank for each experiment

Table 1
2_{III}^{6-3} fractional factorial design. The tested factors were type of extraction solution (A), concentration of the extraction solution, mM (B), pH (C), addition of 4% sodium dodecyl sulphate (SDS) solution (D), temperature, °C (E), and extraction time, hour/s (F). Factor level codes are shown as "−1" or "+1" followed by the real factor level shown between parenthesis. Concentration of soluble Zn is expressed as mean ± standard deviation (mg kg^{-1} feed, n = 3).

Exp.	Factors: Coded (real)						Soluble Zn (mg kg^{-1} feed)
	A: Extraction solution	B: Concentration (mM)	C: pH	D = AB: 4% SDS	E = AC: Temp. (°C)	F = BC: Time (h)	
1	−1 (Tris-HCl)	−1 (10)	−1 (6.5)	+1 (yes)	+1 (20)	+1 (24)	6.2 ± 0.2
2	+1 (Amm. Acetate)	−1 (10)	−1 (6.5)	−1 (no)	−1 (4)	+1 (24)	4.5 ± 0.3
3	−1 (Tris-HCl)	+1 (100)	−1 (6.5)	−1 (no)	+1 (20)	−1 (1)	5.66 ± 0.07
4	+1 (Amm. Acetate)	+1 (100)	−1 (6.5)	+1 (yes)	−1 (4)	−1 (1)	6.9 ± 0.1
5	−1 (Tris-HCl)	−1 (10)	+1 (8.5)	+1 (yes)	−1 (4)	−1 (1)	6.9 ± 0.2
6	+1 (Amm. Acetate)	−1 (10)	+1 (8.5)	−1 (no)	+1 (20)	−1 (1)	3.87 ± 0.09
7	−1 (Tris-HCl)	+1 (100)	+1 (8.5)	-1 (no)	-1 (4)	+1 (24)	10.9 ± 0.3
8	+1 (Amm. Acetate)	+1 (100)	+1 (8.5)	+1 (yes)	+1 (20)	+1 (24)	6.14 ± 0.05

($n = 1$) were performed (in total 32 experiments). Details about the conditions used for experiment 1–8 are presented in Table 1. The experimental design and analysis of data from experiments was performed using R commander plugin for DOE [R foundation for statistical computing, version 3.4, [36,37]. The main effect of each factor (A to F) was calculated using Eq. (1):

$$\text{effect of main factor} = \left(\frac{\sum Y+}{n+}\right) - \left(\frac{\sum Y-}{n-}\right) \quad (1)$$

where "Y +" refers to the responses at level (+1), the "Y −" to the responses at level (−1), the "n +" to the number of data points at level (+1) and "n −" to the number of data points at level (−1).

A two-tailed t-test was used to determine the statistical significance of the main effects at a confidence level of 95% using Eq. (2):

$$t - \text{value of the effect} = \frac{\text{effect of main factor}}{\text{standard error}} \quad (2)$$

Approximately 0.5 g of feed was extracted into 5 mL of extraction solution, for 1 or 24 h, at a temperature of 4 or 21 °C. The extraction solution applied was either Tris-HCl or ammonium acetate, with concentrations of 10 or 100 mM and pH values of 6.5 or 8.5. One milliliter of 4% of SDS was added to some samples (Table 1). The final volume was adjusted to 5 mL in all samples. The samples were extracted in a random order. After the extraction procedure, samples were centrifuged for 10 min at 3000*g* (Eppendorf® Centrifuge 5702, Hamburg, Germany). The samples were fractionated into soluble and non-soluble fractions using a Pasteur pipette. The soluble fractions were filtered through a 0.45 µm disposable syringe filter (Sartorius, Göttingen, Germany) and transferred to new tubes. The non-soluble fractions were dried in an oven for 24 h at 60 °C. The experimental outline of the study is presented in Fig. 1. Total Zn (soluble fractions and non-soluble fractions) was determined using ICP-MS and Zn speciation analysis (soluble fractions) was performed using SEC-ICP-MS.

2.4. Determination of total zinc by ICP-MS

For the determination of total Zn, the feed and feed fractions (i.e. the soluble and the non-soluble fractions) were decomposed using microwave assisted acid digestion based on the procedure previously described [38]. Briefly, approximately 0.2 g of feed was digested using 2.0 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 entire dried non-soluble fractions (~0.5 g). The soluble fractions (500 µL) were 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 total Zn determination was performed by use of an ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) equipped with an autosampler (FAST SC-4Q DX, Elemental Scientific, Omaha,

Table 2
The operating parameters for the ICP-MS and SEC-ICP-MS.

ICP–MS settings (iCapQ)	
Forward power	1550 W
Plasma gas flow	14.0 L min^{-1}
Carrier gas flow	1.02 L min^{-1}
Makeup gas flow	0.80 L min^{-1}
Dwell time	0.1 s per isotope
Isotopes monitored	^{66}Zn, ^{72}Ge

ICP–MS settings (7500cx)	
Forward power	1550 W
Plasma gas flow	15.0 L min^{-1}
Carrier gas flow	0.94 L min^{-1}
Makeup gas flow	0.25 L min^{-1}
Dwell time	0.1 s per isotope
Isotopes monitored	^{127}I, ^{78}Se, ^{66}Zn, ^{59}Co, ^{57}Fe

HPLC settings	
Column	TSKgel G3000SWxl SEC column (30 cm × 7.8 mm, 5 µm particle size) + QC-PAK guard column (7 µm particle size)
Calibration range	1.0×10^4–5.0×10^5 Da
Mobile phase	50 mM Tris-HCl + 3% MeOH (pH 7.5)
Flow rate	0.7 mL min^{-1}
Injection volume	50 µL

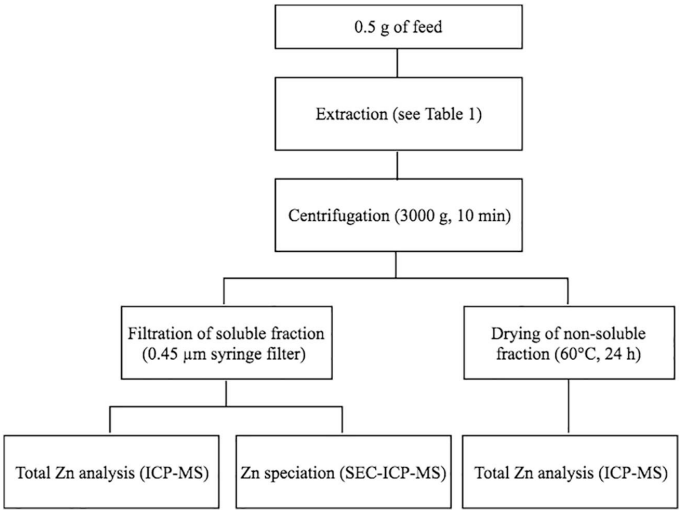

Fig. 1. Methodology flowchart.

USA). The samples were analysed in a random order. A solution of germanium was added on-line for correction of instrumental drift during the analysis. The instrument was optimized using a tuning solution (1 ppb tuning solution B, Thermo Fisher, in 2% HNO_3 and 0.5% HCl) prior to analysis. The instrumental settings are presented in Table 2. Data were collected and processed using the Qtegra ICP-MS software (Thermo Scientific, version 2.1, 2013). For the quantitative determination of total Zn, an external calibration curve (10 to 500 ng mL^{-1}) was used and two certified reference materials were included to assess the accuracy of the method: 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 were in agreement with the certified values. The validated range for Zn determination is from 0.5 to 1400 mg kg^{-1} (DW).

