Feed phosphorus regimes and the effectiveness of a novel phytase on mineralisation and bone health in Atlantic salmon pre- and post-smolt

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Mowi is the largest company among salmon farmers in Norway, with operations in multiple countries and a fully integrated value chain, including the feed division Mowi Feed. Mowi Feed strives to optimise feed ingredients while maintaining sustainability and high quality and continuously seeks to improve fish's health and welfare. Mowi Feed AS funded this project.

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Abbreviations

FM = fish meal

ANF = antinutritional factors

IP6 = inositol hexaphosphate

MAP = mono ammonium phosphate

MCP = mono calcium phosphate

NC = nephrocalcinosis

ANOVA = One-way analysis of variance

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Abstract

Bone health in farmed Atlantic salmon is a challenge affecting fish welfare and the economy. With the increasing use of plant-derived ingredients in fish feeds, impaired mineral availability and utilisation can impact bone health in Atlantic salmon. To address this, the impact of different feed P regimes and including a novel *E. coli* phytase in the feed on bone health for Atlantic salmon pre- and post-smolt was studied. Five experimental diets (Alp, Amc, Pro, Plp, and Pta) were prepared for this trial in freshwater with different levels of FM, total P, source and level of inorganic P, and the addition of phytase. Diet 'Alp' was formulated with mono ammonium phosphate (MAP) as the only inorganic P source. Diet 'Amc' with 50/50 MAP and mono calcium phosphate (MCP), 'Pro' was high FM and 76% MAP, 'Plp' with low FM and 115% MAP, and 'Pta' was low FM, 115% MAP, and inclusion of 2500 FTU/kg phytase. The first three diets had a total P content of 1.5%, while the two latter diets had 1.3%. Atlantic salmon pre-smolts (43 g) were fed the diets for 13 weeks in freshwater, with four replicate tanks for each diet. The tanks were connected to a standard RAS unit. After the freshwater period, the fish were transferred to seawater and fed a standard commercial diet for 3 weeks. Fish were sampled at the start and end of the freshwater and seawater phases. Growth performance, somatic indices, and nephrocalcinosis were monitored. Additionally, the apparent availability, mineral status, vertebral deformities, and mechanical properties were examined to evaluate the effect on bone health. All diets had comparable growth performance, somatic indices, and mineral status. All diets provided fish with the minimum requirement for available P (0.8%), except for Amc (0.73%). Fish fed Amc had twice the occurrence of deformed fish (25%) than the other groups (12.5-15%), suggesting that a slightly lower available P led to increased vertebral deformities. In the low-P diets (Plp and Pta), the Ca:P ratio was lower than in the other groups. Fish fed these diets had a higher occurrence of nephrocalcinosis and indications of softer vertebrae. The present study showed that replacing half of MAP with MCP led to decreased available P and, consequently, to a higher prevalence of vertebral deformities. Further, reduced Ca:P ratio in low P diets led to a higher incidence of nephrocalcinosis and lower mechanical strength. Adding phytase to a low-P diet (Pta) did not impact improving the availability of P. Still, an effect of the phytase was evident in the scales leading to increased mineralisation of Zn.

Introduction

1.1 Salmon Aquaculture in Norway

In 2020, Atlantic salmon accounted for 33% of marine and coastal aquaculture among all finfish species worldwide (FAO, 2022). Among countries producing farmed salmon, Norway is the world's largest. Since the first farmed salmon was set out into a sea pen in a Norwegian fjord outside Trondheim in 1970, salmon aquaculture has grown tremendously. The industry has experienced this growth due to increased global demand, technological advances in aquaculture practices, and increased research into sustainable and efficient feeds and production methods. However, aquaculture still possesses excellent growth potential. Further improvements in fish feed formulations to ensure environmental sustainability by reducing waste and nutrient emissions without compromising fish health and welfare are essential for this expansion. However, the current situation in the industry, with increased mortality and poor health or welfare, is not sustainable if the industry is expected to grow.

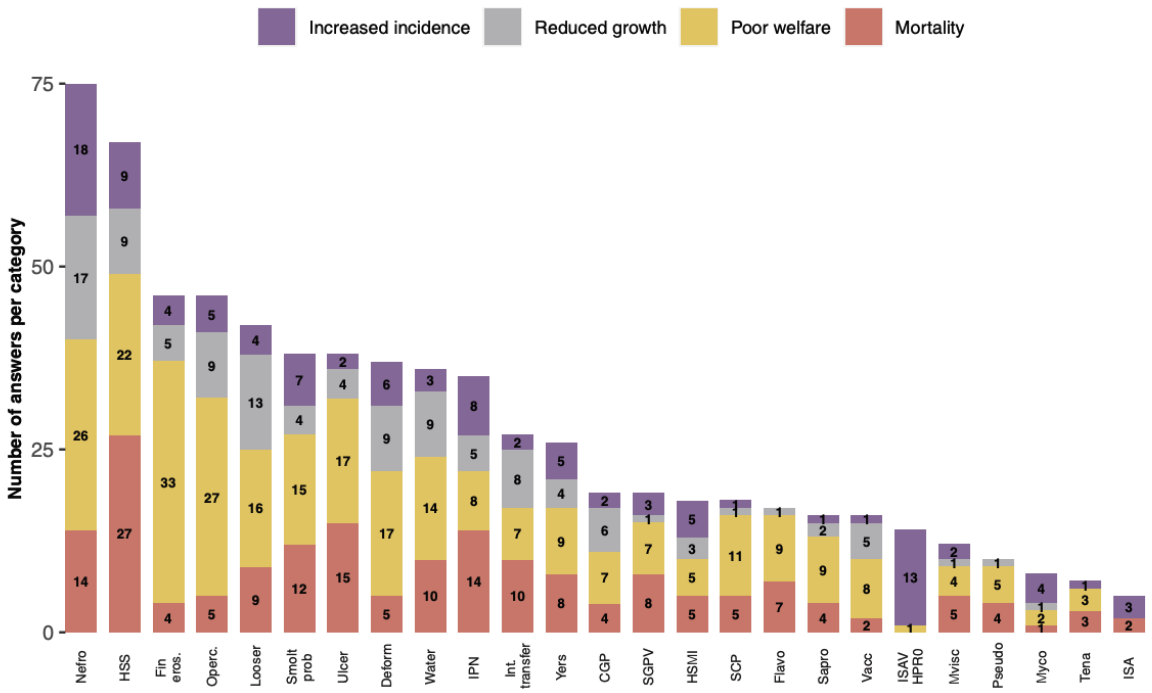


Figure 1. Health problems of juvenile Atlantic salmon production in Norway (Sommerset *et al.*, 2022).

Sommerset *et al.* (2022) reported that the mortality of farmed Atlantic salmon in Norway in 2021 was 33 and 54 million in juvenile and on-growing phases, respectively. Infections caused by bacteria, viruses, and parasites can lead to mortality and various health problems in fish (figure 1). Non-infectious diseases or conditions are also a big problem leading to reduced welfare and increased mortality (figure 1). Specifically, among these non-infectious conditions, such as deformities and nephrocalcinosis, are problems related to mineralisation in the fish. The

mortality rate is commonly used as an indicator of health and welfare along with other indicators, including behaviour, health, environmental factors, and physiological condition (Nilsson *et al.*, 2022). Next to mortality, downgrades cause considerable economic loss to Atlantic salmon farming and indicate prolonged periods of reduced welfare for the salmon. In Norway, approximately 15% of Atlantic salmon is downgraded due to lesions, wounds, maturation, dark spots, and deformities (Wiik-Nielsen, 2023).

1.2 Mineralisation problems in Atlantic salmon farming

1.2.1. Bone health

Morphologically, fish bones consist of dermal head bones, vertebral column and internal skeleton, and scales (Lall and Lewis-McCrea, 2007). In Atlantic salmon, the vertebral column consists of 57-60 vertebra (Kacem, Meunier and Baglinière, 1998), with rims of adjacent vertebra interconnected by intervertebral ligaments derived from the notochord (Nordvik *et al.*, 2005). The notochord is crucial for developing the nervous system and is characterised by hydrostatic pressure responsible for stiffening the body as the fish grows. Each vertebra provides strength and attachment for muscles, while the softer notochord and its derivatives provide necessary mobility between the individual vertebra (Kryvi, 2022). The bones play an essential role in providing support for normal development, posture and during swimming. Bones also serve as an essential site for muscles' attachment, as a reservoir of ions, and as protection of vital organs (Lall and Lewis-McCrea, 2007). The vertebrae are composed of bone tissue, which is connective tissue with crystals of the mineral hydroxyapatite embedded in a strong network of collagen fibres. Bone tissue comprises an organic matrix, minerals, and bone cells. In Atlantic salmon, the bone cells present are bone-forming cells (osteoblasts), multinucleated bone-resorbing cells (osteoclasts), and osteocytes, which are entrapped inside the bone matrix (Nordvik *et al.*, 2005; Gil Martens *et al.*, 2006; Lall and Lewis-McCrea, 2007). In addition, the bones represent an important reservoir of minerals, mainly calcium (Ca), phosphorus (P), zinc (Zn), and manganese (Mn) (Watanabe, Kiron and Satoh, 1997), and the organic matrix is mostly made up of collagen fibrils that provide a stable framework for bone tissue (Wang *et al.*, 2013).

Over the years, increased research on developmental biology in fish has received great attention. These advances have led to faster growth and shorter production cycles, with more focused attention on somatic growth. Meanwhile, less emphasis has been given to proper bone growth, despite bones providing the basic framework (Baeverfjord *et al.*, 2019). Therefore, poor

bone health is a problem in salmon aquaculture, leading to reduced growth and poor welfare (Hansen *et al.*, 2010). Poor bone health is typically observed as deformities in the vertebral column. Bone health issues in Atlantic salmon farming are linked to multiple physiological, environmental, genetic, xenobiotic, and nutritional factors (Waagbø, 2006; Lall and Lewis-McCrea, 2007). The environmental (i.e. abiotic) factors are light intensity, pH, CO_2, temperature, O_2, radiation, salinity, water flow rates, and tank volumes. Nutrient deficiencies, stocking density, growth rates, infections or vaccination, and genetic factors such as mutations, hybridisation or inbreeding are important for the biotic factors. Light intensity, temperature, water velocity, and nutrient imbalances are the most critical factors in bone health. Among the environmental factors, elevated temperatures and photoperiods for growth manipulation are essential factors causing vertebral deformities (Lall and Lewis-McCrea, 2007). Pre- and post-smolts subjected to high-temperature regimes in freshwater or seawater developed a higher prevalence or risk of developing vertebral deformities as adults (Grini *et al.*, 2011; Philip *et al.*, 2023). Fjelldal, Lock, *et al.* (2012) found that post-smolts need higher P supplementation to support normal bone development to accommodate the increased growth rate when Atlantic salmon is reared under continuous light. Less conspicuous deformities in the vertebral column, such as compression-related, vertical- and internal shifts, are hard to detect without an X-ray and have no observable effect on the external morphology of the fish. Therefore, many deformities may follow the fish to market size and cause downgrading losses at harvest (Fjelldal, Hansen and Berg, 2007). However, providing adequate nutrition at critical life stages when manipulating environmental conditions (e.g. during smoltification) is essential.

1.2.2. Nephrocalcinosis

In intensive salmon farming, production conditions may increase the risk of diseases. Nephrocalcinosis (NC) is a production-related condition that leads to the accumulation of mineral deposits, mostly calcium, phosphate, and magnesium, in the kidneys of affected fish. It is regarded as a major problem in the freshwater phase of Atlantic salmon, ranked as the second highest problem leading to increased mortality and reduced growth and welfare (table 1). The mineral deposits can lead to clogging of the tubular system, destruction of the epithelial cells, tissue fibrosis, and inflammation of the surrounding tissue. Clinical signs usually include white, longitudinal stripes on the kidney, swelling of the organ, and in some cases, a decrease in metabolism and energy production. Mortality is generally low; however, it can increase with excess handling and seawater transfer, making it an important welfare indicator in farmed Atlantic salmon (Klykken *et al.*, 2022; Wiik-Nielsen, 2023). The cause of NC is unclear;

however, it has been linked to unbalanced mineral content in the feed and chronic high levels of CO_2 (Wiik-Nielsen, 2023).

Table 1

Most important problems associated with freshwater phase of Atlantic salmon farming in Norway.

Rank	Mortality	Reduced growth	Poor welfare	Increased incidence
1	HSS	Drop-outs	Fin erosion	**Nephrocalcinosis**
2	**Nephrocalcinosis**	**Nephrocalcinosis**	**Nephrocalcinosis**	ISAV-HPR0
3	Drop-outs	**Deformities**	**Operculum deformity**	IPN
4	Inadequate water quality	**Operculum deformity**	HSS	Yersiniosis
5	Issues with smoltification	Inadequate water quality	**Deformities**	Pseudomonas sp.

Notes: Table adapted from (Wiik-Nielsen, 2023), with reports from salmon producers in Norway. HSS (haemorrhagic smolt syndrome), ISAV-HPR0 (non-virulent infectious salmon anaemia virus), IPN (infectious pancreas necrosis).

1.3. Changing feed composition and bone health

One of the most exciting and challenging advances in salmon farming has been the increased use of plant-based ingredients in fish feeds. This allows the feed producers to increase the use of vegetable oil and protein and lower the use of the scarcer resources such as fish oil and fishmeal in a more modern salmon diet. In 1990, the average salmon feed in Norway was produced from approximately 90% marine sources, 9% vegetable sources, and 1% micro-ingredients (Aas, Ytrestøyl and Åsgård, 2019). However, despite its high nutritious value and palatability, marine sources have become more expensive and less sustainable (Hussain *et al.*, 2015), leading researchers and feed manufacturers to search for a cheaper and more sustainable protein commodity. Fish feed formulations today contain less FM, higher levels of concentrated plant-based proteins, vegetable oils, fish oils, vitamins, minerals, amino acids, and supplements as pigment and inorganic P sources to meet the nutrient requirements of the fish. According to a 2020 update, the salmon feed was produced from approximately 22% marine sources, 73% vegetable ingredients, and 4% micro-ingredients, including vitamin and mineral additions, feed P, astaxanthin, and crystalline amino acids (Aas, Åsgård and Ytrestøyl, 2022). Increasing micro-ingredients is essential to provide the same nutritional benefit when replacing marine sources.

The quality of fish feed depends on its nutrient composition and balance and effective utilisation and bioavailability to the fish. Plant-based materials inherently contain anti-nutritional factors (ANF), which are known to reduce the bioavailability of minerals and protein to the fish, thereby contributing to reduced uptake and mineralisation (Thompson, 1993), and aquaculture pollution when excreted into the environment (Baruah *et al.*, 2004). Such ANFs interfere with

food utilisation and affect the health and production of animals (Makkar, 1993). Some affect protein utilisation and inhibit digestion, some are metal ion scavengers, some are antivitamins, and others, such as mycotoxins and nitrates, belong to neither of the groups mentioned (Makkar, 1993).

1.4. Phytic acid, mineral availability, and digestibility

1.4.1. Phytic acid

One of the metal ion scavengers called phytic acid (IP6) is a significant problem associated with using plant-based protein ingredients in fish feed. IP6 consists of a sugar called myoinositol, to which six phosphate groups are covalently linked, hence the name IP6 (Angel *et al.*, 2002). Free phytic acid is often called phytate (calcium salt of IP6) or phytin (calcium/magnesium salt of IP6). It varies in the presence of metal ions and physiological pH (Oatway, Vasanthan and Helm, 2001). IP6 is the primary P reserve in many plants, accounting for up to 85% of total P (Reddy, Sathe and Salunkhe, 1982; Vats and Banerjee, 2004). IP6, as an ANF, affects the utilisation of minerals and contains P in a form unavailable to monogastric animals (Eeckhout and De Paepe, 1994; Francis, Makkar and Becker, 2001). Using phytate-P ingredients such as soybean meal has been associated with nutritional stressors that can induce inflammation and changes in gut health (Baeverfjord and Krogdahl, 1996). Consequently, further processing of soybean meal into soy protein concentrate (SPC) was designed to lower the antinutrient content and increase the protein level (Escaffre, Kaushik and Mambrini, 2007), and is currently the ingredient used in the largest amount (21% of the feed ingredients) (Aas, Åsgård and Ytrestøyl, 2022). Despite this processing method, SPC still contains lower mineral content and ANFs, reducing mineralisation (Storebakken, Shearer and Roem, 2000). As a result, phytic acid can directly reduce the availability of feed P, with fish retaining only 40% of modern commercial fish feed (Sugiura, 2018). IP6 has a high density of negatively charged phosphate groups, which allows them to form complexes with mineral cations such as potassium (K), magnesium (Mg), Ca, Zn, iron (Fe), and copper (Cu) and form poorly soluble complexes. Therefore, phytate interferes with mineral metabolism, especially since several minerals' uptake and body status are primarily regulated in the gastrointestinal tract or liver (Lall, Kaushik and Schrama, 2021). Due to its chelating properties, it can reduce the availability of i,.e. Zn and Mg (Denstadli *et al.*, 2006) and Ca (Fredlund *et al.*, 2006). In addition to affecting mineral availability, phytate can form phytate-protein complexes that may hinder protein digestion and amino acid absorption in the small intestine. It may also indirectly stabilise proteins through interactions with the surrounding water medium due to the kosmotropic effects

of the phosphate anions (Oatway, Vasanthan and Helm, 2001; Baruah *et al.*, 2004; Selle *et al.*, 2012). Phytate may also form complexes with lipids and carbohydrates that impairs their accessibility in fish feeds, making it necessary to add dietary nutrients to overcome nutritional deficiencies (Priya *et al.*, 2023).