2.5. Zinc speciation by SEC-ICP-MS

The SEC-ICP-MS method was developed based on principles described elsewhere [20,39]. Further optimisation was done in this study to the analysis of a fish feed. The soluble fractions were analysed using a 1260 HPLC coupled with a 7500cx ICP-MS (Agilent Technologies, Santa Clara, USA) and a SEC column (TSKgel G3000SWxl, Tosoh, Stuttgart, Germany). The mobile phase solution was prepared by dissolving an appropriate amount of tris(hydroxymethyl)aminomethane to reach the desired ionic strength (50 mM) in an aqueous 3% (v/v) MeOH solution, followed by adjustment of pH to 7.5 with HCl (37% w/w). The samples were analysed in a random order. The instrument was tuned according to manufacturer's instructions. The instrumental settings for the HPLC and ICP-MS are listed in Table 2.

Prior to speciation analysis of the soluble fractions, a molecular weight calibration was performed using thyroglobulin (660 kDa, monitoring ^{127}I), glutathione peroxidase (84 kDa, monitoring ^{78}Se), Zn/Cu superoxide dismutase (32 kDa, monitoring ^{66}Zn), myoglobin (17 kDa, monitoring ^{57}Fe), vitamin B12 (1.36 kDa, monitoring ^{59}Co). The standards were prepared with a concentration of 100 ng element mL^{-1} in Milli-Q® water. For the quantitative determination of Zn species an external calibration curve of the Zn/Cu SOD standard (5 to 200 ng Zn mL^{-1}) was applied, and species were quantified by peak areas. The calibration curve was analysed at the beginning and at the end of the analytical sequence. The 50 ng Zn mL^{-1} standard was analysed at the middle of the sequence. All sample extracts were spiked with 0.5 µL of vitamin B12 (1000 ng mL^{-1}) prior to analysis in order to correct for retention times shifts.

The chemical nature of the Zn species in the soluble fractions was further investigated. The soluble fractions of experiment 7 ($n = 3$) were split in two parts, one was heated and the other was kept as is. The soluble fractions were heated at 90 °C for 10 min using a heat block (Bibby Scientific Stuart, Stone, Staffordshire) as described by Temara and colleagues [40]. The heated and non-heated extracts were evaluated by the SEC-ICP-MS method as previously described.

3. Results and discussion

3.1. Total zinc in feed

The average total Zn concentration was 110 ± 8 mg kg^{-1} of feed ($n = 10$). The target value was used to calculate the recovery of the extraction experiments.

3.2. Effect of extraction factors by fractional factorial design

The concentration of soluble Zn was different in the various experimental runs proposed by the 2^{6-3} fractional factorial design (Table 1). The highest and lowest Zn recoveries were obtained under the conditions dictated by experiments 7 and 6 respectively (Table 1).

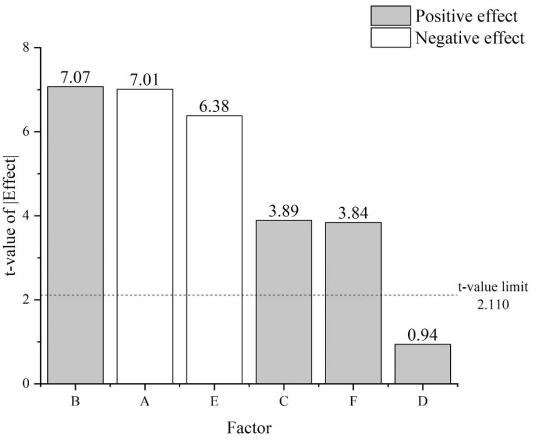

Fig. 2. Pareto chart with the magnitude of the main effects using the concentration of soluble Zn as response. Horizontal axis shows the factors ordered accordingly with their magnitude; type of extraction solution (A), concentration of the extraction solution, mM (B), pH (C), addition of 4% sodium dodecyl sulphate (SDS) solution (D), temperature, °C (E), and extraction time, hour/s (F); the vertical axis shows the *t*-value of the absolute effect. The reference line is the *t*-value limit ($\alpha = 0.05$; d.f. = 17). A *t*-value of |effect| above the reference line indicates a significant contribution of this factor to the response ($p < 0.05$). In grey, the effects with positive *t*-value and, in white, the effects with negative *t*-value. The standard error was 0.1448 for each main factor.

The former condition consisted of 100 mM Tris-HCl, pH 8.5 at a temperature of 4 °C for 24 h and the latter of 10 mM ammonium acetate, pH 8.5 at a temperature of 20 °C for 1 h.

The effect of the main factors on the response is presented in the form of a Pareto chart (Fig. 2). The results show that the factors having a statistically significant effect ($p < 0.05$) on Zn extraction were type of extraction solution (A), molar concentration of the extraction solution, mM (B), pH (C), temperature, °C (E) and extraction time, hour/s (F). The addition of 4% sodium dodecyl sulphate (SDS) solution did not have a significant effect on the Zn extraction and such finding was also reported elsewhere [41]. The same results suggest that to maximize the concentration of Zn extracted, factors such as type of extraction solution (A) and temperature, °C (E) should be kept at the low (−1) level, which implies using Tris-HCl as extraction solution and performing the extraction at 4 °C. Furthermore, factors such as molar concentration of the extraction solution, mM (B), pH (C) and time, hour/s (F) should be kept at the high (+1) level. This means the extraction should be performed using 100 mM Tris-HCl, pH 8.5 at a temperature of 4 °C for 24 h. Altogether, these extraction conditions correspond to those described by experiment 7 (Table 1). As a set of experimental conditions was obtained in the initial fractional factorial design, no more experiments were performed.

3.3. Zinc extraction recovery

Zinc recovery (%) was determined for the soluble and non-soluble fractions by calculation of the ratio of Zn obtained for each fraction compared to total Zn in the feed (110 mg kg^{-1} feed) (Table 3). The variation obtained was acceptable taking into consideration the measurement uncertainty of the method, which is 20%. The sum of both fractions was calculated by adding the average value found in soluble Zn (%) and non-soluble Zn (%). The overall recovery of Zn ranged from 83 to 124%.

Between 4 and 10% of Zn was extracted into the soluble fraction of the feed (Table 3). The extraction method is a critical step in element

Table 3
Total Zn in the soluble and non-soluble fractions (%) and the calculated sum Zn (%). Soluble Zn and non-soluble Zn values are expressed as mean ± standard deviation (%, n = 3).

Exp.	Soluble Zn (%)	Non-soluble Zn (%)	Sum Zn (%)
1	5.6 ± 0.1	105 ± 12	111
2	4.1 ± 0.2	82 ± 19	83
3	5.15 ± 0.06	112 ± 5	117
4	6.3 ± 0.1	118 ± 6	124
5	6.2 ± 0.1	108 ± 14	114
6	3.52 ± 0.08	105 ± 3	109
7	9.9 ± 0.2	98 ± 6	108
8	5.58 ± 0.05	102 ± 7	108

speciation analysis. This is mainly due to the challenges of providing high extraction recovery as well as preserving the integrity of the original species during the extraction process simultaneously [42,43]. There is a number of speciation protocols, which include the use microwave and ultrasound assisted extraction [44,45]. However, our study focus on the use of mild extraction conditions to keep the chemical species intact. Microwave and ultrasound-assisted extraction could affect the species integrity [44,45]. Consequently, both microwave and ultrasound assisted extraction were not included in the extraction methodology.

Mild extraction conditions were applied to keep the integrity of the chemical species intact, which may compromise the extraction recovery in the soluble fraction. Furthermore, Zn ion (Zn^{+2}) can easily bind to other compounds which are less soluble in water (i.e. phytic acid, sulphides) [46,47]. The lower solubility found in this study could be due to Zn binding to other compounds present in the fish feed and thereby forming water insoluble Zn species.