1.4.2. Feed phosphates

Due to phosphorus (P) from IP6 being mainly unavailable to fish and in diets where P from fishmeal is insufficient, the addition of expensive and non-renewable inorganic phosphate is included to ensure adequate available feed P (Hernández, Satoh and Kiron, 2005; Lee *et al.*, 2020). Inorganic phosphates are a dietary supplement that increases fish development and P nutrition; however, their high inclusion in fish feeds may contribute to environmental pollution. There are multiple sources of inorganic phosphates, and solubility is an important criterion, as only dissolved phosphate is available for intestinal absorption. Mono ammonium phosphate (MAP; $NH_4H_2PO_4$), monocalcium phosphate (MCP; $Ca(H_2PO_4)_2$), dicalcium phosphate (DCP; $CaHPO_4$), monosodium phosphate (MSP; NaH_2PO_4) and potassium phosphate (MKP; KH_2PO_4) are used in fish feeds. MAP has previously been found to have a significantly higher P digestibility (90%) compared to MCP (70%) (Morales *et al.*, 2018). The primary global source of P used in crop farming and animal feeds is mined phosphate rock. Phosphate rock is a non-renewable resource facing a depletion crisis; hence it is imperative to enhance P utilisation efficiency in salmon farming, considering bioavailability, retention, and recovery of effluent P (Wu *et al.*, 2016; Ytteborg *et al.*, 2016; Yuan *et al.*, 2018). Effluents and pollution potential of formulated feeds increase steadily when substituting fish meal for plant-based material. This is mainly due to the poor bioavailability of phytic acid, which increases P excretion to the environment leading to eutrophication (Debnath *et al.*, 2005). In a report regarding P waste generation and recycling prospects, the authors recognised that the most significant P contributor to inland water had changed from crop farming to aquaculture (Liu *et al.*, 2020). This speaks volumes about the increased P emissions from aquaculture and emphasises the need for better P utilisation and recycling. To transition into more efficient and suitable P sources, a study by Ytteborg *et al.* (2016) showed that P hydrolysed from herringbone by-product was as efficient as commercially available sodium phosphate salts. On the one hand, new sources of supplying P in the feed are an alternative to inorganic feed P. On the other hand, including specialised enzymes that break down the ANFs is an effective alternative for better utilisation of low fishmeal feeds, as seen in other species (Adeshina *et al.*, 2023; Dias and

Santigosa, 2023). In addition, it contributes to the economic and environmental sustainability of aquaculture production.

1.5. Phytase and its application in fish feeds

Phytic acid is a heat-stable anti-nutrient, and Atlantic salmon lack adequate enzymes to release P by hydrolysis. Myoinositol (1,2,3,4,5,6)-hexaphosphate phosphohydrolase or phytase, catalyses the hydrolysis of phosphomonoester bonds from phytic acid resulting in the liberation of free inorganic orthophosphates available for absorption in animals (Mullaney and Ullah, 2003; Kumar *et al.*, 2012). This consequently decreases the chelation capacity of phytate and is the most efficient method to reduce phytic acid while retaining the mineral content of the grains (Kumar *et al.*, 2012; Gupta, Gangoliya and Singh, 2015). Dietary phytases can make P more available, lessening the need for supplemental inorganic P. Phytases are widespread and can be found in plants, microorganisms, and animal tissues. There are several different types of phytases, and they usually vary depending on temperature and pH. As reviewed in Priya *et al.* (2023), the activity of commercial microbial phytases in the pH range of 2.5-5.5, making them suitable for salmon; however, the optimum thermal tolerance range is usually between 45-60°C, making the feed extrusion process a limiting factor in phytase application in extruded fish feeds. Phytase is generally applied to the feed after extrusion. In addition, the phytase activity strongly depends on the water temperature and is generally more efficient in warm water environments (Priya *et al.*, 2023). In Nile tilapia, a warm water fish species, dietary phytase could completely substitute inorganic P with no negative impacts (Adeshina *et al.*, 2023). There is, however, limited published information on phytases for cold-water fish species. This remains an area to be explored; hence a phytase with efficiency at lower temperatures may be more suitable for salmonids (Dersjant-Li *et al.*, 2014). Previously, several trout studies have been performed using microbial or fungal phytases with little or no effect at low temperatures (11°C) (Forster *et al.*, 1999; Dalsgaard *et al.*, 2009; Yigit *et al.*, 2018). More recently, an enhanced *Escherichia coli* phytase has been studied in rainbow trout fed a low-P plant-based diet at low temperatures (11°C and 15 °C) (Lee *et al.*, 2020). Adding this phytase at a dose of 2500 FTU/kg improved growth performance and retention of P and N, giving promise to applying phytase in salmonid diets.

1.6. Nutrition and bone health

Several nutrients are suggested to play a role in bone development (Lall and Lewis-McCrea, 2007), of which P is the most studied (Watanabe, Kiron and Satoh, 1997). Still, microminerals such as Zn, Mn and selenium (Se) also have a role to play in bone development and remodelling

(Baeverfjord *et al.*, 2019). Phosphorus is essential in skeletal development, growth, and remodelling (Yogev *et al.*, 2020), and fish depend on the dietary P supply for bone mineralisation (Lall and Lewis-McCrea, 2007). P deficiencies impair growth, feed utilisation, and bone mineralisation and density and can lead to skeletal abnormalities (Baeverfjord *et al.*, 2019). Other micronutrients, such as fatty acids and vitamins (D, A, K, E, and C), are also essential for normal growth and bone formation (Lall and Lewis-McCrea, 2007).

1.6.1. Mineral nutrition

The fish body is composed of 60-90% water. Upon removal of this water, through either heating or freeze-drying, most of the resulting dry matter comprises organic elements such as carbon, oxygen, nitrogen, hydrogen, and sulfur. These elements are essential for forming proteins, fats, and carbohydrates. Following the combustion of the organic elements, ash remains, composed solely of inorganic elements, namely minerals. Fish require minerals, which are essential to sustain vital physiological and biochemical functions and normal life processes (Lall, Kaushik and Schrama, 2021), and optimal levels are crucial for the growth and maintenance of normal fish health (Watanabe, Kiron and Satoh, 1997; Lall, Kaushik and Schrama, 2021). The essential minerals can be divided into macrominerals and microminerals based on their function and concentration in fish tissues.

Macrominerals

Macrominerals occur in concentrations of the order of magnitude of g/kg wet weight. They include the electrolytes sodium (Na), potassium (K) and chlorine (Cl), and the structural bone minerals Ca, Mg, and P. Electrolytes are essential for osmoregulation and acid-base equilibrium in fish and are abundant in seawater. Essential bone macrominerals include Ca, Mg, and P. Ca and P are critical structural elements for developing and maintaining the skeletal system and other functions. They are deposited as tricalcium phosphate $Ca_3(PO_4)_2$ that eventually changes to form hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, which is deposited in the organic matrix during mineralisation (Lall, Kaushik and Schrama, 2021). Ca is the most abundant mineral in bones, and fish usually have unlimited access to Ca from the water via branchial absorption (Baeverfjord *et al.*, 2019). Mg is a critical element with many essential physiological functions in the body, both intracellular and extracellular. Extracellular Mg is vital to normal nerve conduction, muscle function, and skeletal tissue metabolism. In addition, Mg is essential in the body as a specific activator or cofactor in at least 300 enzymatic reactions (El-Mowafi and Maage, 1998). Signs of deficiency include reduced growth, NC, and vertebral deformity in rainbow trout, as reviewed in Lall et al. (2021).

Table 2

Macro- and micro mineral concentration in whole body, plasma, scales, and vertebrae in Atlantic salmon (*Salmo salar*)

Macrominerals	Whole body (g/kg ww)	Plasma (mmol/L)	Scales (% dw)	Vertebrae (%, dw)
Ca	3-5.5	3 ± 0.4	190 ± 3	212 ± 22
Mg	0.3-0.5	1 ± 0.2	2 ± 0.3	2 ± 1
P	4-5	3.5 ± 1.7	94 ± 5	101 ± 11
Microminerals	Whole body (mg/kg ww)	Plasma (µmol/L)	Scales (mg/kg dw)	Vertebrae (mg/kg dw)
Mn	1.5-3	1.3 ± 0.1	-	11.2 ± 2.9
Fe	10-20	10.9 ± 0.5	-	30 ± 3.7
Cu	1-3	25.6 ± 3.3	-	-
Zn	25-60	319 ± 86	-	168.3 ± 14.9
Se	0.2-0.4	3.3 ± 0.7	-	-

Notes: Values adapted from (Antony Jesu Prabhu, Schrama and Kaushik, 2016).

Microminerals

Essential microminerals occur in concentrations of mg/kg wet weight. They include manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), iodine (I), cobalt (Co) and selenium (Se). The role of micro minerals in the body is immense, as they are involved in cellular metabolism, the formation of skeletal structures, the maintenance of colloidal systems, the regulation of acid-base equilibrium, and a variety of other physiological functions. Zinc is the most abundant and important micromineral for salmonid bone health. As reviewed in Baeverfjord *et al.* (2019), Zn impacts bone formation and collagen synthesis and improves Ca deposition in the extracellular bone matrix. Suboptimal levels of Zn negatively affect mineral status in Atlantic salmon and bone health. Recently, the optimal dietary levels of Zn (180 mg/kg diet) and Se (0.65 mg/kg diet) in low FM diets were determined in post-smolts (Antony Jesu Prabhu *et al.*, 2020; Sartipi Yarahmadi *et al.*, 2022). In an even more recent study by Philip *et al.* (2023), the authors found a preventive effect of supplementing high levels of Zn (200 mg/kg) and Se (0.7 mg/kg) on the incidence and severity of vertebral deformities at 12°C. Baeverfjord *et al.* (2019) reviewed that Mn is essential for forming an extracellular matrix and regulating bone resorption. An enzyme dependent on Cu, lysyl-oxidase, is important for the biosynthesis of collagen and deposition of Ca and P in bones (Tomaszewska *et al.*, 2017), and deficiency can lead to impaired bone growth.

1.6.2 Mineralization and vertebral deformities in Atlantic Salmon

Mineral status in fish is primarily determined by the concentration of minerals in whole fish or specific tissues. Therefore, the concentration of a mineral includes the sum of the mineral in function and the mineral in storage. The mineralisation process is an intricate and organised process that enables the formation of hydroxyapatite crystals in precise amounts within an extracellular matrix of collagen fibrils (Wang *et al.*, 2013).

As described earlier, fish bone is a complex structure with several essential functions. Another principal function is to resist mechanical forces and fractures. The strength of bone depends on the quantity, but most importantly, the quality of bone tissue. Among important qualitative measures is the regular arrangement of collagen fibrils and mineralisation. The interplay between collagen and hydroxyapatite is essential for the mechanical properties of bone, notably its toughness and stiffness. Impairment of the mechanisms underlying the generation of collagen fibrils as the bone grows may lead to mechanical failure, tissue fibrosis, and, ultimately, bone deformities (Baeverfjord *et al.*, 2019). Due to the nature of salmonids' swimming pattern, movement between adjacent vertebral bodies is generally limited to lateral flexion. The lateral flexion generates a mechanical load and may, in some cases, affect the normal development of bone tissue. This can occur by suboptimal mineralisation in the vertebrae with a high mechanical load during swimming. Hence, the mechanical load generated might affect the normal development of bone tissue. As farmed salmon is selected for fast growth and high muscle mass, insufficient minerals combined with the mechanical load imposed by the lateral musculature during swimming synergistically cause vertebral deformities in those high-load areas (Fjelldal *et al.*, 2009). Witten *et al.* (2009) categorised commonly observed vertebral deformities in Atlantic salmon. It includes 20 types in five categories of deformations (described in detail elsewhere; image 6). Other bone deformities observed in Atlantic salmon are, i.e. compressed snout (pug-head), jaw deformities, and short operculum (Lall and Lewis-McCrea, 2007).

1.7 Thesis aim, objectives and hypotheses

The project aimed to determine the effects of different feed P regimes and the inclusion of an enhanced E. coli phytase in the feed for Atlantic salmon pre- and post-smolt and to compare mineral status and bone health parameters.

The objectives of the project were to:

- Evaluate the effect of type and inclusion level of inorganic feed phosphates in Atlantic salmon.
- Evaluate the effect of a low-temperature phytase on mineral availability and utilisation in Atlantic salmon.
- Impact of different feed P regimes on bone health in Atlantic salmon.

The experiment was based on the following hypotheses:

$H0_1$: Substituting half of MAP with MCP in the feed for Atlantic salmon pre-smolt reared in a RAS does not affect the mineral status and bone health.

$H1_1$: Substituting half of MAP with MCP in the feed for Atlantic salmon pre-smolt reared in a RAS affects mineral status and bone health.

$H0_2$: Including an enhanced low-temperature phytase (*Escherichia coli*) in the feed for Atlantic salmon pre-smolt reared in a RAS does not affect the mineral status and bone health.

$H1_2$: Including an enhanced low-temperature phytase (*Escherichia coli*) in the feed for Atlantic salmon pre-smolt reared in a RAS affects mineral status and bone health.

2. Materials and methods

2.1. Ethical statement

The feeding trial and sampling were conducted at LetSea Research and Development (Sandnessjøen, Norway). All the sampling procedures were conducted on euthanised fish by Finquel vet. (MS222) overdose.

2.2. Experimental diets and feeding

Five experimental diets were formulated by MOWI Feed and produced at the feed production facility at NOFIMA, Bergen. The feeds were extruded. All five diets had the same digestible protein and energy levels, and the pellet size was 2.4 mm and 3.5 mm when fed to pre- and post-smolt, respectively. The diets have different inclusion of fishmeal (FM), total P, available P, source and amount of inorganic P, and the addition of an exogenous phytase. The diets were addressed by a three-letter code: Alp, Amc, Pro, Plp, and Pta. Feed formulation and analysed proximate composition is presented in Table 3. All diets were formulated to meet the dietary P requirement of Atlantic salmon as per NRC (2011), with a total P level ranging from 13 to 15 g/kg feed. Diets 'Alp' and 'Amc' was formulated to contain 15 g/kg total P with mono ammonium phosphate (MAP) alone or a 50:50 mix of MAP and mono calcium phosphate (MCP) as the inorganic P supplement, respectively. Diet 'Pro' was formulated to contain the same total P levels as Alp and Amc; however, with lower supplementation of MAP (75%) but an increased level of fish meal. The 'Plp' diet was formulated to have low total P (13 g/kg feed) compared to Alp, Amc and Pro but still meet the available P needs through increased AMP supplementation. The 'Pta' diet was top coated along with the oil post-extrusion with an enhanced *Escherichia coli* phytase (Quantum Blue; AB Vista, Marlborough, UK) with an estimated inclusion level of 2500 FTU/kg, where one FTU is defined as the amount of enzyme which liberates one µmol of inorganic phosphate from sodium phytate at pH 5.5 and 37°C in 1 min.

Fish were fed using a 24-hour continuous feeding program with an automatic feeding cylinder and an estimated degree of overfeeding at 12%. Uneaten feeds were collected and weighed to estimate the feed intake. The experimental diets ceased at the end of the freshwater phase. To measure the apparent availability of minerals, yttrium oxide was added to the feed as an inert marker for all five diets. When switching to seawater, all animals were given the same feed.

Table 3

Feed formulation, proximate composition, and mineral composition of experimental diets

	Alp	Amc	Pro	Plp	Pta
Feed formulation (in %)					
Fishmeal (L_T)	30.00	30.00	37.21	17.50	17.50
Soy protein concentrate	20.00	20.00	20.00	30.00	30.00
Wheat gluten	10.23	10.23	5.10	13.06	13.06
Pea protein concentrate	1.20	1.20	1.20	1.20	1.20
WHT B	7.26	7.24	5.64	4.90	4.90
Dehulled beans	7.50	7.50	7.50	7.50	7.50
Fish oil	14.07	14.07	13.79	15.33	15.33
Plant oil (rapeseed)	5.72	5.72	5.91	5.44	5.44
Vitamins and minerals	0.792	0.792	0.789	0.871	0.871
MAP	1.504	0.752	1.146	1.766	1.766
MCP	0	0.774	0	0	0
Astaxanthin	0.075	0.075	0.075	0.075	0.075
Yttrium oxide	0.07	0.07	0.07	0.07	0.07
Phytase (FTU/kg)	0	0	0	0	2500
Analysed nutrient composition of the feeds					
Proximate composition (g/100g ww)					
Protein	46	48	46	48	48
Lipid	23.1	23.2	21.7	22.9	22.9
Ash	7.5	7.9	7.6	6.5	6.4
Dry matter	91	92	90	93	94
Phytase (FTU/kg)	<50	<50	<50	<50	1820
Mineral composition of experimental diets					
Macrominerals (g/kg ww)					
Ca	15	17	17	11	10
Na	4.5	4.6	5.0	3.0	2.9
K	10.0	10.0	9.1	11.0	11.0
Mg	2.0	2.1	1.8	2.1	2.2
Total P	15	15	15	13	13
Ca:P	1.0	1.1	1.1	0.85	0.77
Available P	*8.5*	*7.3*	*8.3*	*8.2*	*8.1*
Microminerals (mg/kg ww)					
Mn	43	43	40	49	51
Fe	190	210	220	240	200
Cu	7.9	8.3	7.4	8.3	8.4
Zn	160	170	160	160	160
Se	0.79	0.87	0.88	0.58	0.57

Notes: Feed formulation, proximate composition, and mineral content of experimental diets. Ingredients are listed as percentages of whole feed. In addition, the analysed macro- and micromineral content of the experimental diets is presented in the order of g/kg ww and mg/kg ww, respectively.

2.3 Experimental conditions, fish, and husbandry

2.3.1 Experimental animals

Kvarøy Smolt AS delivered the pre-smolt (SalmoBreed Strain) with an average start weight of 43.3 ± 7.0 g. The trial was initiated on 6000 Atlantic salmon parr (*Salmo salar*) individually weighed and randomly allocated into twenty 2000 L tanks with 300 fish per tank and quadruple tanks per dietary treatment, all with a feed collection system (Image 1) as described by Helland, Grisdale-Helland and Nerland, (1996).

Image 1: Experimental tanks installed with feed collection system used in the study. Image Credit: Ernst Hevrøy.

2.3.2 Husbandry Conditions and Water Quality

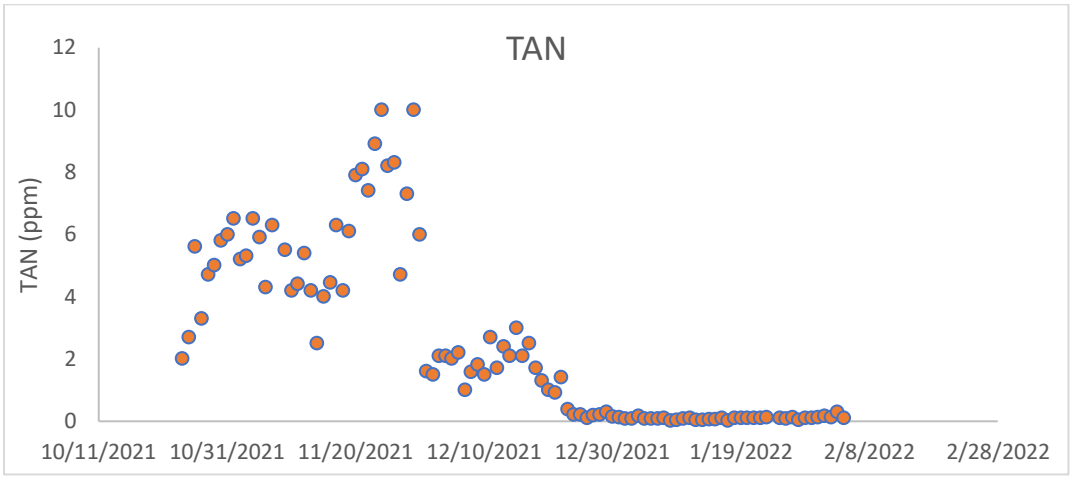
The trial was conducted in a fixed bed (biofilter) recirculating aquaculture system (Billund Aquaculture) with a UV-disinfection system (UltraAqua) and a mechanical drum filter. Environmental parameters such as pH, total ammonium nitrogen (TAN), nitrite, salinity, and alkalinity were monitored daily. These values are presented in Table 3.

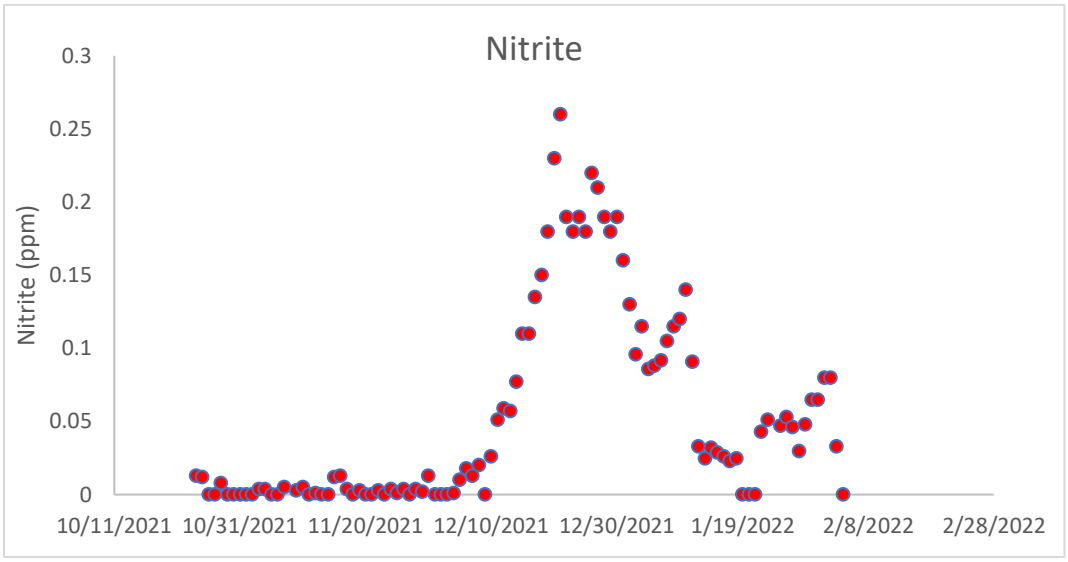
Table 4

Water quality parameters.

	pH	TAN	Nitrite	Salinity	Alkalinity
FW (22.10.22-13.01.22)	7.0 ± 0.2	3.1 ± 2.8	0.06 ± 0.07	0.6 ± 0.6	116 ± 50
SW (14.01.22-04.02.22)	7.9 ± 0.1	0.11 ± 0.05	0.04 ± 0.02	29.3 ± 6.4	217 ± 42

Notes: an overview of daily environmental data monitored. Values are presented as mean ± SD for the respective period with salinity as ‰, and alkalinity, nitrite, and TAN as ppm.





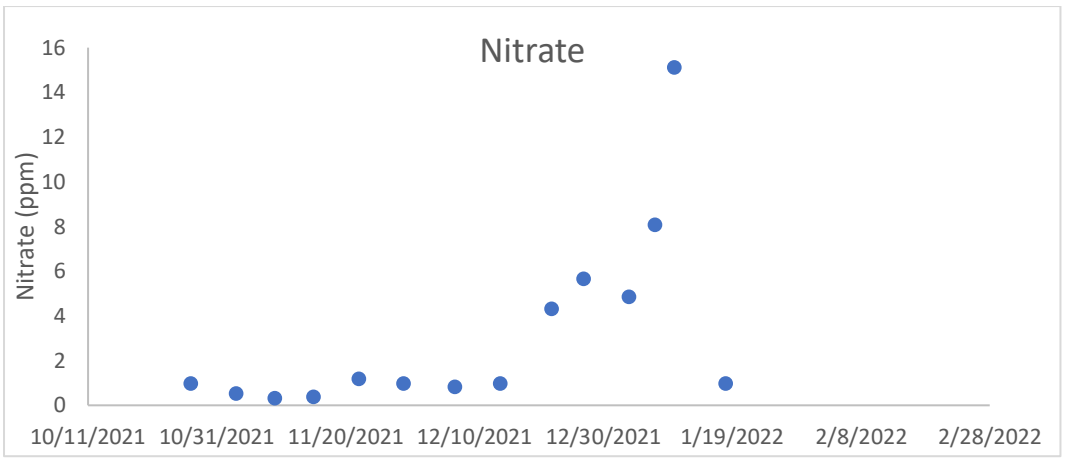


Figure 2: Daily measurements of TAN, nitrite, and nitrate. Nitrate was not measured during the seawater phase.

Temperature and oxygen levels were monitored continuously, and the average oxygen saturation levels were 103% across all tanks. The temperature ranged from 10-13°C in freshwater and 10°C in seawater. In addition, levels of nitrate, CO_2, and turbidity were

monitored weekly. All samples for water quality analysis were taken from an empty tank within the system and analysed colorimetrically. Fish were reared in freshwater for 13 weeks and seawater for three weeks. Salinity levels of the system were increased to 1-2 ppt between December 1st 2021, and January 13th 2022. In response to the small increase in salinity, nitrite levels were substantially increased. This increase suggests a difficulty in the bacterial conversion of nitrite to nitrate; however, levels were still within the safe limit. This explains freshwater's high standard deviations for TAN and nitrite (figure 2). At the end of the freshwater trial, salinity levels were gradually increased over five days (i) 1 ppt, (ii) 8 ppt, (iii) 13 ppt, (iv) 27 ppt, (v) 32 ppt, as seen in figure 3. In SW, the salinity was approximately 32‰ for three weeks. Seawater was collected through a pipe from 140 meters depth in the fjord.

In FW, fish were exposed to a light regime of 24:0 (light: dark), 18:6, 12:12, and again at 24:0 for 6, 1, 2, and 4 weeks, respectively. An 18:6 light regime is commonly used to stimulate the start of the declining day length, and a 12:12 regime mimics a winter photoperiod to stimulate smoltification. Lastly, a 24:0 regime is widely practiced in salmon farming, allowing "around-the-clock" feeding and enhancing growth.

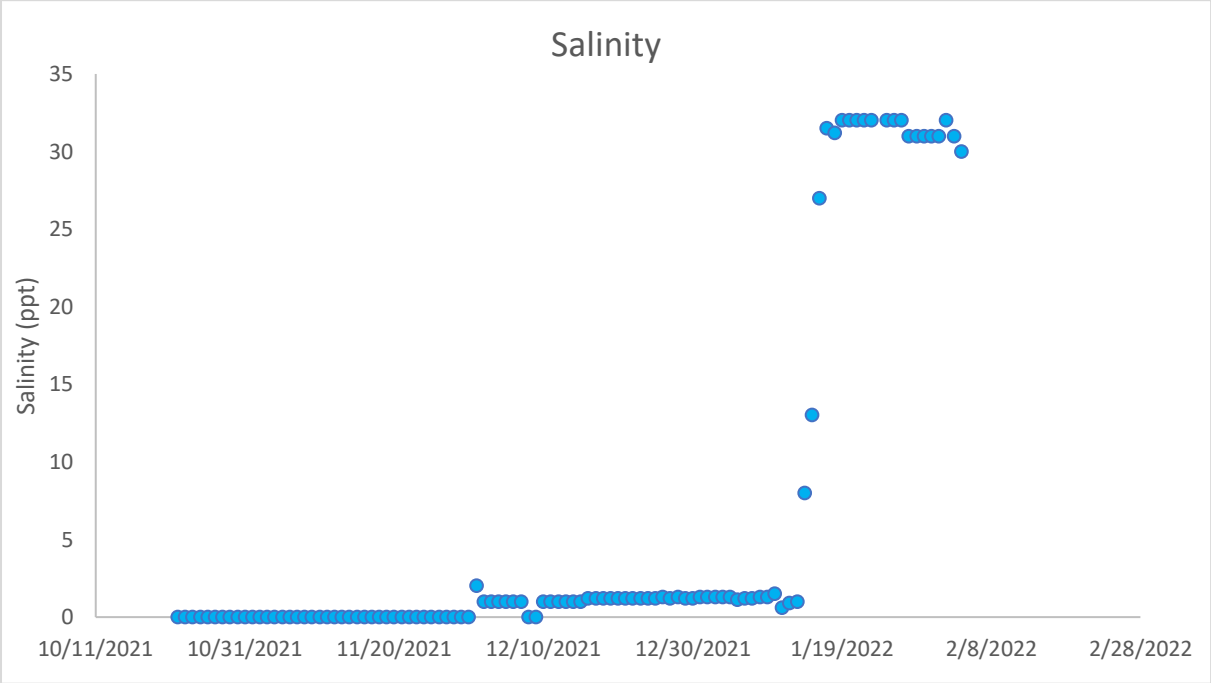


Figure 3: Salinity levels in freshwater and seawater during the trial are presented as ppt (‰) with daily measurements.

2.4 Sampling procedure of fish and tissues

During the trial period, fish were weighed and measured three times, following a 24-hour fast; (i) before the trial (initial sampling), (ii) after 12 weeks in freshwater (FW sampling), and (iii) after three weeks in seawater (SW sampling). During the start of the trial, an initial sampling

was performed where 35 fish were pooled and stored at -20°C to analyse whole fish mineral content. Starting the trial, 6000 fish were measured for weight and fork length, and distributed to the different tanks (300 fish/tank). At the end of the FW phase, 150 fish were sampled, including 10 fish for whole body, 10 for x-ray imaging, and four for tissue samples. All the fish were stripped for faeces. The remining 150 fish/tank were kept for the seawater phase of the experiment.

Apart from whole fish, plasma and tissues were collected in the FW and SW sampling. Blood was drawn from four fish per tank, 16 fish per diet, by puncture of the ventral caudal peduncle in a lithium heparinised 4 mL Vacuette tubes (REF 454029, Austria) and placed on ice (Image 2). Blood from each fish was centrifuged at 4000g (4°C) for 7 min with a VWR Mega Star 600R centrifuge (Germany) to separate plasma from the blood. Plasma was stored on dry ice and shipped to the Institute of Marine Research (Bergen, Norway) for further analysis.

Image 2: Blood sampling. Image credit: Ernst Hevrøy.

Fish scales from both sides were scraped using a scalpel from four fish per tank (Image 3), pooled into two tubes for mineral and ash analysis, and placed on ice. This was done in both the FW and SW sampling. The fish were dissected to measure the weight of the liver, heart, viscera, and gutted weight was measured to calculate somatic indices.

Image 3: Scale sampling. Image credit: Ernst Hevrøy.

Four fish per tank were examined for the prevalence and degree of nephrocalcinosis in the FW sampling (Image 4). Kidneys were scored by visual examination as 0 (normal kidney), 1 (minor swollen kidney and few white nodules), 2 (swollen kidney, grey colour, several white nodules, and irregular surface), and 3 (swollen kidney, grey colour, large areas of interconnected nodules, and irregular surface) (Nilsson *et al.*, 2022).