3.4. Zinc speciation analysis of feed by SEC-ICP-MS

Different types of columns and mobile phases were tested in order to identify the most robust technique for Zn speciation analysis, i.e. a method that preserves the integrity of the metal binding species. First, various anion-exchange settings were applied for the separation of Zn species. However, the obtained chromatograms showed poor resolution and severe peak broadening. Hence, anion-exchange chromatography was disregarded as a chromatographic separation technique for Zn species in fish feed extracts. This finding is consistent with a previous study, according to Persson et al. (2009), anion-exchange chromatography showed poor chromatographic results for Zn compounds from barley grains [20]. Reversed phase chromatography (RPC) was applied for the separation of Zn species but the obtained chromatograms also showed poor resolution and peak broadening. Hence, RPC was also disregarded as a chromatographic separation technique for Zn species in fish feed extracts. In IEC and RPC, the separation is based on electrostatic forces [48] and this may cause effects on the native chemical structures in the separation creating artefacts and misleading information [49]. This may be due to de-stabilization of the metal binding species and the weak binding capacity of some metals, such as Zn [46]. When applying SEC, the stability of the Zn species markedly improved, and therefore SEC-ICP-MS was chosen as a method for Zn speciation. The SEC-ICP-MS method gave semi-quantitative results for the Zn species detected using the external calibration curve of the Zn/Cu SOD standard (Table 4). Furthermore, the method provided qualitative results regarding molecular size of Zn species present in the feed extracts, by comparison of elution times of Zn species with the elution times of the molecular weight calibration standards (Fig. 3 and Table 4).

The soluble fractions of experiment 1 to 8 were evaluated by SEC-ICP-MS. Both, number of peaks and total Zn in the soluble fraction were used as parameters to select the set of Zn extraction conditions. The results from the SEC-ICP-MS analysis show the presence of several Zn species. The different extraction conditions affected the type and amount of species present in the extract (Table 3). Extraction conditions applied in experiment 1, 4, 5, 7 and 8 extracted peaks 1 to 4. However, when using the extraction conditions of experiment 2, 3 and 6, peak 1 was not detected. The ratio of each peak was calculated based on the sum of all peaks and it is presented in Table 3. Peak 1, peak 2, and peak 3 were the least abundant (1–6%) and peak 4 was the most abundant (84–95%). Fig. 3 shows the Zn profile of the soluble fraction of a feed extract using the extraction conditions of experiment 7 ($n = 3$) obtained by SEC-ICP-MS. The chromatograms from the three replicates of experiment 7 are overlapping, thus indicating good repeatability (Fig. 3).

The SEC-ICP-MS method gave qualitative information regarding the size range of the Zn species present in the soluble fraction. The molecular weight calibration was performed using thyroglobulin (660 kDa, Rt ~ 9.4 min), glutathione peroxidase (84 kDa, Rt ~ 12.9), Zn/Cu superoxide dismutase (32 kDa, Rt ~ 14.6), myoglobin (17 kDa, Rt ~ 17.2), vitamin B12 (1.36 kDa, Rt ~ 19.5). On the chromatograms, it was observed the elution of peak 1 (Rt ~ 8.6 min) and those Zn species have a high molecular weight (≥ 600 kDa). Additionally, peaks 2 and 3 peaks were observed (Rt ~ 15.7 and 16.6 min) and the Zn species in this case are medium molecular weight 1 (Mw ~ 32–17 kDa). Peak 4 (Rt ~ 18 min) indicates the presence of Zn species with low molecular weight (Mw ~ 17 kDa–1.36 kDa).

In SEC, the molecules separation is based on molecule size. Hence, each peak might contain several compounds with similar molecular weight [50].

Structural information about the Zn-containing compounds present in peak 1 to 4 would give complementary data about the Zn species. However, one limiting factor in further method development is the lack of standards to study Zn compounds [19,51]. The Zn compounds were further investigated providing complementary information of the chemical nature of the Zn species in the soluble fractions. The Zn compounds found in the soluble fraction originate from different sources, as the feed samples contain both animal and plant ingredients. The different ingredients and the molecular weight range of the Zn peaks suggest that the observed Zn peaks could be metalloproteins. One of the most studied metalloproteins is the ubiquitous metallothioneins (MTs). The MTs are thermally stable proteins, so a heating step is a commonly used protocol to confirm their presence [52]. Thus, the soluble fractions of experiment 7 ($n = 3$) were heated. The chromatographic profile obtained from heated and non-heated extracts were compared, and the chromatographic profiles were similar. The compounds eluting in peak 1, 2, 3 and 4 were heat stable, suggesting that the compounds are MTs. The MTs are known to be the only proteins which are heat stable and have metal association ability [53]. This supports our suggestion of the Zn compounds being MTs.

4. Conclusions

In the present study, the effect of different conditions for the extraction of Zn from fish feed was studied using a FFD approach. Eight experiments were carried out and the effect of six different factors on the extraction of Zn was determined. The highest recovery for Zn in fish feed was obtained when using 100 mM Tris-HCl, pH 8.5 at a temperature of 4 °C for 24 h and four peaks were found under these extraction conditions. The application of mild extraction conditions and SEC were found to be appropriate to keep the Zn species intact. The speciation profile of Zn in the soluble fractions was evaluated using a SEC-ICP-MS method developed to study Zn species in a fish feed. This analytical method will be used to characterize Zn species present in feeds.

Table 4
Zinc speciation analysis by SEC-ICP-MS (mean ± standard deviation, n = 3, mg kg $^{-1}$ feed).

	Peak 1 (≥ 600 kDa, Rt ~ 8.6 min)		Peak 2 (32–17 kDa, Rt ~ 15.7 min)		Peak 3 (32–17 kDa, Rt ~ 16.6 min)		Peak 4 (17–1.36 kDa, Rt ~ 18 min)	
Exp.	mg kg $^{-1}$ feed	Area (%)	mg kg $^{-1}$ feed	Area (%)	mg kg $^{-1}$ feed	Area (%)	mg kg $^{-1}$ feed	Area (%)
1	0.33 ± 0.03	4	0.505 ± 0.005	6	0.442 ± 0.004	6	6.75 ± 0.06	84
2	n.d.	0	0.263 ± 0.005	3	0.339 ± 0.002	4	8.6 ± 0.1	93
3	n.d.	0	0.44 ± 0.01	5	0.447 ± 0.003	5	8.7 ± 0.2	91
4	0.138 ± 0.002	1	0.349 ± 0.004	4	0.376 ± 0.004	4	8.9 ± 0.2	91
5	0.181 ± 0.004	2	0.278 ± 0.005	3	0.354 ± 0.002	4	8.3 ± 0.1	91
6	n.d.	0	0.294 ± 0.001	3	0.383 ± 0.001	4	9.0 ± 0.5	93
7	0.0635 ± 0.0004	1	0.171 ± 0.006	2	0.295 ± 0.001	3	9.7 ± 0.2	95
8	0.29 ± 0.06	4	0.326 ± 0.006	4	0.343 ± 0.005	4	7.13 ± 0.07	88

n.d. = not detected.