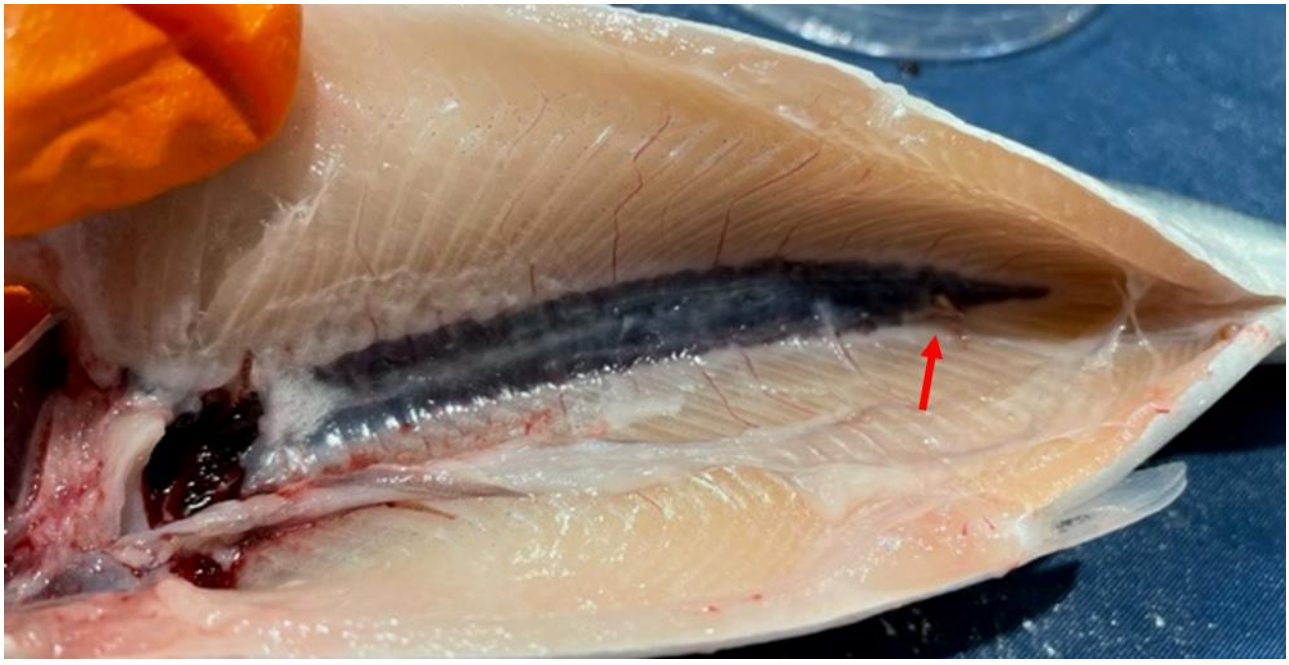


Image 4: Kidney showing mineral deposits at the junction of the urinary tubes (red arrow). Image credit: Ernst Hevrøy.

In the FW sampling, faeces were stripped from the distal part of the gut by applying gentle pressure from the ventral fin to the anus (Image 5). 150 fish per tank were stripped to obtain enough faeces to determine mineral content and digestibility.

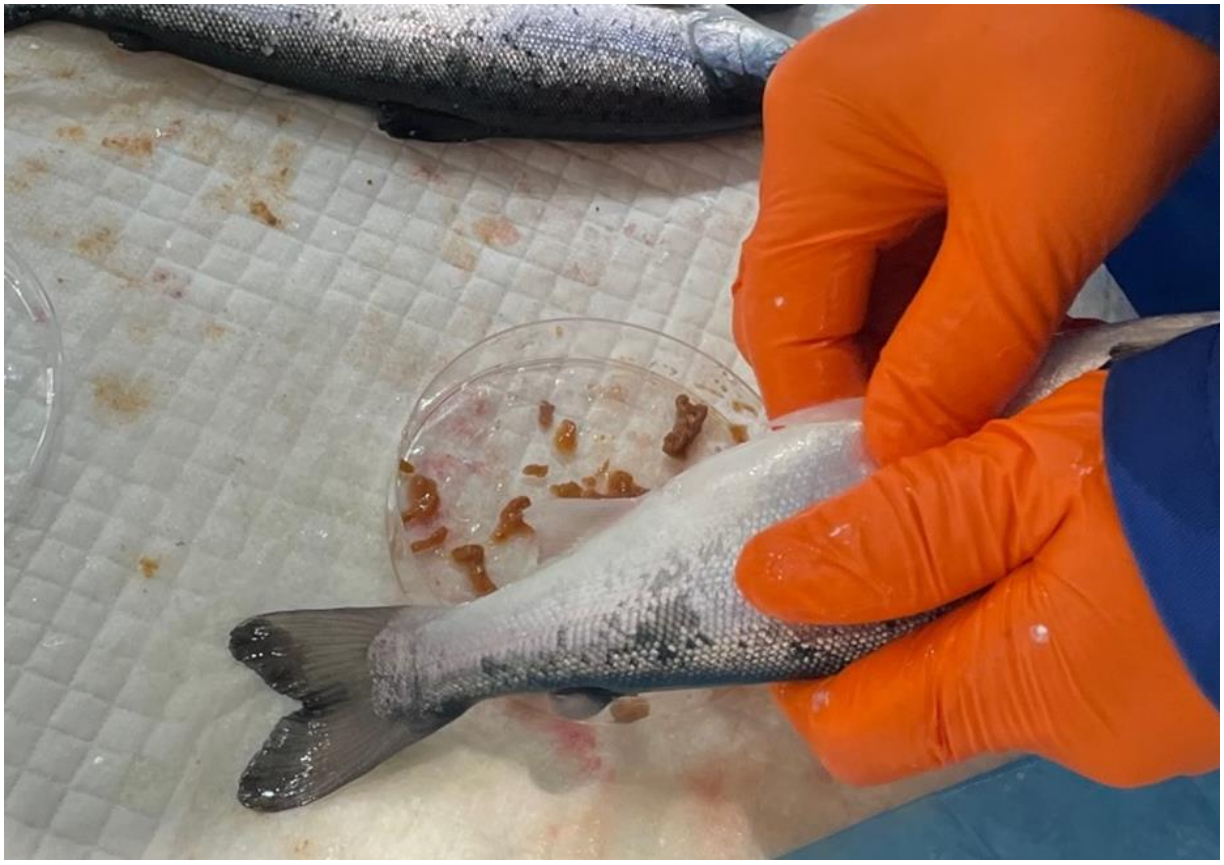


Image 5: Stripping faeces from the distal intestine for apparent availability assessment. Image credit: Ernst Hevrøy.

Ten fish per tank from the transfer trial were carefully pooled and stored at -20°C and later shipped to the Institute of Marine Research (Matredal, Norway) for x-ray imaging, analysis of vertebral deformities, and determination of vertebral mechanical strength.

Table 5

Overview of samples and mineral analyses performed on fish.

	Nb. Of samples analysed	Collection of sample	Parallels	Metal	Alkali	Yttrium
Initial sampling						
Whole body	1	Pooled	2	X	X	
Freshwater sampling						

Feed	5		2	X	X	X
Whole body	20	Pooled	2	X	X	
Faeces	20	Pooled	2	X	X	X
Plasma	80	Individual	1	X	X	
Scales	20	Pooled	2	X	X	
Whole fish (X-ray)	200	Individual				
Mechanical strength	240	Individual				
Vertebrae	80	Individual	1	X	X	
Seawater sampling						
Feed	1		2	X	X	X
Whole body	20	Pooled	2	X	X	
Plasma	80	Individual	1	X	X	
Scales	20	Pooled	2	X	X	

Notes: The number of samples analysed in the initial, freshwater, and seawater sampling is presented in Table 5. Unless specified elsewhere, all analyses were performed at the Institute of Marine Research (Bergen, Norway).

2.5 Analytical methods

2.5.1 Homogenization and freeze-drying

Feed samples were taken from the experimental diets in the 2.4 mm and 3.5 mm feeds and the common feed used after seawater transfer. Each feed sample was homogenised (Knife Mill Grindomix GM 300) and distributed into different tubes to analyse for minerals, yttrium, protein, fat, ash, and dry matter content.

The faeces and vertebrae were weighed on a digital scale (Mettler Toledo Precision Balance MS6002TS/00) and freeze-dried for 72 hours using a FreeZone Bulk Tray Dryer (LabConCo, Kansas) to determine the dry matter. The principle behind freeze-drying is based on the three states that water can exist in solid, liquid, and gas. The three phases can be transformed or coexist. In a high vacuum state, the sublimation principle allows water in the pre-frozen material to bypass the melting phase and directly sublimate to water vapour, which is then removed, thus achieving the purpose of freeze-drying. After freeze-drying, the samples were homogenised by pestle and mortar into an even powder and kept at room temperature until analysis.

Whole fish were ground using a meat grinder (GiantFood, China) and homogenised using a food processor and were treated the same as for faeces and vertebrae.

2.5.2 Mineral analysis

Microwave digestion (UltraWAVE / UltraCLAVE) of samples

For the digestion of freeze-dried sample material and feed, 0.20-0.25 g was weighed in a 15 mL quartz tube, with 2mL nitric acid 69% Suprapur (HNO_3) and 0.5 mL of deionised water (Milli-Q water) added afterwards. The samples were capped and placed on a positioning rack in the Milestone Ultrawave (UltraWAVE, Milestone, Sorisole, Italy) with a Teflon container containing 130 mL Milli-Q water and 5 mL hydrogen peroxide (H_2O_2). When digesting wet material, such as plasma and scales, a broader type of 15 mL and 35 mL quartz tubes were used, respectively. The addition of Milli-Q water was optional for wet samples. Both plasma and scales were digested in a Milestone Ultraclave (UltraCLAVE, Milestone, Sorisole, Italy) with a Teflon container containing 300 mL Milli-Q water and 10 mL H_2O_2.

After digestion, all samples were diluted to 25 mL with Milli-Q water in a volumetric flask and transferred to a 50 mL falcon tube.

2.5.3 Dilution of sample material

After transferring the diluted sample to a 50 mL falcon tube, depending on the matrix, it was further diluted with 5% nitric acid. An overview of the dilution process is provided in Table 6.

Table 6

Overview of the dilution process.

	Plasma	Whole body	Scales	Faeces	Vertebrae	Feed
Metal	1x	1x	10x	20x	20x	1x
Alkali	1x	5x	50x	50x	50x	5x
Yttrium	-	-	-	100x	-	20x

Notes: Dilutions of various tissues are presented in table 6.

2.5.4 Multi-element determination by inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS) is an analytical technique used to determine multiple elements in a sample, combining an ion-generating argon plasma source (approximately 7000°C) and mass spectrometry detection. First, a liquid sample is injected into a nebuliser for aerosolisation. An internal standard is pumped in along with the sample before nebulisation to mitigate matrix effects. The resulting aerosol is then filtered to remove larger droplets, and the remaining aerosol is introduced into the plasma torch, where it is ionised. The ions are separated based on their mass-to-charge ratio and detected as hits/second. Finally, an

ion detector converts these ions into an electrical signal and is read by computer software (Qtegra^{TM} iCap Q, Thermo Scientific, version 2.14.5122.158). An internal standard precedes the samples to ensure instrument quality.

For the determination of the elements Mn, Fe, Cu, Zn, and Se (ICP-METAL), the following standards were used: multi-element standard (Spectrascan: 1000 mg/L Al, Fe, Mg, Zn, 50 mg/L As, Ba, Cu, Mn, Se, Sr and 10 mg/L Ag, Cd, Co, Cr, Mo, Ni, Pb, U, V), Rhodium (Spectrascan 1012 ± 2 mg/L in 5% HCl), Thulium (Merck Sertipur 1000 ± 3 mg/L in 2-3% HNO_3) and Germanium (Spectrascan 1000 ± 3 µg/L in H_2O).

When determining the elements Ca, Na, K, Mg, and P (ICP-ALKALI), the following standards were used: Spectrascan multi-standard: Na (500 ± 3 mg/L), Mg (250 ± 1 mg/L), K (500 ± 3 mg/L), Ca (250 ± 1 mg/L) og P (500 ± 3 mg/L), and Scandium (Sc) Spectrascan 1006 ± 3 mg/L in 5% HNO_3. The following standard was used when determining yttrium: Spectrascan (1001 ± 4 µg/mL Yttrium) in 2% HNO_3.

The certified reference materials (CRM) used for mineral analysis (ICP-METAL) were oyster tissue (OT, CRM 1566, NIST) and lobster hepatopancreas (TORT 3, National Research Council, Canada). For analysis of macro-minerals (ICP-ALKALI), the standard reference materials (SRM) used were skimmed milk powder (BD150, ERM) and bovine liver (1577c, NIST). An ICP-MS iCap Q (Thermo Fischer) with collision cell and FAST SC-4DX autosampler was used for element determination. The instrument was tuned before analysis with a tuning solution (1 ppb tuning solution B, Thermo Fisher, in 2% HNO_3 and 0.5% HCl).

2.5.5 Determination of dry matter and ash content

To determine the dry matter, faeces, whole fish, and vertebrae were freeze-dried and weighed on a digital scale before and after the process, as described previously.

Dry matter in feed was analysed using a drying oven (Termaks TS 8056 Drying Oven). Approximately 3.5 grams were weighed in a ceramic bowl able to withstand high temperatures, which is particularly important for subsequent analysis of ash content. An aluminium container would suffice if the samples were only subjected to the drying oven. To determine ash content in scales and feed, approximately 3.5 grams were weighed on a digital scale before and after combustion in a muffle furnace (Thermolyne 30400 Furnace) at 550°C for 16-18 hours. If the material has been previously processed in a drying oven, the same material is used for determining ash content directly. Ash content refers to the amount of inorganic material left in a sample after burning at high temperatures.

2.5.6 X-ray imaging

From the FW sampling, ten fish from each tank were placed on a 10 MP digital X-ray tablet and photographed by a portable X-ray unit (Gierth TR 90/30 peak). Images were analysed manually for vertebral deformities and recorded based on the classification system as shown in image 6 (Witten *et al.*, 2009). Fusion of one or two vertebrae to the urostyle and the base of the skull is not included in the deformity count as described in Drábiková *et al.* (2022).

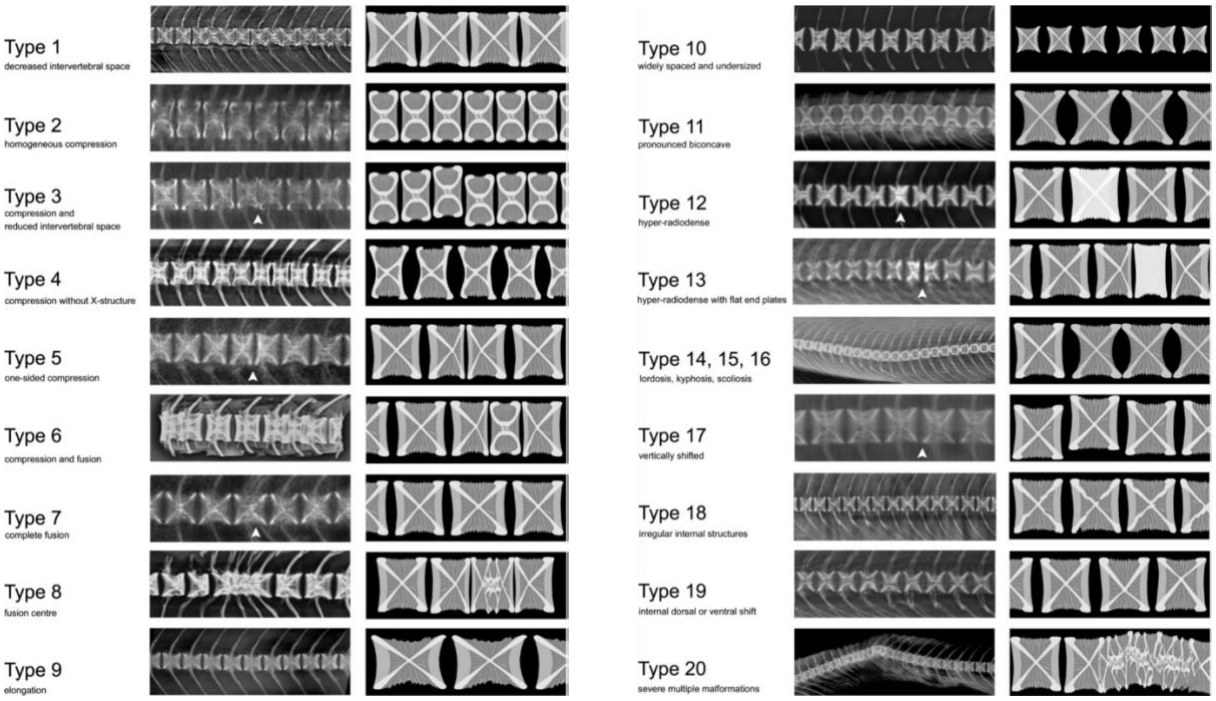


Image 6: Radiographs and schematic representations of vertebral body malformations in Atlantic salmon. Adapted from (Witten *et al.*, 2009). Type 1-decreased intervertebral space. Type 2-homogeneous compression. Type 3-compression and reduced intervertebral space (white arrowhead). Type 4-compression without X-structure. Type 5-one-sided compression (white arrowhead). Type 6-compression and fusion. Type 7-complete fusion (white arrowhead). Type 8-fusion centre. Type 9-elongation. Type 10-widely spaced and undersized. Type 11-pronounced biconcave. Type 12-hyper-radiodense (white arrowhead). Type 13-hyper-radiodense with flat end plates (white arrowhead). Types 14-16-lordosis, kyphosis, scoliosis. Type 17-vertically shifted (white arrowhead). Type 18-irregular internal structures. Type 19-internal dorsal or ventral shift. Type 20-severe multiple malformations.

2.5.7 Vertebral centrum compression

Vertebral centrum compression tests were conducted on four fish per tank at the Institute of Marine Research (Matredal, Norway). Following Fjelldal *et al.* (2005, 2006), three vertebral bodies (V43-V45) were located and dissected for each fish. They were subsequently cleaned of excessive muscle, notochord, neural and haemal arches, and spines were removed (Drábiková

et al., 2021). Vertebral bodies were submerged in distilled water and briefly dried on paper before the compression test. Fresh vertebrae give more consistent results than vertebrae dried to a constant weight at 100 °C, as the bone behaves similarly to the bone in vivo (Drábiková *et al.*, 2021).

Each vertebral body was measured in cranial-caudal length, dorsal-ventral height, and lateral diameter to the nearest 0.01 mm using a slide calliper before compression. Load deformity testing was measured with a texture analyser (TA-HD plus Texture Analyser, Stable Micro Systems Ltd., Surrey, UK) by compressing a single vertebral centrum along its cranial-caudal axis with a steadily advancing piston (test speed at 0.01 mm/s). When the vertebra is compressed, it is possible to record its behaviour under compressive load and to identify the extent of deformation and resistance properties. Each vertebra is placed on a flat surface, and a flat cylinder probe is lowered onto the sample to a given force or distance. The test was terminated when the vertebra reached a compression strain of 35%. Mechanical properties were calculated using the Texture Exponent Software (Stable Micro Systems Ltd., Surrey, UK).

Using various mechanical parameters, it is possible to evaluate the strength and stability of bones. The main mechanical property is stress, which describes vertebral resistance to an externally applied force. Another important property is strain, which is the ratio of change in vertebral height compared to the original size. Young´s modulus of elasticity can be derived from these properties, usually expressed in pressure per unit area (stiffness), measured in megapascals (MPa) units (Drábiková *et al.*, 2022), as seen in Figure 4.

The stress-strain curve can be divided into elastic and plastic regions. The elastic region is where the vertebra shows a linear relationship between strain and stress, while the plastic region is where the vertebra no longer offers a constant stress-strain proportionality (Hamilton *et al.*, 1981). The modulus of elasticity represents the slope of the elastic region in a stress-strain curve. A 0.2% offset is used when calculating the mechanical properties to account for the elasticity of the vertebra. When the vertebra is subjected to a load, it will deform and recover slightly when removed. The 0.2% offset line will account for this elasticity and ensure the mechanical properties are calculated based on the material´s true properties rather than its temporary deformation. Toughness (MPa x %) represents the area under the stress-strain curve preceding the failure point (MPa), which is the amount of force and deformation absorbed by the vertebra before it fails or pure plastic deformation occurs (Hamilton *et al.*, 1981).

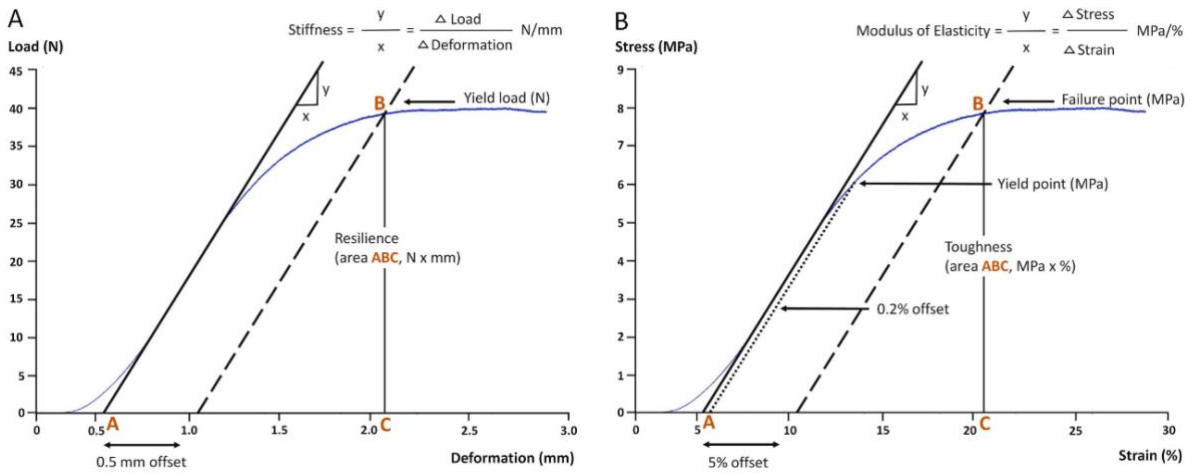


Figure 4: Load-displacement (A) and stress-strain (B) curves. The figure is from (Drábiková *et al.*, 2022), showing load-displacement (A) and stress-strain (B) curves for Atlantic salmon vertebra when not adjusted and adjusted for size, respectively. Graph (A) shows the relationship between the deformation in mm (x-axis) and the load in Newton (y-axis) (Fjelldal *et al.*, 2004). Yield load (N), stiffness (N/mm), and resilience (N x mm) of the bone all measure distinct properties. Yield load demonstrates the overall strength of the bone structure, stiffness is linked to mineralisation, and resilience is the amount of energy required to break the bone. Additionally, the distance between the offset line and yield load (x-axis) indicates the brittleness of the bone (Hamilton *et al.*, 1981). Graph (B) shows the relationship between strain (x-axis) and stress (y-axis). The solid line represents the modulus of elasticity (MPa/%), the first dashed line (0.2% offset) represents the yield point (MPa), the second dashed line (5% offset) describes the failure point (MPa), and the area under the curve represents the toughness (MPa x %) (Drábiková *et al.*, 2022).

2.6 Data analysis

Tanks were used as experimental units for all data sets (n=4). All data are given as mean ± standard deviation (SD). The data were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant differences posthoc test in GraphPad Prism (version 8.0.1) to assess the statistical significance between data. In addition to comparing all five diets, two triplet comparisons were performed using the Pro diet as the reference group. The two triplet comparisons were performed to assess the difference between inorganic P source (Alp, Amc and Pro) and the effect of a low-temperature E. coli phytase (Pro, Plp, and Pta). The level of statistical significance was set at $P \leq 0.05$. Plasma and vertebrae were analysed using a nested one-way ANOVA on individual fish data with tanks as a random factor. The whole body, faeces, and scales were analysed using a one-way column ANOVA with tanks considered statistical replicates.

2.7 Calculations

Growth parameters

Feed intake (FI) was calculated as follows:

Feed intake (g) = Feed given (g) – uneaten feed collected (g)

The feed conversion ratio (FCR) was calculated as follows:

$FCR = FI_{diet} / (BW_{final} - BW_{initial})$,

Where FI_{diet} is the amount of feed eaten in grams, $BW_{initial}$ is the body weight at the start of the period, and BW_{final} is the body weight at the end.

The thermal growth coefficient (TGC) was calculated as follows:

$TGC = 1000 (BW_{final}^{1/3} - BW_{initial}^{1/3}) / (T \times days)$

Where T is the water temperature in °C and days is the number of days between the start and end of the period.

Apparent availability

The apparent availability (AA) was determined for minerals according to the equation below:

$$AA\ (\%) = 100 - \left(\frac{100(yttrium\ in\ diet)}{(yttrium\ in\ faeces)} * \frac{(mineral\ in\ faeces)}{(mineral\ in\ diet)}\right.$$

3. Results

3.1. Survival

During the trial period, eleven mortalities were registered, of which five fish were due to unknown causes, four fish jumped out of the tanks, and two were from mechanical injuries. All the mortalities registered were accidental or random occurrences unrelated to the dietary treatments. This accounts for a 99.8% survival rate.

3.2. Growth performance

Body weight gain (BWG) was calculated at the end of the freshwater phase (BWG-FW), after three weeks in seawater (BWG-SW), and as a total (BWG-FW + BWG-SW). The same was done for FI, FCR, and TGC. When comparing Alp, Amc, and Pro, a significant effect was seen in BWG-FW and BWG-total. BWG-FW was significantly higher in Alp and Pro than Amc ($p < 0.001$, ANOVA), and had a significantly higher BWG total than Alp and Amc ($p < 0.001$, ANOVA). When comparing Pro, Plp, and Pta, a significant effect was observed in BWG-FW, which was significantly higher in Pro than Plp ($p < 0.05$, ANOVA), but not when compared to Pta.

When comparing the diets Alp, Amc, and Pro for FI-FW, all were significantly different from each other ($p < 0.0001$, ANOVA). When comparing the diets Pro, Plp, and Pta, Pro's mean value was significantly higher than both Plp and Pta ($p < 0.01$, ANOVA). No effect was observed between diets in FI-SW. Total feed intake (TFI) between the diets Alp, Amc, and Pro, was significantly higher in Pro compared to both Alp and Amc ($p < 0.001$, ANOVA). When comparing the diets Pro, Plp, and Pta, TFI was significantly higher in Pro compared to both Plp and Pta ($p < 0.01$, ANOVA).

A significant effect was observed in FCR-FW when comparing Pro, Plp, and Pta, showing Pro with a higher mean than both Plp and Pta ($p < 0.01$, ANOVA). The same effect was seen in FCR total.

No statistically significant effects between diets (Alp, Amc, Pro, Plp, and Pta) were observed when comparing the thermal growth coefficient (TGC).

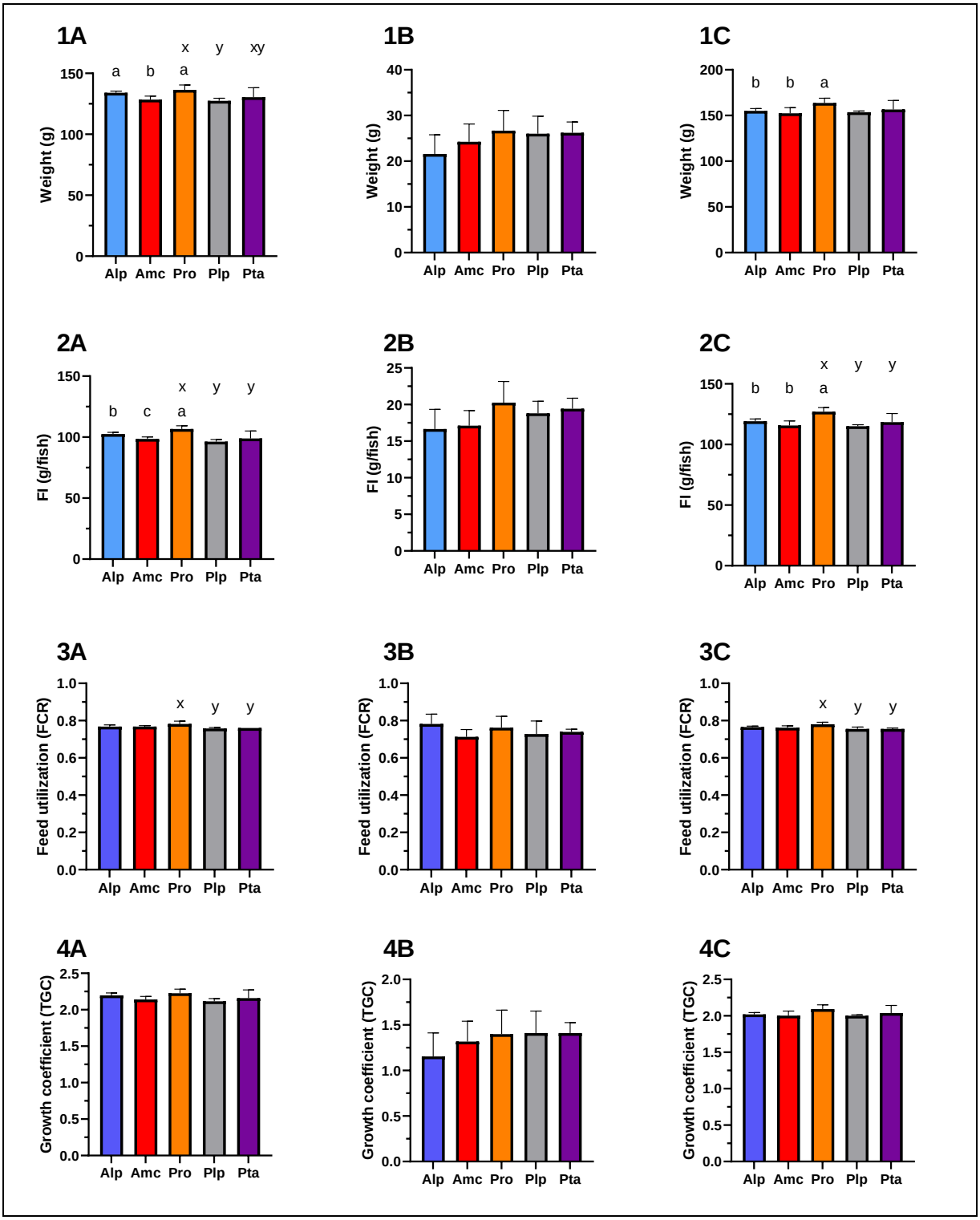


Figure 5 shows the growth performance indicators, 1. body weight gain (BWG), 2. feed intake (FI), 3. feed conversion ratio (FCR), 4. thermal growth coefficient (TGC) in A, freshwater phase (FW); B, seawater phase (SW) and C, for the entire trial period (total). Formulas and units of measurement used for calculations are presented in section **2.7**. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.3. Occurrence of nephrocalcinosis (observational data)

The occurrence of nephrocalcinosis in Atlantic salmon smolt at the end of the freshwater phase is presented in Figure 6. In general, the severity of nephrocalcinosis was low. However, the prevalence of nephrocalcinosis (score 1) was highest in diets with low P (Plp and Pta). In contrast, the diets with high P (Alp, Amc and Pro) had the lowest occurrence of nephrocalcinosis. In addition, score 2 was observed in diet Alp and Plp, and there was no occurrence of score 3 among the different diets, hence not included in the figure.

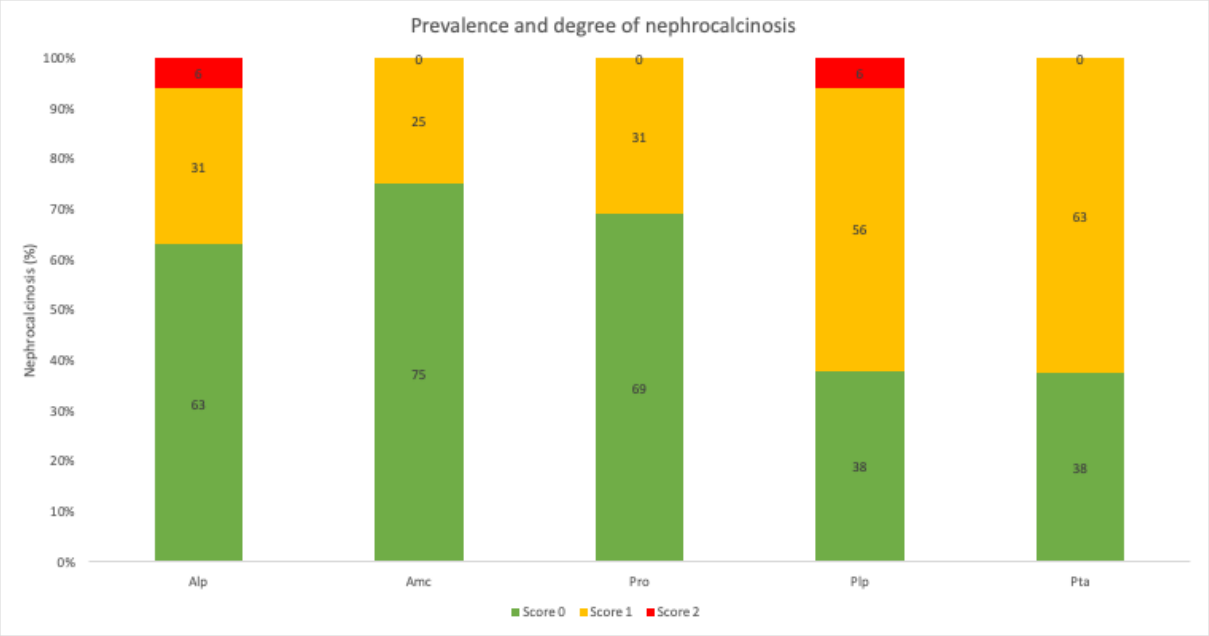


Figure 6 represents observational data of the prevalence and score of nephrocalcinosis (0-3) between diets Alp, Amc, Pro, Plp, and Pta at the end of the freshwater phase. The scoring system is based on the absence of, a low, medium, or high degree of visual calcification in the kidney.