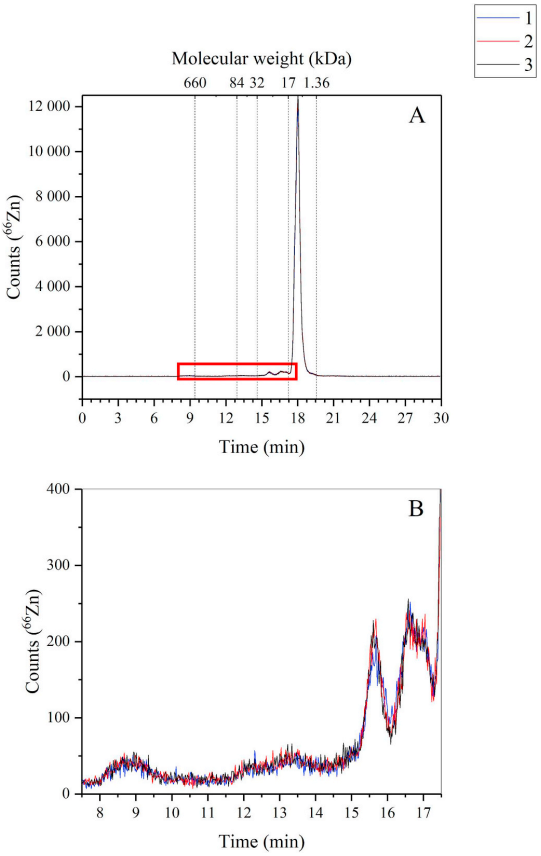

Fig. 3. Chromatogram of Zn species in the soluble fraction of feed, extracted according to experiment 7, and analysed by SEC-ICP-MS (n = 3); (A) Shows a complete Zn profile; (B) Shows an enlargement of Zn profile from 8 to 17 min; Blue, red and black lines represent the three replicates of experiment 7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

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Paper II

P. Antony Jesu Prabhu, Thea Stewart, Marta Silva, Heidi Amlund, Robin Ørnsrud, Erik-Jan Lock, Rune Waagbø and Christer Hogstrand

Zinc uptake in fish intestinal epithelial model RTgutGC: Impact of media ion composition and methionine chelation

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Zinc uptake in fish intestinal epithelial model RTgutGC: Impact of media ion composition and methionine chelation

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ABSTRACT

Apical uptake of zinc as ionic Zn(II) or as Zn-methionine (Zn-Met) was studied in RTgutGC cell line *in vitro* under media compositions mirroring the gut luminal ionic concentration of freshwater (FW) and seawater (SW) acclimated salmonids. Viability of the RTgutGC cells exposed to experimental media preparations showed a time-dependent decrease in SW treated cells, with the effect being significant at 48 h ($P < 0.01$), but not at 12 h or 24 h. Half effective concentration of Zn exposure over 12 h (EC_{50}, in μM) was not differentially affected by media composition (FW, 59.7 ± 12.1 or SW, 83.2 ± 7.2; mean ± SE, $P = 0.43$). Zinc (^{65}Zn) influx in RTgutGC was not different between FW or SW treated cells, but increased significantly in the presence of methionine (2 mM, L-Met or DL-Met). An interaction effect was observed between Zn concentration and media ionic composition on the impact of Met on apical Zn uptake (L-met, $P < 0.001$; DL-met, $P = 0.02$). In the presence of Met, apical Zn uptake in SW medium was significantly lower compared to FW, but only at higher Zn concentrations (12 and 25 μM, $P < 0.01$). Further, Met facilitated Zn uptake was reduced in cells treated with an amino acid transport system blocker with the effect being more significant and stereospecific in SW ionic conditions. The findings of this study showed that (i) Zn speciation in the presence of Met improved apical Zn uptake in RTgutGC cells and Zn-Met species were possibly taken up through Met uptake system. (ii) The effect was differentially affected by the ionic composition of the medium. Implications and limitations of the observations towards practical Zn nutrition of salmonids are discussed.

1. Introduction

While essential as a nutrient, zinc (Zn) is also a potential toxicant and an environmental contaminant of concern [1]. Although fish are able to acquire waterborne Zn via gills, diet is regarded to be the major source of Zn [2]. Knowledge on gastrointestinal (GI) uptake of Zn is therefore significant in fish nutrition and aquatic toxicology [3]. The GI tract is a highly versatile and multi-functional organ in fish [4]. In addition to the primary function of nutrient uptake, the GI tract also serve osmoregulatory functions [5]. The ionic composition of the gut luminal content in seawater (SW) fish varies from that of freshwater (FW) fish due to the fact that marine fish drink and selectively precipitate ions to facilitate water uptake in the hyperosmotic seawater medium [6]. To date, knowledge on the impact of ionic composition of the gut luminal contents on nutrient uptake at the intestinal epithelium is limited. Impact of lumen composition on the GI uptake of Zn was found to be complex and affected by interactions with other ions and ligands at various stages of absorption [3,7].

In aquaculture, formulated fish feeds are supplemented with Zn additives, as the bioavailability of endogenous Zn in feed ingredients is low due to the presence of anti-nutritional factors [8]. The Zn additives used can be categorised into inorganic salts and chelated forms. Inorganic Zn salts of sulphate, chloride or oxide are used, where sulphate is the most studied and relatively more bioavailable form of inorganic Zn to fish [9]. Among chelated forms, Zn chelated with specific amino acids eg. methionine [10,11], glycine [12] or a mix of amino acids have been studied [13,14]. In the aforementioned studies, the chemical form of Zn additive and interactions anti-nutrients have been the focus of investigation towards enhancing bioavailability of dietary Zn [8,9]. In some of these studies, dietary supply of amino acid chelated Zn was found to be more bioavailable than inorganic salts of Zn [11,14]. Nevertheless, results are inconsistent and subject to high variability

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[9,15], such that it remained inconclusive even in a radiotracer study [16]. Physiological studies on Zn uptake in fish using *in vitro* brush border vesicles [17] and *in vivo* perfusion models [3] have revealed that amino acids with high binding affinity for Zn(II) can improve Zn uptake, but the mechanism for which is less understood.

Similar to mammals, GI uptake of Zn in fish is also believed to be orchestrated by more than one transport system involving solute carrier families Slc30 (Znt) and Slc39 (Zip); and potentially L-Type Calcium Channel (LTCC) and divalent metal transporter-1(DMT1) [7,18]. In the intestine, dietary Zn binds to the mucus of the intestinal epithelium, and is transported into the epithelial cells either as the Zn(II) ion or bound to amino acids [1,15]. Uptake of Zn(II) through Zip4 is of vital nutritional significance in mammals; however, the relative efficiency and functional importance of Zip mediated Zn(II) uptake versus amino acid facilitated Zn uptake in GI tract remains to be well understood in mammals, and even more so in fish [1]. The complexity of the environment and multiple dietary interactions have been major constraints in understanding the limiting factors of dietary Zn bioavailability in fish nutritional studies. In this regard, strengthening our knowledge on uptake mechanisms is required to better understand and predict dietary metal bioavailability from feed matrices to fish under varying environmental conditions [19,20].

Until recently, enterocyte cell models were not available to study nutrient uptake mechanisms for fish nutrition research. However, now an intestinal epithelial cell line (RTgutGC) exhibiting apical and basolateral characteristics has been established [21,22]. RTgutGC cell line has been proposed as a physiologically adequate fish intestinal epithelial model, equivalent to the Caco-2 cell line for human intestinal epithelium [22]. Since then, RTgutGC cells have been well characterised with structural and functional features like forming a monolayer, mucous secretion, tight junction and desmosomes formation between adjacent cells, develop trans-epithelial resistance and polarize over time to exhibit epithelial characteristics [21,23,24]. RTgutGC cells have been used to study metal uptake characteristics for environmental monitoring of potential metal toxicants like silver and its nanoparticles [23,25,26]. However, uptake of nutritionally relevant metals have not been investigated in the RTgutGC model. In this study, we examined the apical uptake of Zn and Zn-Met species as affected by media composition mirroring the luminal ionic concentrations found in freshwater (FW) and seawater (SW) acclimatised salmonids using the RTgutGC cell line.

2. Material and methods

2.1. Cell culture

RTgutGC cells (obtained in kind from Professor Dr. Kristin Schirmer, Dept. of Environmental Toxicology, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Switzerland) were aseptically cultured in Leibovitz' L-15 medium (Invitrogen/Gibco, Switzerland) containing 5% fetal bovine serum (FBS, Eurobio, France) and 1% gentamycin sulfate (BioWhittaker™/Lonza, Belgium) at 19 °C in normal atmosphere as previously described by [22]. The cells were routinely grown in 75 cm^2 cell culture flasks and when confluent after 2 weeks, they were either split to new flasks or harvested to be used in experiments. Cells from the confluent flasks were harvested by washing twice with 1 ml Versene EDTA solution (Invitrogen/Gibco, Switzerland) and detached using 0.7 ml of trypsin (0.25% in phosphate-buffer saline, PBS, Biowest, Nuaille´, France). The trypsin reaction was stopped by adding 10 ml of L15/FBS medium. The resulting cell suspension was centrifuged at 1000 rpm, 19 °C for 3 min. The density of the harvested cells was estimated by manual counting using haemocytometer. The cells were diluted to required volume in L15/FBS and seeded to each well in 24 well plates (Falcon™ Polystyrene Microplates) at a density of 5×10^4 cells $well^{-1}$ and incubated at 19 °C for 48 h prior to experiments.

Table 1
Chemical and ionic composition of the experimental media tested.

Chemical composition (mM)	L15/ex	Freshwater (FW)	Seawater (SW)	Values from literature reports	
Sodium nitrate	155	155	155		
Potassium nitrate	6.2	6.2	6.2		
Magnesium sulfate	3.8	19.5	51.1		
Calcium nitrate	1.5	5.4	5.4		
HEPES	5.0	5.0	5.0		
Magnesium chloride	–	15.0	44.9		
Sodium pyruvate	5.7	5.7	5.7		
Galactose	5.7	5.7	5.7		
pH	7.1	7.4	7.4		
Ionic strength	178.0	258.0	400.0		
Ionic composition (mM)				FW‡	SW§
Calcium, Ca^{2+} *	1.6 ± 0.1	5.3 ± 0.2	5.1 ± 0.2	2 - 4	60 - 185
Magnesium, Mg^{2+} *	3.9 ± 0.3	32.5 ± 0.7	89.4 ± 2.5	15 - 25	50 - 135
Potassium, K^+ *	8.2 ± 1.2	8.6 ± 1.1	7.6 ± 1.3	8 - 10	3 - 14
Sodium, Na^+ *	160 ± 3	157 ± 2	153 ± 3	100 - 150	100 - 225
Nitrate, NO_3^- **	164	172.4	172.4		
Sulfate, SO_4^- **	3.8	18.7	48.6		
Chloride, Cl^- **	1.5	31.5	94.5	25 - 35	60 - 120

*analysed (n = 3); **nominal. L15/ex, adapted from [29]; FW and SW, conceptually formulated to mimic ionic composition of intestinal luminal fluid in freshwater [27,28]‡ and seawater [6] § acclimated salmonids. Ionic strength was calculated using the software Visual MINTEQ.

2.2. Exposure media composition

Two experimental media (i) FW and (ii) SW were conceptually designed from [27,28,6] to closely represent the luminal ionic composition of the freshwater (FW) and seawater (SW) acclimated salmonids, respectively (Table 1). One other medium (L15/ex), adapted from [29], was used as reference to test the viability of cells when treated with experimental media compositions. The composition of the reference exposure medium was based on the ionic concentration of complete L-15 medium used to culture the RTgutGC cells without amino acids or serum and was shown to be able to maintain viability of the cells up to 72 h [21]. The nominal and analysed ionic concentrations of exposure media are presented in Table 1. The concentrations of ions were analysed using a PE NexION 350D ICP-MS instrument following the method as described in [25].

2.3. Cell viability assay

The metabolic activity of the cells, measured with the Alamar blue assay, was used to indicate cell viability. Cell suspension in complete L15/FBS was seeded to 96-well plates at a density of 4×10^4 cells $well^{-1}$ and incubated at 19 °C for 24 h before exposure to different experimental media. After 24 h, the L15/FBS medium was removed from each well, rinsed twice with phosphate buffered saline (PBS) and treated with L15/ex, FW or SW medium with total nominal zinc concentrations of 0, 25, 50, 100 and 150 μM; the respective analysed Zn concentrations were 0.2, 25.2, 52.1, 105.8 and 158.3 μM. The viability of cells was examined at 12 h, 24 h and 48 h post-exposure to experimental media by incubating in dark for 1 h at 19 °C with the Alamar blue reagent (10 μL $well^{-1}$) and absorbance recorded at 570 nm, with 600 nm as a reference wavelength using a spectrophotometric plate reader.

2.4. Zinc (^{65}Zn) influx assays

2.4.1. Effect of ionic composition of the medium

The RTgutGC cells were seeded to 24 well plates at a density of 5×10^4 cells well^{-1} in complete L15/FBS medium, and incubated at 19 °C for 48 h. Subsequently, the medium in the wells were removed, rinsed thoroughly with PBS, treated with respective experimental medium and allowed to acclimatise for 20 min. Later, the cells were treated with the same medium with ^{65}Zn(II) (as $ZnCl_2$; approx. 4 kBq/ml; Perkin-Elmer, USA) added at different concentrations (added as $ZnSO_4$ solution, molecular biology grade, Sigma) and incubated at 19 °C for 15 min. The medium was then recovered from the well, rinsed with ice cold medium (with 200 μM Zn, pH 7.4) and quench buffer (with 5 mM EGTA, pH 7.4) for 5 min to remove any adsorbed ^{65}Zn(II). The monolayer of cells adhered to the bottom of the wells were digested with 100 μl of 2% hot SDS detergent and the cellular material recovered completely. The cell digests were then counted for radioactivity using 1282 Compugamma Laboratory Gamma Counter, LKB Wallac. The counts per minute (cpm) obtained were corrected for background activity and radioactive decay, and converted using specific activity calculations following the formulae of Glover and Hogstrand [3]. In each treatment group, 6 wells were used; 3 wells were exposed to ^{65}Zn containing medium, whereas 3 others were exposed to equivalent concentrations of Zn from $ZnSO_4$ only. The latter were used for determining the protein concentration of cells (after homogenisation with 500 μl of 0.5 M NaOH) using Bradford assay kit (Bio-Rad) with BSA as the standard. The rate of uptake was expressed as pmoles Zn min^{-1} mg^{-1} protein.

2.4.2. Impact of methionine on Zn uptake

Zn uptake by RTgutGC cells were examined in FW and SW media compositions in the presence of L-methionine (L-Met, Sigma) or DL-methionine (DL-Met, Alfa Aesar). The cells were exposed to nominal concentrations of 3.07, 6.14, 12.27 and 24.55 μM ^{65}Zn(II) in FW and SW media (i) without amino acids (control) or with 2 mM of (ii) L-Met or (iii) DL-Met. The pH of all experimental media preparations were adjusted to 7.4 using 0.5 M NaOH. The preparation of cells prior to experimental exposure and the assay conditions were as described in 2.4.1. The exposure period was 15 min and experiments were performed in triplicate (n = 3) with three technical (well) replicates per experiment. The influx of ^{65}Zn(II) was calculated as described in 2.4.1.

2.4.3. Impact of amino acid transport inhibitor on zinc uptake

Cells were seeded in 24 well plates as described in 2.4.1 and exposed to FW or SW medium with a nominal concentration of 10 μM ^{65}Zn(II) either without amino acids (i) or with 2 mM of (ii) L-Met, (iii) D-methionine (D-Met, Sigma) or (iv) DL-Met. The exposure to the above media preparations were made in the presence or absence of 10 mM of an amino acid transport inhibitor (2-Aminobicyclo [2.2.1] heptane-2-carboxylic acid, BCH, Sigma). The exposure period was 15 min and assay conditions were as described in 2.4.1. The experiment was performed in triplicate (n = 3) with three technical (well) replicates each time.