3.4. Somatic indices

Somatic indices of Atlantic salmon at the end of the freshwater phase are presented in Table 7. No significant effects were observed between the different experimental groups.

Table 7

Somatic indices of Atlantic salmon in freshwater.

Parameter	Alp	Amc	Pro	Plp	Pta	P-value
HSI	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	n.s.
CSI	0.17 ± 0.03	0.17 ± 0.03	0.18 ± 0.03	0.18 ± 0.03	0.19 ± 0.04	n.s.
VSI	7.0 ± 1.8	7.3 ± 0.9	7.1 ± 0.7	7.3 ± 1.1	7.3 ± 1.3	n.s.

Notes: HSI (hepato-somatic index), CSI (cardio-somatic index), and VSI (visceral-somatic index) are presented as mean ± S.D. One-way ANOVA was performed to evaluate the statistical difference between

diet groups. Significant differences were evaluated by Tukey's honestly significant differences (HSD) test and means marked by "ns" are non-significant.

3.5. Apparent availability coefficients (AAC) of minerals.

3.5.1 Macrominerals

The AAC of macrominerals at the end of the freshwater phase is presented in Figure 7. A significant effect was observed in AAC of P among the macrominerals analysed. The AAC of P was significantly higher in Alp compared to Amc ($p < 0.05$, ANOVA) and significantly higher in Plp and Pta compared to Pro ($p < 0.05$, ANOVA).

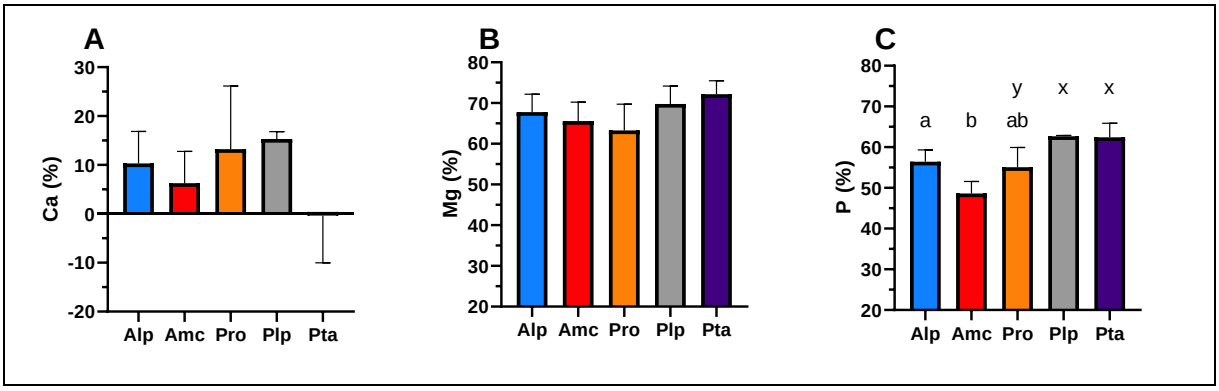


Figure 7 shows macrominerals' apparent availability coefficients (AAC) at the end of the freshwater phase A. calcium (Ca), B. magnesium (Mg), and C. phosphorus (P). Data represent calculations based on the mineral content in the feed, faeces, and an inert marker presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.5.2 Microminerals

The apparent availability coefficients (AAC) of microminerals at the end of the freshwater phase are presented in Figure 8. Among the AACs, a significant effect was observed for Fe and Zn. The AAC of Fe was significantly higher in Pro compared to Alp and Amc ($p < 0.01$, ANOVA), and the AAC of Zn was significantly higher in Pta compared to Pro ($p < 0.01$, ANOVA), but not when compared to Plp.

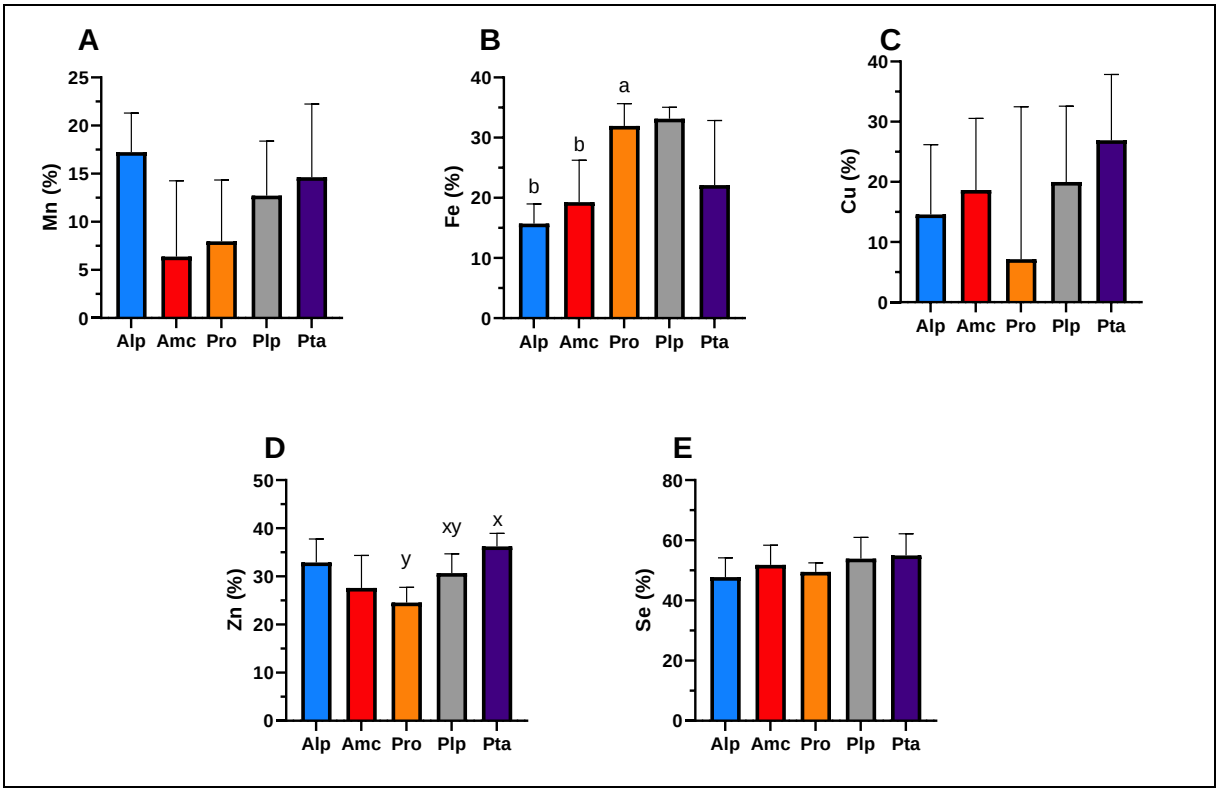


Figure 8 shows the apparent availability coefficients (AAC) of microminerals at the end of the freshwater phase, A. manganese (Mn), B. iron (Fe), C. copper (Cu), D. zinc (Zn), E. selenium (Se). Data represent calculations based on the mineral content in the feed, faeces, and an inert marker, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.6. Mineral status in freshwater (FW).

3.6.1 Dry matter (DM) and ash

The dry matter content of the whole body and vertebrae was determined after freeze-drying of the sampled tissues in FW. No significant differences were found between the experimental diet groups.

Table 8

DM and ash of analysed samples.

	Alp	Amc	Pro	P-value	Pro	Plp	Pta	P-value
Freshwater								
Whole body DM	32.7 ± 0.2	32.6 ± 0.3	32.5 ± 0.3	n.s.	32.5 ± 0.3	32.5 ± 0.5	33.0 ± 0.4	n.s.
Vertebrae DM	49.5 ± 0.9	49.3 ± 0.7	49.7 ± 1.3	n.s.	49.7 ± 1.3	49.2 ± 1.5	48.8 ± 0.9	n.s.
Scales ASH	11.5 ± 1.3	10.6 ± 0.3	10.7 ± 0.4	n.s.	10.7 ± 0.4	11.0 ± 0.4	10.3 ± 0.5	n.s.

Notes: Dry matter and ash for the analysed samples in FW, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found (n.s.).

3.6.2a Macromineral status of the whole body

The macromineral concentration of the whole body at the end of the freshwater phase is presented in Figure 9. No significant effects of the dietary treatments were observed among the macrominerals analysed.

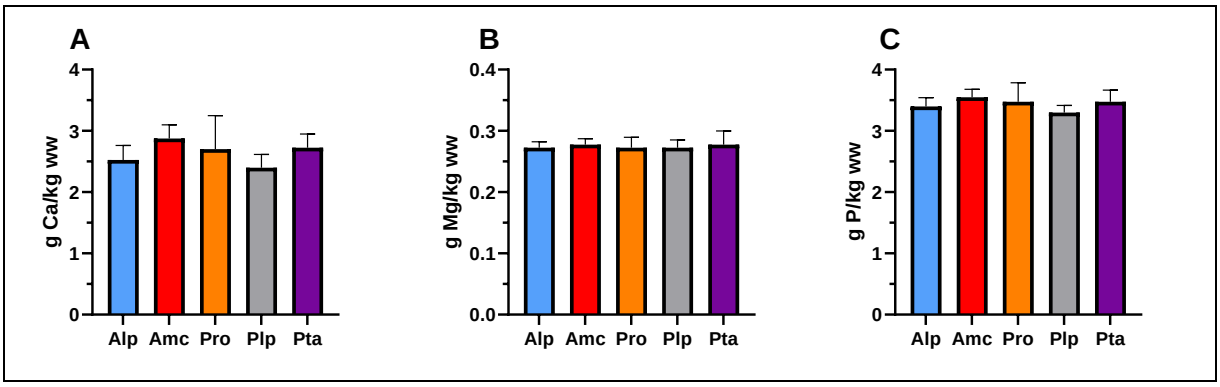


Figure 9: Macromineral status of the whole body in FW, A. calcium (Ca), B. magnesium (Mg), and C. phosphorus (P). Data represent pooled samples of 40 fish per diet group, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.6.2b Micromineral status of the whole body

The micromineral concentration of the whole body at the end of the freshwater phase is presented in Figure 10. Among the microminerals analysed, the only significant effect of the dietary treatments was observed in selenium (Se) when comparing the groups Pro, Plp, and Pta. The concentration of Se in the whole body was significantly higher in Pro compared to both Plp and Pta ($p < 0.0001$, ANOVA).

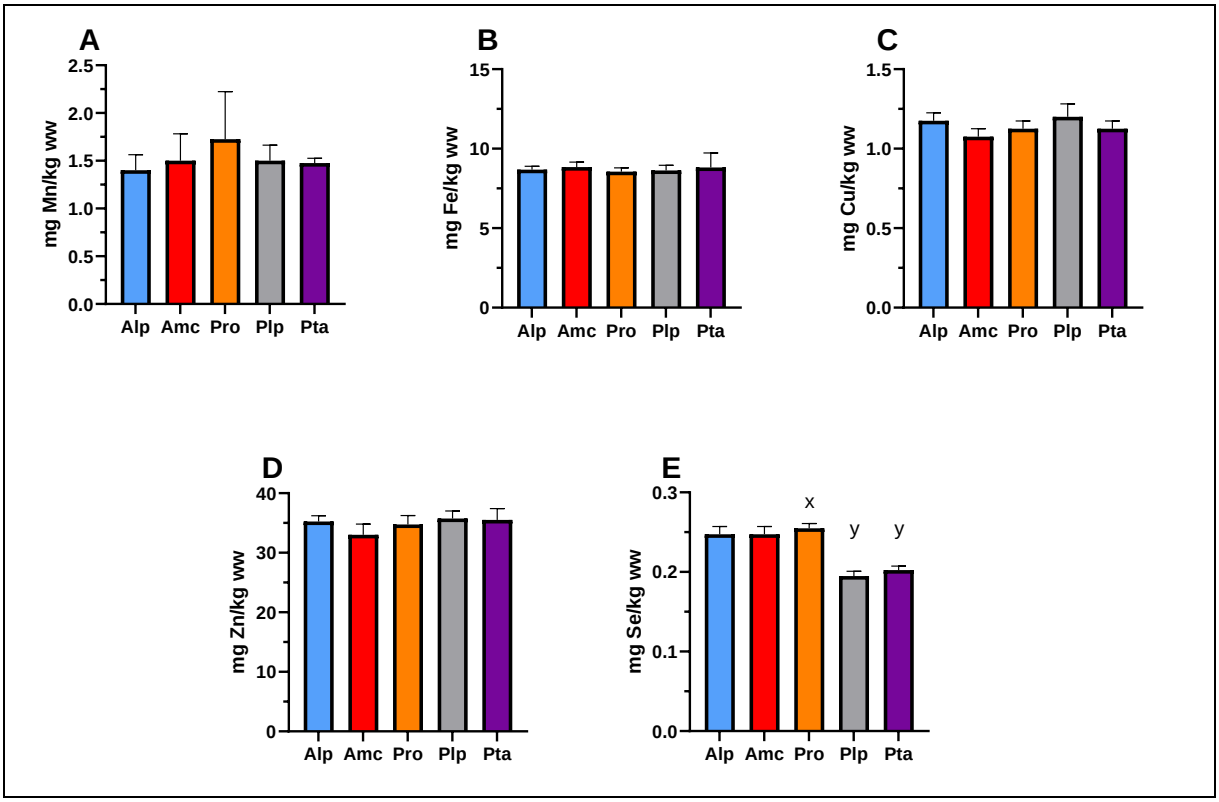


Figure 10: Micromineral status of the whole body in FW, A. manganese (Mn), B. iron (Fe), C. copper (Cu), D. zinc (Zn), E. selenium (Se). Data represent pooled samples of 40 fish per diet group, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.6.3a Macromineral status of plasma

The macromineral content of plasma at the end of the freshwater phase is presented in Figure 11. Among the macrominerals analysed, no significant effects were observed ($p > 0.05$, ANOVA).

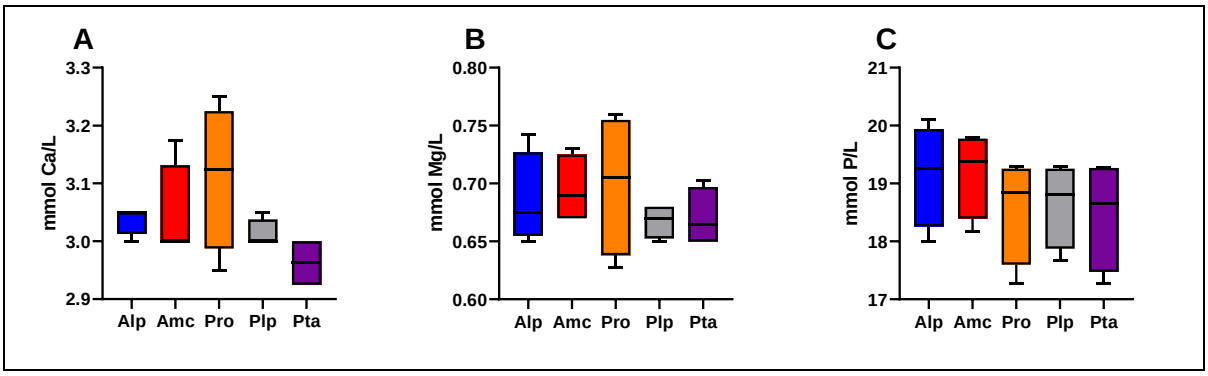


Figure 11: Macromineral status of plasma in FW, A. calcium (Ca), B. magnesium (Mg), and C. phosphorus (P). Data is presented as a boxplot of n=16 observations from 4 tanks per diet group. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp,

and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.6.3b Micromineral status of plasma

The micromineral concentration of plasma at the end of the freshwater phase is presented in Figure 12. Among the microminerals analysed, the only significant effect of the dietary treatments was observed in selenium (Se) when comparing the groups Pro, Plp, and Pta. The concentration of Se in the plasma was significantly higher in Pro compared to both Plp and Pta ($p < 0.001$, ANOVA).