2.4.4. Data analyses

The data presented in this manuscript are mean of three repeated observations (n = 3), analysed using GraphPad Prism version 7 for Windows, GraphPad Software, California, USA. Data on EC_{50} for Zn exposure in FW and SW medium were estimated using a four parameter, variable slope model. The test of significance between EC50 in FW and SW media was obtained through fit-comparison option available in GraphPad Prism. Rest of the data were analysed by two-way ANOVA followed by Tukey's multiple comparison test; whenever the interaction effect was significant, one-way ANOVA was employed to make group-wise comparisons using Tukey's multiple comparison test.

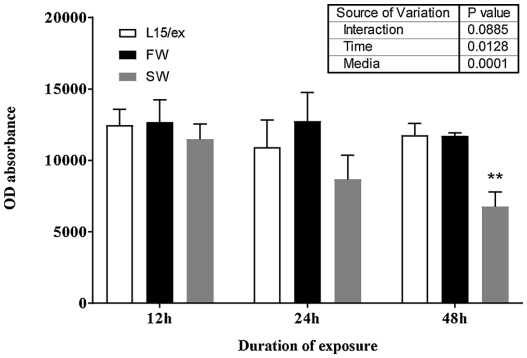

Fig. 1. Viability of RTgutGC cells (measured with Alamar blue assay) exposed to FW (black bar) and SW (grey bar) experimental media relative to L15/ex (white bar) as reference medium. Data are represented as mean ± SD (n = 3) of 12, 24 and 48 h exposure periods. Two-way ANOVA showed significant effect of both media composition ($P < 0.01$) and time ($P < 0.05$). Asterisk (*) indicates significant difference at each time point as obtained through Tukey's multiple comparison test (**, $P < 0.01$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3. Results

3.1. Cell viability assay

The metabolic activity of the cells exposed to the FW and SW media compositions were on par with the cells exposed to the reference medium (L15/ex) at 12 h, and declined thereafter in SW treated cells in a time-dependent manner (Fig. 1). The exposure to increasing concentrations of Zn in the medium reduced the metabolic activity of the cells in a dose dependent manner (Fig. 2). Analysis of the cell viability data using a four parameter, variable slope model indicated that the EC_{50} for Zn (as μM, mean ± SE) was not significantly different between RTgutGC cells exposed to FW (59.7 ± 12.1) or SW (83.2 ± 7.2) media (P = 0.43).

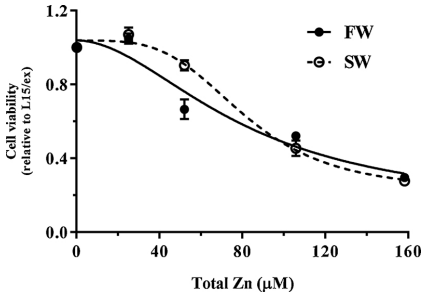

Fig. 2. Concentration dependent decrease in metabolic activity of RTgutGC cells exposed to total zinc concentrations from 0 to 160 μM in FW (filled circle, solid line) or SW (open circle, dashed line) after 12 h exposure, normalised to L15/ex. Four parameter, variable slope model used to calculate EC_{50} for Zn (mean ± SE, in μM) in RTgutGC cells exposed to FW (59.7 ± 12.1) and SW (83.2 ± 7.2) media, however the difference was not statistically significant (P = 0.43). The test of significance was performed in GraphPad Prisim by the option to compare best-fit values of specific parameter (EC_{50}) between two data sets.

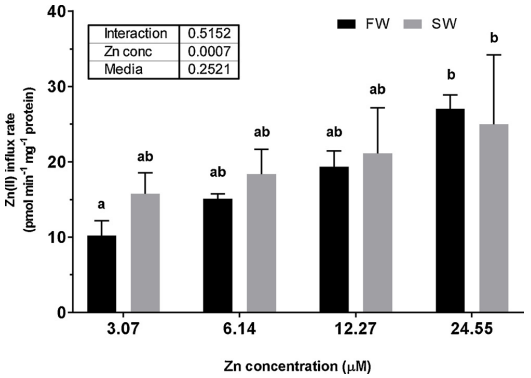

Fig. 3. Impact of ion composition in the medium on Zn influx in RTgutGC cells (mean ± SD; n = 3): Zn influx under FW (black bars) and SW (grey bars) media. Data were analysed through two-way ANOVA, followed by Tukey's multiple comparison test. The P-values of ANOVA are presented in insets and the post-hoc differences among groups are represented as superscript letter above the bars. Bars with different are statistically different ($P < 0.05$).

3.2. Effect of media composition and methionine chelation on apical Zn uptake

The influx of ^{65}Zn increased with increasing Zn concentration in the media ($P < 0.001$). The difference in the ion composition between FW or SW media did not have a significant impact the influx of ^{65}Zn in RTgutGC cell line (Fig. 3). Methionine (L- or DL-) inclusion in the media at 2 mM concentration significantly influenced the apical influx of Zn in RTgutGC cells (Fig. 4). Two-way ANOVA showed significant interaction effect (L-met, $P < 0.001$; DL-met, $P = 0.02$) between Zn concentration and media ionic composition on the impact of methionine on Zn uptake. Post-hoc comparison following one-way ANOVA showed that Zn uptake in the presence of methionine (L-Met or DL-Met) was significantly lower in SW treated cells at higher Zn concentrations (12 and 25 μM) ($P < 0.05$). Cells exposed to amino acids along with the amino acid transporter blocker (BCH) showed a significant ($P < 0.001$) reduction in ^{65}Zn uptake when compared to cells untreated with BCH. The effect was more pronounced and stereospecific in SW than in FW conditions (Fig. 5).

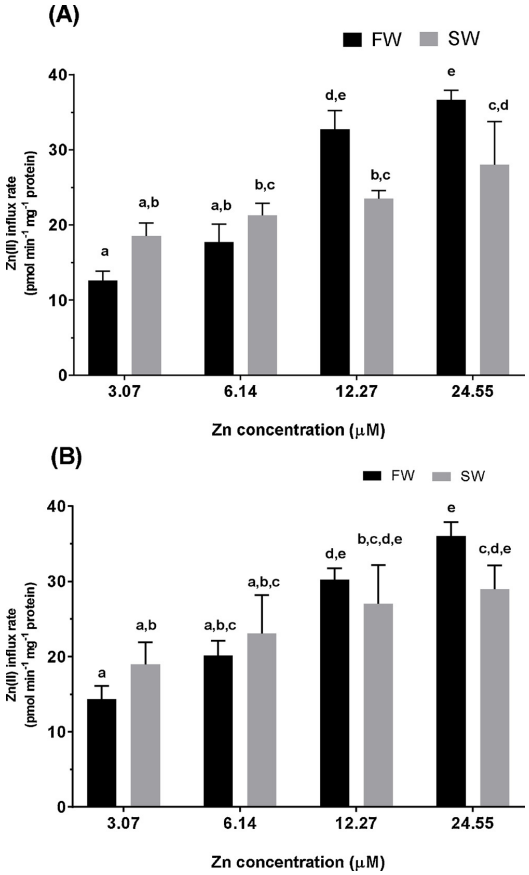

Fig. 4. Impact of methionine chelation on Zn influx in RTgutGC cells (mean ± SD; n = 3): Zn influx under FW (black bars) and SW (grey bars) medium (A) with 2 mM L-methionine; (B) 2 mM DL-methionine. Data were initially analysed by two-way ANOVA, which showed a significant interaction effect. Therefore, one-way ANOVA was performed followed by Tukey's multiple comparison test. The post-hoc differences among groups are represented by superscript letter above the bars. Bars with different letters differ significantly ($P < 0.05$).