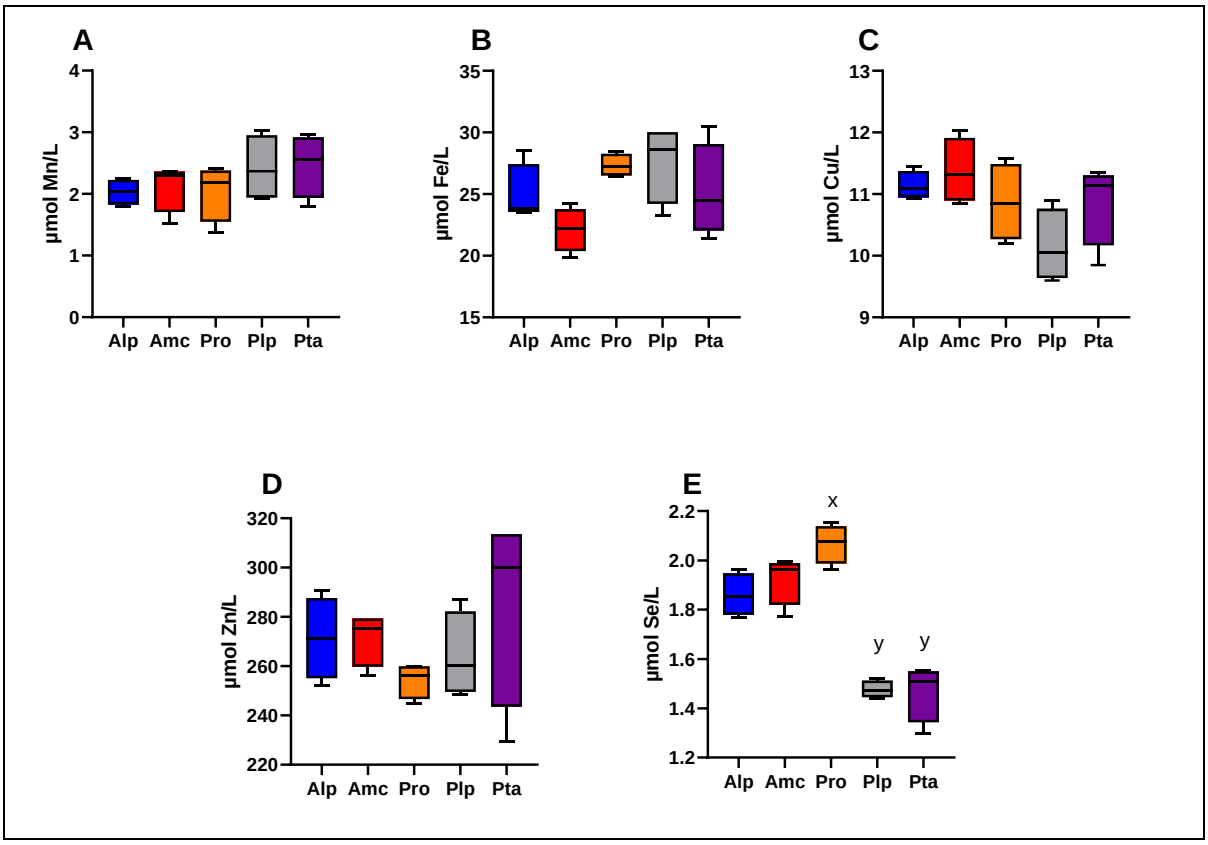


Figure 12: Micromineral status of plasma in FW, A. manganese (Mn), B. iron (Fe), C. copper (Cu), D. zinc (Zn), E. selenium (Se). Data is presented as a boxplot of n=16 observations from 4 tanks per diet group. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.6.4a Macromineral status of scales

The macromineral concentration of the scales at the end of the freshwater phase is presented in Figure 13. No significant differences were observed between the groups for the macrominerals analysed.

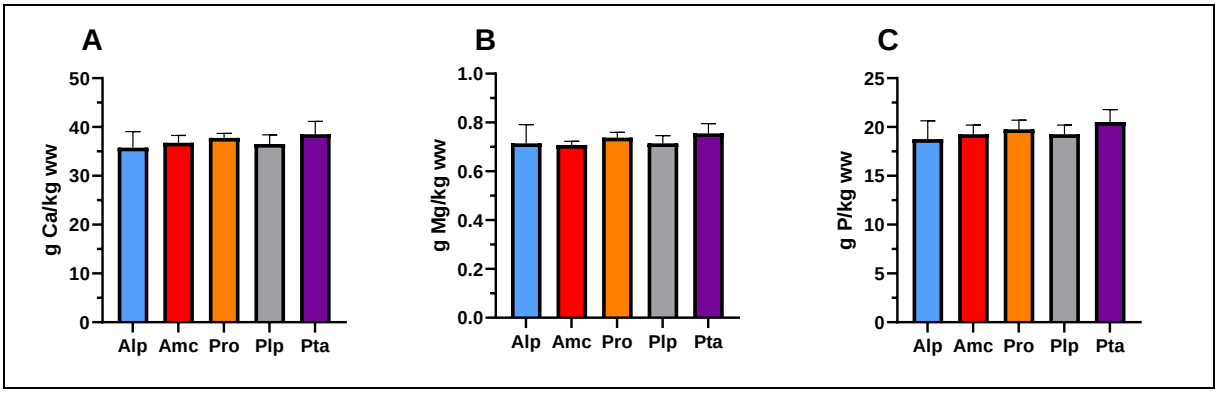


Figure 13: Macromineral status of fish scales in FW, A. calcium (Ca), B. magnesium (Mg), and C. phosphorus (P). Data represent pooled samples of 16 fish per diet group, presented as mean ± SD. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.6.4b Micromineral status of scales

The micro-mineral concentration of the fish scale at the end of the freshwater phase is presented in Figure 14. Among the different micro-minerals analysed, the concentration of copper (Cu) in the scales was significantly higher in Pta compared to Plp ($p < 0.05$, ANOVA) but not compared to Pro. The concentration of zinc (Zn) was significantly higher in Pta compared to both Plp and Pro ($p < 0.01$, ANOVA). Selenium (Se) concentration was significantly higher in Pro compared to both Plp and Pta ($p < 0.0001$, ANOVA). The only significant difference in scale microminerals between Alp, Amc, and Pro was a significantly higher level of Fe in Pro compared to Alp or Amc.

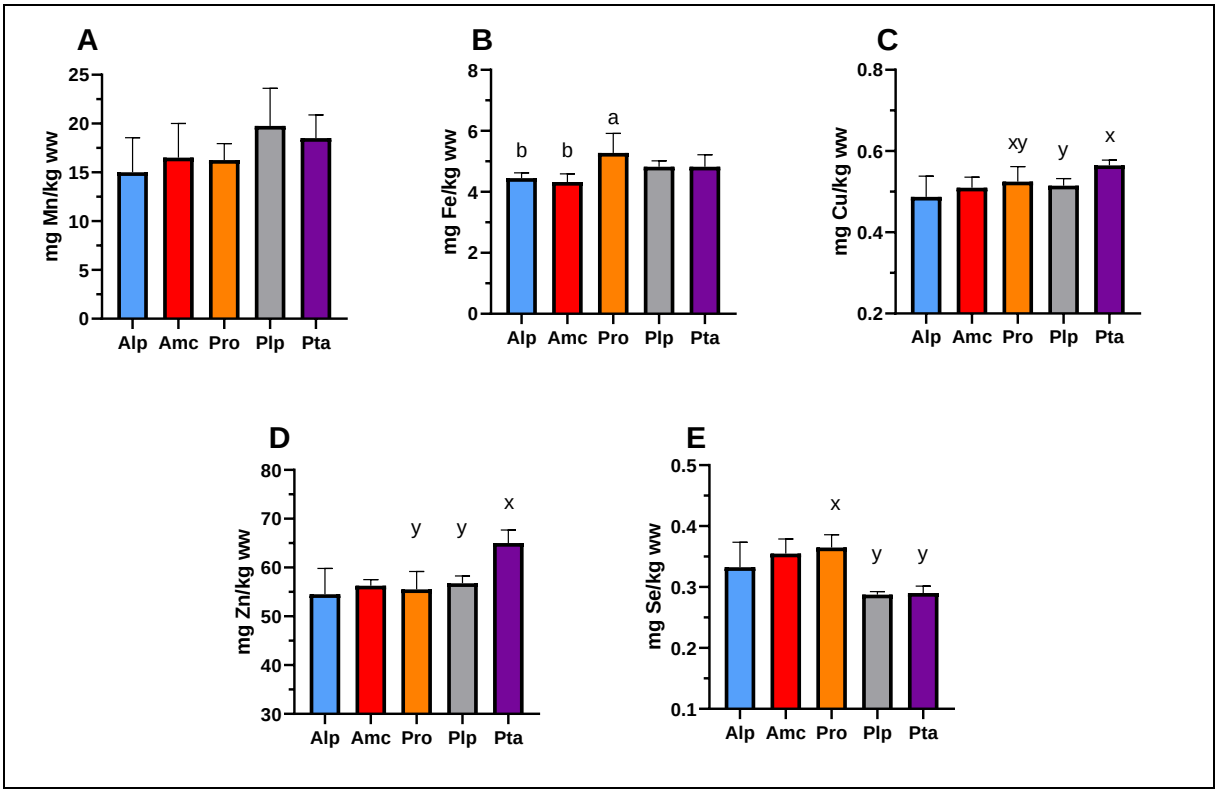


Figure 14: Micromineral status of fish scales in FW, A. manganese (Mn), B. iron (Fe), C. copper (Cu), D. zinc (Zn), E. selenium (Se). Data represent pooled samples of 16 fish per diet group, presented as mean ± SD. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.6.5a Macromineral status of vertebrae

The macromineral content of the vertebrae at the end of the freshwater phase is presented in Figure 15. Among the macrominerals analysed, no significant effects were observed ($p > 0.05$, ANOVA).

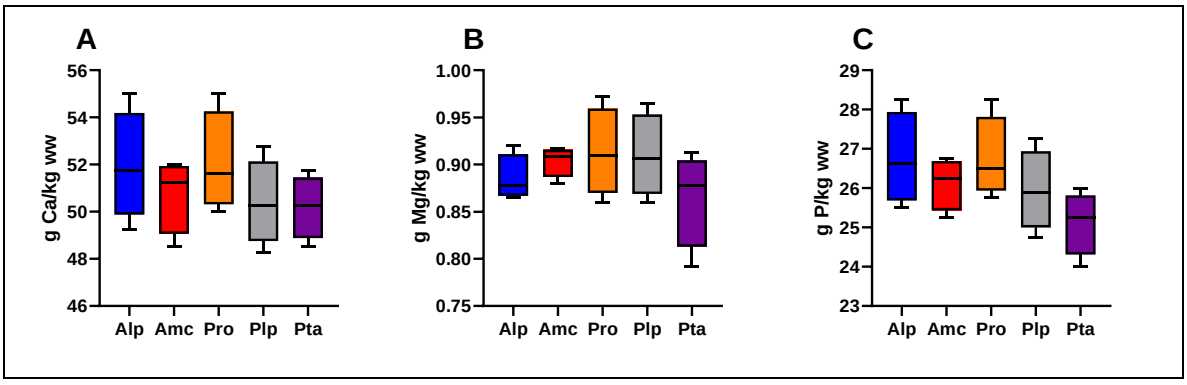


Figure 15: Macromineral status of vertebrae in FW, A. calcium (Ca), B. magnesium (Mg), and C. phosphorus (P). Data is presented as a boxplot of n=16 observations from 4 tanks per diet group. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp,

and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.6.5b Micromineral status of vertebrae

The micromineral concentration of the vertebrae at the end of the freshwater phase is presented in Figure 16. Among the microminerals analysed, the only significant effect of the dietary treatments was observed in selenium (Se) when comparing the groups Pro, Plp, and Pta. The concentration of Se in the vertebrae was significantly higher in Pro compared to both Plp and Pta ($p < 0.0001$, ANOVA).

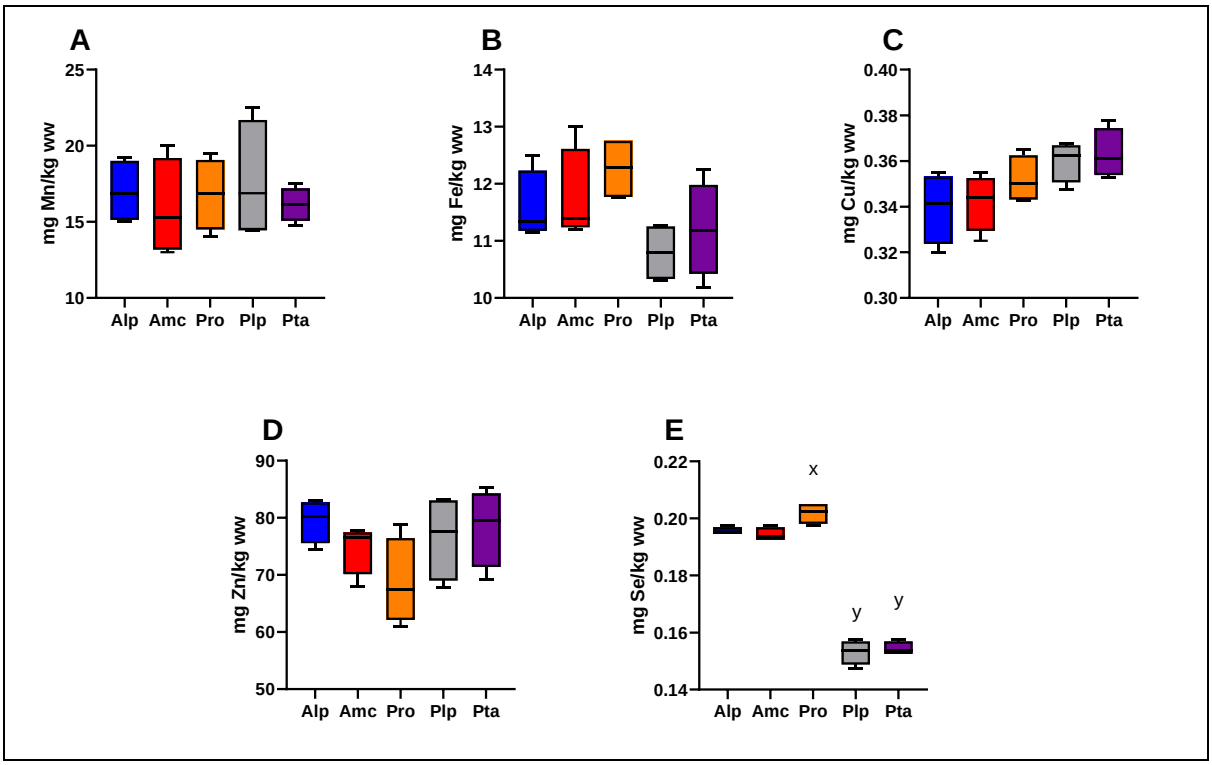


Figure 16: Micromineral status of vertebrae in FW, A. manganese (Mn), B. iron (Fe), C. copper (Cu), D. zinc (Zn), E. selenium (Se). Data is presented as a boxplot of n=16 observations from 4 tanks per diet group. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.7. Mineral status in seawater (SW)

3.7.1 Dry matter and ash

The dry matter content of the whole body and the ash content of scales in SW is presented in Table 9. No significant effects were found.

Table 9

Whole body DM and scale ash at the end of SW phase.

	Alp	Amc	Pro	P-value	Pro	Plp	Pta	P-value
Seawater								
Whole body DM	31.7 ± 0.7	31.3 ± 0.8	31.6 ± 0.6	n.s.	31.6 ± 0.6	31.6 ± 0.2	31.1 ± 0.2	n.s.
Scales ASH	11.0 ± 0.8	10.2 ± 1.2	10.8 ± 0.7	n.s.	10.8 ± 0.7	10.4 ± 0.6	11.1 ± 0.4	n.s.

Notes: dry matter and ash for the analysed samples in SW, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found (n.s.).