4. Discussion

Ionic composition in the gastrointestinal (GI) lumen of fish can vary depending on the environment (salinity induced hypo- or hyper-osmoregulation), feeding status (time after a meal) and diet composition (ion concentration of the diet). In rainbow trout, *in vitro* metal uptake studies have used artificial saline preparations to closely mimic the ionic composition found in the GI lumen [30–33]. However, large variations seem to exist in the Ca^{2+} and Na^{+} concentrations between the artificial saline and actual measurements in rainbow trout. The Na^{+} concentration in the intestinal luminal content of rainbow trout 10–20 h post-meal and 24 h after sudden change to seawater varies from about 100–225 mM [6]; whereas, in the mucosal saline used for the *in vitro* studies the Na^{+} concentration ranges between 0 and 60 mM [30–33]. Similarly, the Ca^{2+} concentration in the mucosal saline used in studies in rainbow trout [33] and other marine/seawater adapted fish was about 5 mM [30–32]; whereas, in intact marine fish, the luminal content was reported to vary from 60 to 185 mM [6]. Higher Ca^{2+} levels tend to precipitate chloride and sulfate, and hence it might be a methodological consideration with *in vitro* studies to use less Ca^{2+}. Moreover, in marine fish, the high Ca^{2+} entering the GI lumen through drinking of seawater is precipitated as calcium carbonate aided by Ca^{2+} induced bicarbonate secretions from the intestinal epithelium [34]. In this context, it is not known if bicarbonate secretion is active in RTgutGC cell line and this merits further investigation. In the present study, although the Na^{+} concentrations were closer to the range of those found in seawater living rainbow trout during digestion of a meal [6], the Ca^{2+} concentrations were considerably lower (see Table 1). The effect of luminal Ca^{2+} on intestinal zinc uptake is complex; it stimulated epithelial Zn uptake in rainbow trout, but inhibited post-intestinal accumulation of Zn [7]. It is therefore possible that the difference in Ca^{2+} concentrations used in the present study and that in the seawater rainbow trout intestine influenced our results but it is difficult to predict the directionality of this uncertainty.

Zinc transport across cellular and intracellular membranes takes place through Zn transport proteins (Zip and Znt) [18]. Transport of Zn into the cytosol is mediated by members of the slc39 (Zip) transporter family, while movement of Zn away from the cytosol, either into organelles or out of the cell, is achieved through members of the slc30 (Znt) transporter family [35]. In mammals, Zip1–6 and Zip14 are

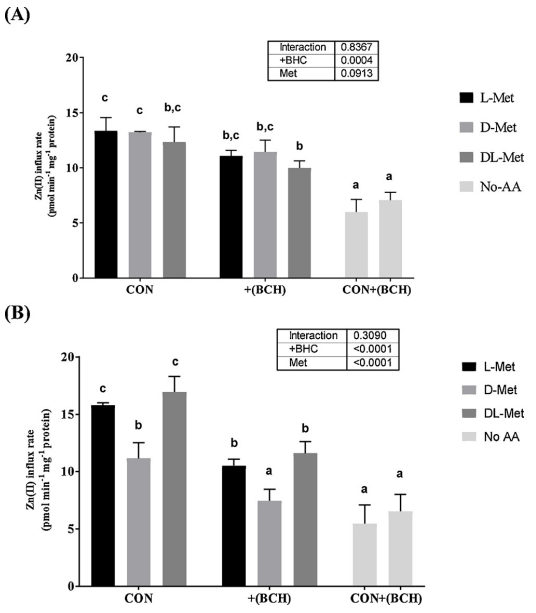

Fig. 5. Impact of amino acid transport inhibitor (2-Aminobicyclo [2.2.1] heptane-2-carboxylic acid, BCH, 10 mM) on zinc influx in RTgutGC cells exposed to FW (A) and SW (B) medium. L-methionine (L-Met), D-methionine (D-Met) or DL-methionine (DL-Met) added at 2 mM concentrations. No-AA (without methionine, negative control); CON, control with methionine; +BCH, with BCH. Data presented as mean ± SD (n = 3) analysed using two-way ANOVA, with Met and BCH as main effects (No-AA treatments were not included in the two-way ANOVA model). P-values of the main effects and their interaction are provided as insets. The differences between groups as obtained by Tukey's multiple comparison test are shown as superscript letter over the bars. Bars with different letters are significantly different ($P < 0.05$).

involved in Zn uptake from extracellular fluid; Zip4 located at the apical surface and Znt1 at the basolateral membrane of intestinal epithelia are of vital importance for uptake of dietary Zn [36,37]. The mRNA and protein levels of key Zn transporters were differentially expressed in the duodenum of pigs fed $ZnSO_4$ or Zn-chitosan chelate as the dietary Zn source; the $ZnSO_4$ group, with more ionic Zn(II) had significantly higher Zip4 and Zip5 protein abundance [38]. Although Zip transporters are involved in the uptake of ionic Zn(II), other divalent metal transport systems (DMT1, CTR1, TRPV6) [39,40] and amino acid mediated pathways may also contribute to Zn uptake in mammals [41,42]. However, in fish Trpv6 is not expressed in the intestine and the importance of Dmt1 and Ctr1 for intestinal Zn uptake are not known. It has been suggested that amino acid-linked Zn transport occurs in fish intestine [3,17]. Zn uptake in brush-border membrane vesicles was correlated to mono-histidine species [17] and the bis-histidine Zn complex was bioavailable to rainbow trout *in vivo* [3]. The effects of histidine and cysteine on the uptake of Zn by mammalian erythrocytes have also been suggested to be mediated by the bis-complex of metal-amino acid species [42]. The donor ligand hypothesis and/or the transported chelate hypothesis have been suggested as the two plausible means of amino acid aided metal transport in fish [3,17,20,43]. The donor-ligand hypothesis assumes that amino acid ligand aids is shuttling metals from inhibitory ligands in the luminal chyme to dedicated metal transporters; whereas, in the transported-chelate hypothesis an alternative transport pathway which accepts the metal-amino acid chelate as a substrate is proposed. In rainbow trout brush border membrane vesicles, lack of stereospecific action of histidine (L- or D-) upon apical Zn(II) uptake suggested of the donor-ligand exchange, but was not in support of the transported chelate hypothesis [17]. However, the possibility of fish amino acid transport system being less stereospecific was also suggested, contrary to mammals [44]. Nevertheless, the impact of different stereoisomers is important to be studied as the efficiency of organic Zn additive used in fish feeds can be related to the stereoisomer used to chelate. Moreover, it is of relevance in understanding the possibility of methionine supplements (L- or DL-) used in fish feeds improving Zn uptake. By studying the uptake of copper (Cu) from Cu-histidine in the presence of an array of potential histidine transport system inhibitors, Glover et al. [20] suggested a distinct transport system for Cu-histidine chelates in rainbow trout brush border membrane vesicles, *in vitro*. Recently, manganese (Mn) from Mn-lysine complex has been suggested to be transported by amino-acid uptake pathways (y^+ and $b^{0,+}$), different from the ionic Mn^{2+} uptake pathway in primary rat intestinal epithelial cells [45]. In the present study, Zn uptake increased in the presence of methionine and it was reduced upon simultaneous exposure to BCH, a potent blocker of Na^+-dependent methionine transport systems in intestinal epithelial cells [46,47]. These data suggest that the Zn-methionine chelate is transported through an amino acid mediated uptake pathway, similar to that suggested for Cu-histidine and Mn-lysine [20,45]. Therefore, the transported chelate hypothesis merits further investigation.

In mammals, amino acid uptake systems are pH sensitive and are either Na^+-dependent (B° and $B^{0,+}$), or Na^+-independent ($b^{0,+}$, L, and y^+); whereas, systems $B^{0,+}$, $b^{0,+}$, and y^+ are used by cationic amino acids, systems B° and L are specific for neutral amino acids [46,48]. Understanding of the different amino acid uptake pathways and the mode of action of the amino acid transport blocker used (BCH) is required to comprehend the differential effects of BCH on Zn uptake in RTgutGC cells exposed to FW and SW medium. BCH competes with methionine as a substrate of transport systems, which includes both the Na^+-dependent system (B° and $B^{0,+}$) and a part of the Na^+-independent (L-type) amino acid transport systems [46]. While the usefulness of BCH to study amino acid uptake systems is documented, no reports are available to refer if BCH has the ability to chelate Zn. We hereby show that BCH alone was neither able to significantly increase or decrease the uptake of Zn(II), which implies that Zn chelation with BCH was not favored under the test conditions. In fish, the Na^+-mediated components of amino acid transport in the intestine are dependent on luminal Na^+ concentration, whereas only the non-mediated components are functional in the absence of luminal Na^+ [49,50]. Although the Na^+ concentration and pH of the medium was not different between FW and SW, the effect of BCH in reducing Zn uptake in the presence of methionine was more potent in cells exposed to SW medium. This observation could possibly be due to a differential contribution of the Na^+-independent system in amino acid uptake under FW and SW luminal conditions. Indeed, in the European seabass (*Dicentrarchus labrax*), the contribution of the saturable Na^+-independent component was much higher for epithelial transport of methionine, compared to other amino acids namely glycine or alanine [51]. Further studies targeting specific Zn(II) and putative Zn-amino acid chelate transport systems are required to better understand the underlying mechanisms.

With increasing inclusion of plant derived protein sources used in salmonid feeds, availability of dietary Zn is reduced and requires higher supplementation levels than those recommended by NRC [52] to meet the Zn requirement of salmonids [53]. Due to environmental concerns, the European Food Safety Authority (EFSA) opinion suggested reduction in the maximum permissible Zn levels in salmonid feeds from 200 to 150 mg kg^{-1} complete feed and also laid emphasis on improving the availability of dietary Zn to limit Zn emissions [54]. In this context, our present study provides basic data in support of Zn-Met chelation to improve apical Zn uptake in the enterocytes, but also the possibility of a physiological limitation under luminal conditions of high ionic

concentration. The lack of functional adaptability in RTgutGC cells to SW ionic conditions due to short exposure and lack of convincing data to discern if the cells exhibited uptake at initial rate or saturation kinetics are potential limitations of this study. RTgutGC exhibited a transient induction in mRNA expression of Na^+/K^+-ATPase (α-subunit) only up to 24 h upon exposure to a high ionic SW medium [21]. Therefore, the uptake characteristics displayed by the RTgutGC cells herein can be more comparable to rainbow trout enterocytes exposed to seawater challenge and not that displayed by a seawater adapted fish. Our previous research on zebrafish suggests that there is no or limited systemic control of zinc uptake across the intestine, in the sense of humoral regulation, and the intestinal epithelium is responding directly to the Zn availability in the gut [55]. This improves the relevance of an *in vitro* system for studies on Zn uptake; nevertheless, a cell culture will always be a model of reality. Hence, translating the results from this *in vitro* uptake study in an enterocyte cell line to intestinal Zn uptake *in vivo* should be viewed with these constraints in mind.

The findings of this study towards understanding intestinal Zn uptake under different ionic conditions and dietary Zn forms will be of high practical interest in fish nutrition. To conclude, using RTgutGC we found no evidence for a difference in Zn uptake in media representing the intestine of FW and SW salmonids. However, Zn uptake in the presence of methionine was influenced by the ionic concentration in the media.

Declarations of interest

None.

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Paper III

Marta S. Silva, Saskia Kröckel, P. Antony Jesu Prabhu, Wolfgang Koppe, Robin Ørnsrud, Rune Waagbø, Pedro Araujo and Heidi Amlund

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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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.

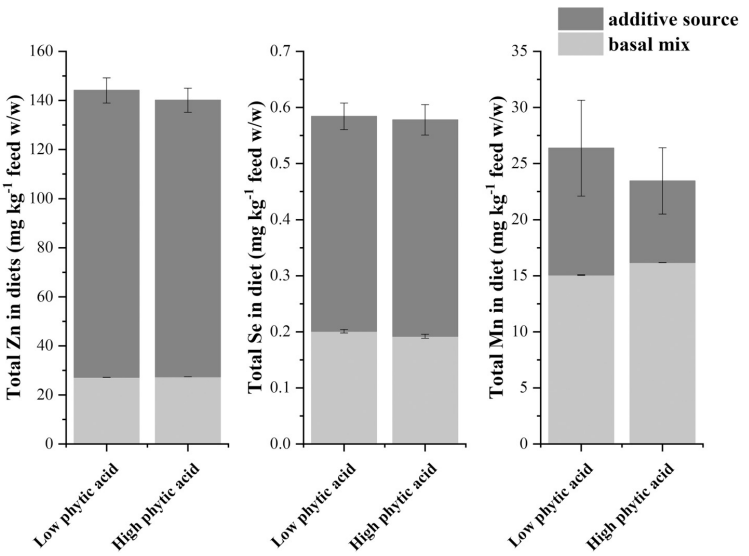

Fig. 1. Total concentration (mg kg^{-1} feed w/w) of zinc (Zn), selenium (Se) and manganese (Mn) in the low phytic acid diets (n = 8) and in the high phytic acid diets (n = 8); total concentration values are presented as average ± standard deviation; the light grey represents the concentration of Zn,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

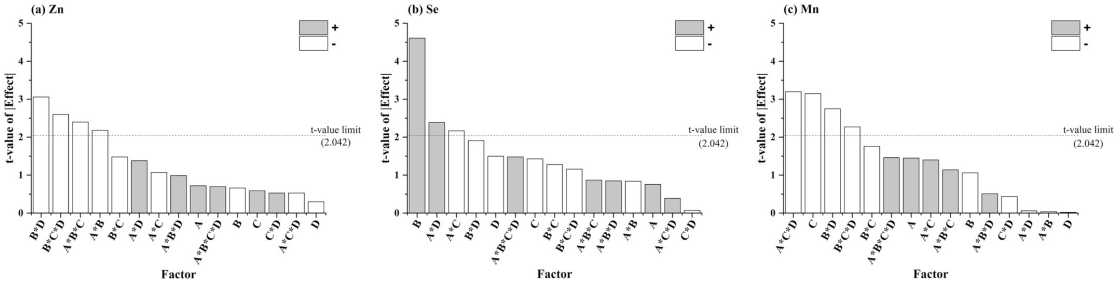

Fig. 2. Pareto chart showing the t-value of the effect using individually apparent availability (AA, %) of zinc (Zn) (a), selenium (Se) (b) and manganese (Mn) (c); the horizontal axis shows the factors and interactions ordered according to their magnitude, the factors are Zn additive source (A), Se additive 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;

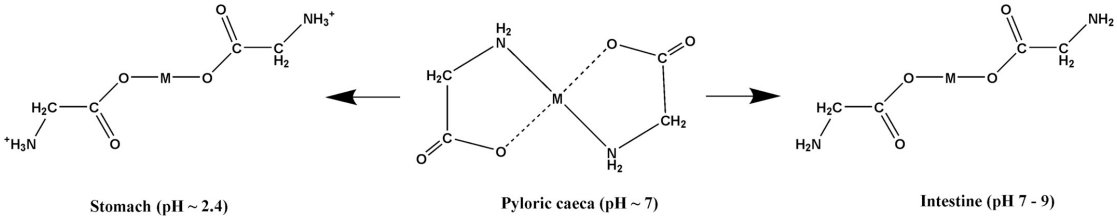

Fig. 3. – Changes in chemical conformation of glycine chelate during the fish intestinal tract: stomach (pH ~ 2.4), pyloric caeca (pH ~ 7) and intestine (pH 7–9).

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 ± 0.1 μmol g^{-1} ($n = 2$) and 12.0 ± 0.1 μmol 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 ± 0.1 μmol g^{-1} to 12.0 ± 0.1 μmol 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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Paper IV

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***In vitro* digestion method to evaluate solubility of dietary zinc, selenium and manganese in Atlantic salmon (*Salmo salar*) diets**

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