3.7.2a Macromineral status of the whole body

The macromineral concentration of the whole fish after three weeks in seawater is presented in Figure 17. Among the macrominerals analysed, no significant effects were observed between the groups.

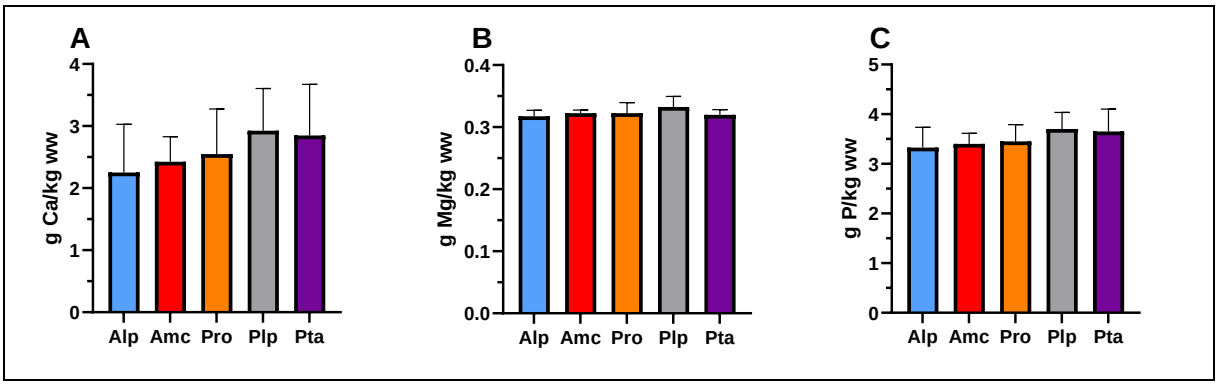


Figure 17: Macromineral status of the whole body in SW, A. calcium (Ca), B. magnesium (Mg), and C. phosphorus (P). Data represent pooled samples of 16 fish per diet group, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.7.2b Micromineral status of the whole body

The micromineral concentration of the whole body after three weeks in seawater is presented in Figure 18. Among the microminerals analysed, the only significant effect of the dietary treatments was observed in selenium (Se) when comparing the groups Pro, Plp, and Pta. However, the concentration of Se in the whole body was significantly higher in Pro compared to both Plp and Pta ($p < 0.0001$, ANOVA).

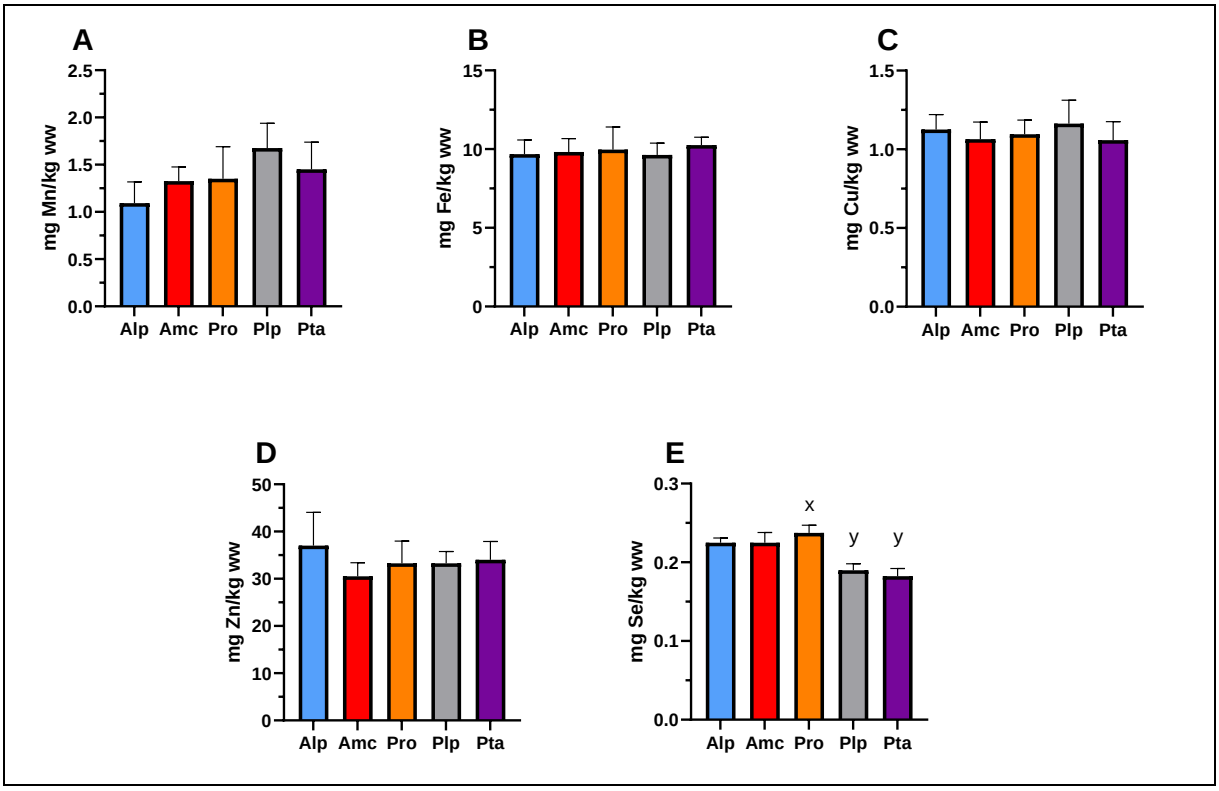


Figure 18: Micromineral status of the whole body in SW, A. manganese (Mn), B. iron (Fe), C. copper (Cu), D. zinc (Zn), E. selenium (Se). Data represent pooled samples of 16 fish per diet group, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.7.3a Macromineral status of plasma

The macromineral content of plasma after three weeks in seawater is presented in Figure 19. Among the macrominerals analysed, no significant effects were observed ($p > 0.05$, ANOVA).

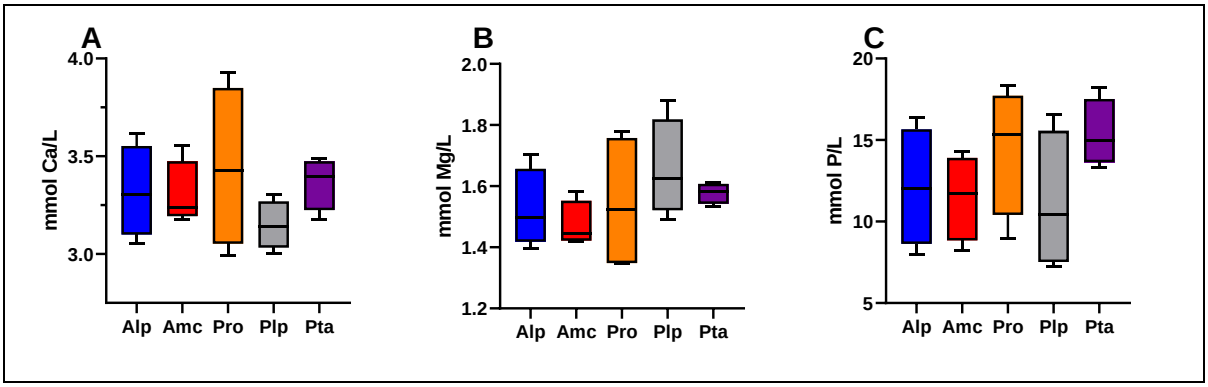


Figure 19: Macromineral status of plasma in SW, A. calcium (Ca), B. magnesium (Mg), and C. phosphorus (P). Data is presented as a boxplot of n=16 observations from 4 tanks per diet group. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.7.4a Macromineral status of scales

The macromineral concentration of the scales after three weeks in seawater is presented in Figure 20. No significant effects of the dietary treatments were observed among the macrominerals analysed.

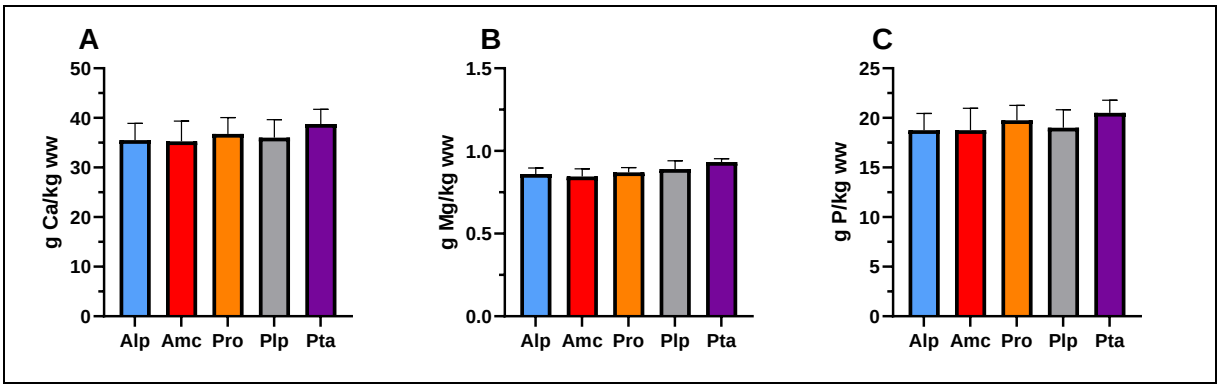


Figure 20: Macromineral status of fish scales in SW, A. calcium (Ca), B. magnesium (Mg), and C. phosphorus (P). Data represent pooled samples of 16 fish per diet group, presented as mean ± SD. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

3.7.4b Micromineral status of scales

The micromineral concentration of the scales after three weeks in seawater is presented in Figure 21. Among the microminerals analysed, the only significant effect of the dietary treatments was observed in selenium (Se) when comparing the groups Pro, Plp, and Pta. However, the concentration of Se in the scales was significantly higher in Pro compared to both Plp and Pta ($p < 0.05$, ANOVA).

3.7.5 Calcium to phosphorus ratio (Ca:P)

The Ca:P ratio of the analysed tissues is presented in Table 10. When comparing the experimental diet groups, only one significant effect was found. The Ca:P ratio of vertebrae at the end of the freshwater phase was significantly higher in Pta compared to Plp ($p < 0.05$, ANOVA).

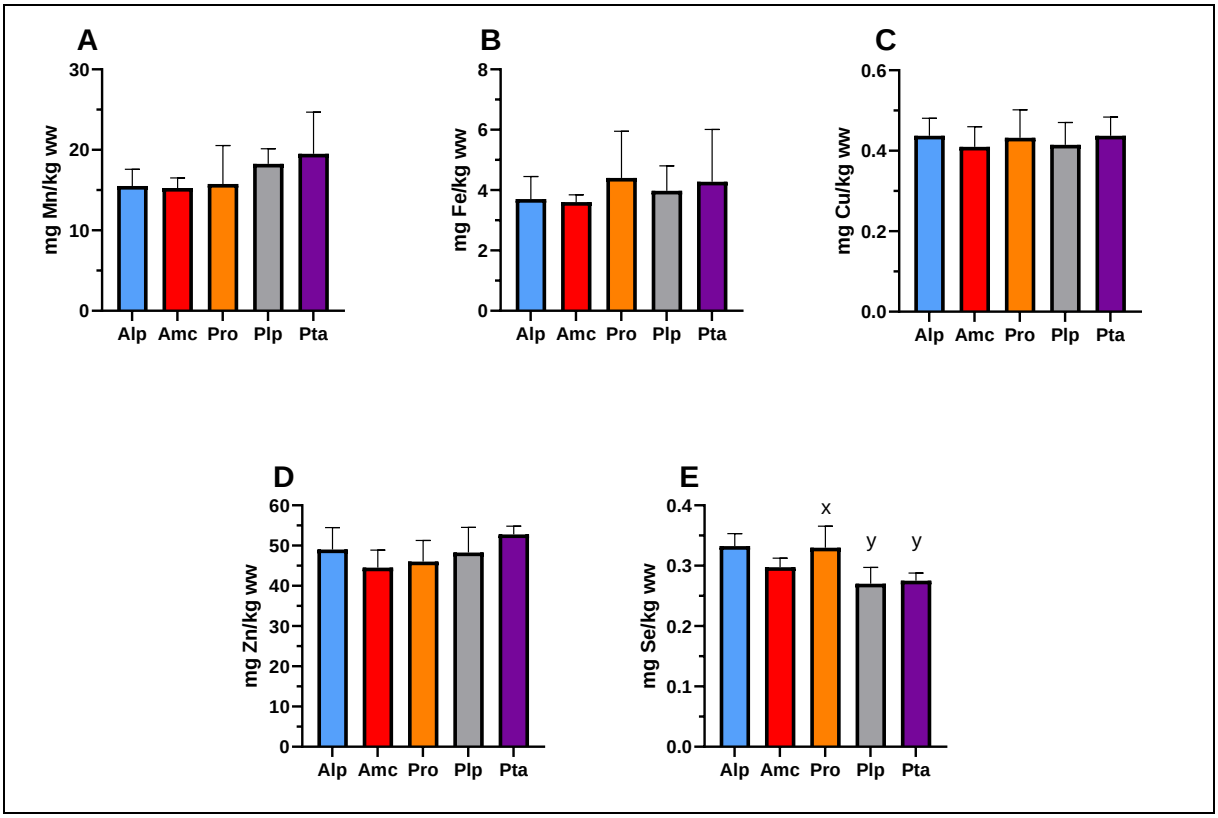


Figure 21: Micromineral status of fish scales in SW, A. manganese (Mn), B. iron (Fe), C. copper (Cu), D. zinc (Zn), E. selenium (Se). Data represent pooled samples of 16 fish per diet group, presented as mean ± SD. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found.

Table 10

Ca:P ratio of analysed samples in FW and SW.

	Alp	Amc	Pro	P-value	Pro	Plp	Pta	P-value
Freshwater								
Whole body	0.74 ± 0.05	0.81 ± 0.05	0.77 ± 0.09	n.s.	0.77 ± 0.09	0.73 ± 0.05	0.79 ± 0.03	n.s.
Scales	1.91 ± 0.02	1.91 ± 0.02	1.92 ± 0.04	n.s.	1.92 ± 0.04	1.90 ± 0.01	1.88 ± 0.03	n.s.
Vertebrae	1.94 ± 0.02	1.94 ± 0.03	1.95 ± 0.01	n.s.	1.95 ± 0.01^{xy}	1.94 ± 0.01^{y}	2.00 ± 0.02^{x}	0.03
Seawater								
Whole body	0.66 ± 0.16	0.71 ± 0.07	0.73 ± 0.14	n.s.	0.73 ± 0.14	0.78 ± 0.12	0.77 ± 0.14	n.s.
Scales	1.89 ± 0.01	1.88 ± 0.02	1.86 ± 0.03	n.s.	1.86 ± 0.03	1.89 ± 0.01	1.89 ± 0.03	n.s.

Notes: triplet comparisons of calcium to phosphorus ratio (Ca:P) for all relevant tissues in freshwater and seawater, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found (n.s.).

3.8. X-ray radiography and deformities

The prevalence of deformed fish and the types of deformities observed in this trial are presented in Table 11. Fish fed the diet Amc had the highest prevalence of deformed fish compared to the other diets. A few compression and fusion-related deformities were observed; however, the most prevalent deformity was a dorsal/ventral shift of the vertebral bodies (type 19).

Table 11

Prevalence and type of deformity observed in the freshwater phase of Atlantic salmon

	Alp	Amc	Pro	Plp	Pta
Prevalence of deformed fish (%)	12.5	25	12.5	12.5	15
Total deformed vertebra	11	20	8	10	8
Type 3: compression and reduced intervertebral space	2	2	2	4	0
Type 5: one-sided compression	1	5	2	0	2
Type 6: compression and fusion	1	0	0	0	0
Type 7: complete fusion	4	2	0	0	0
Type 12: hyper-radiodense	0	1	0	0	0
Type 15: kyphosis	0	0	0	1	0
Type 17: vertically shifted	0	1	2	1	1
Type 19: internal dorsal or ventral shift	3	9	2	4	5

Notes: Prevalence and deformity type was determined based on a subjective evaluation of 40 fish per diet group. Classification of deformities is based on image 6 (Witten et al., 2009).

Regional identity of vertebra deformity

Localisation and prevalence of vertebral deformities at the end of the freshwater phase are presented in Figure 22. Most deformities across all experimental diet groups occurred between V23-V34, as seen in Figure 22. Concordant with results from Table 11, fish fed the diet Amc had the highest prevalence of deformities.

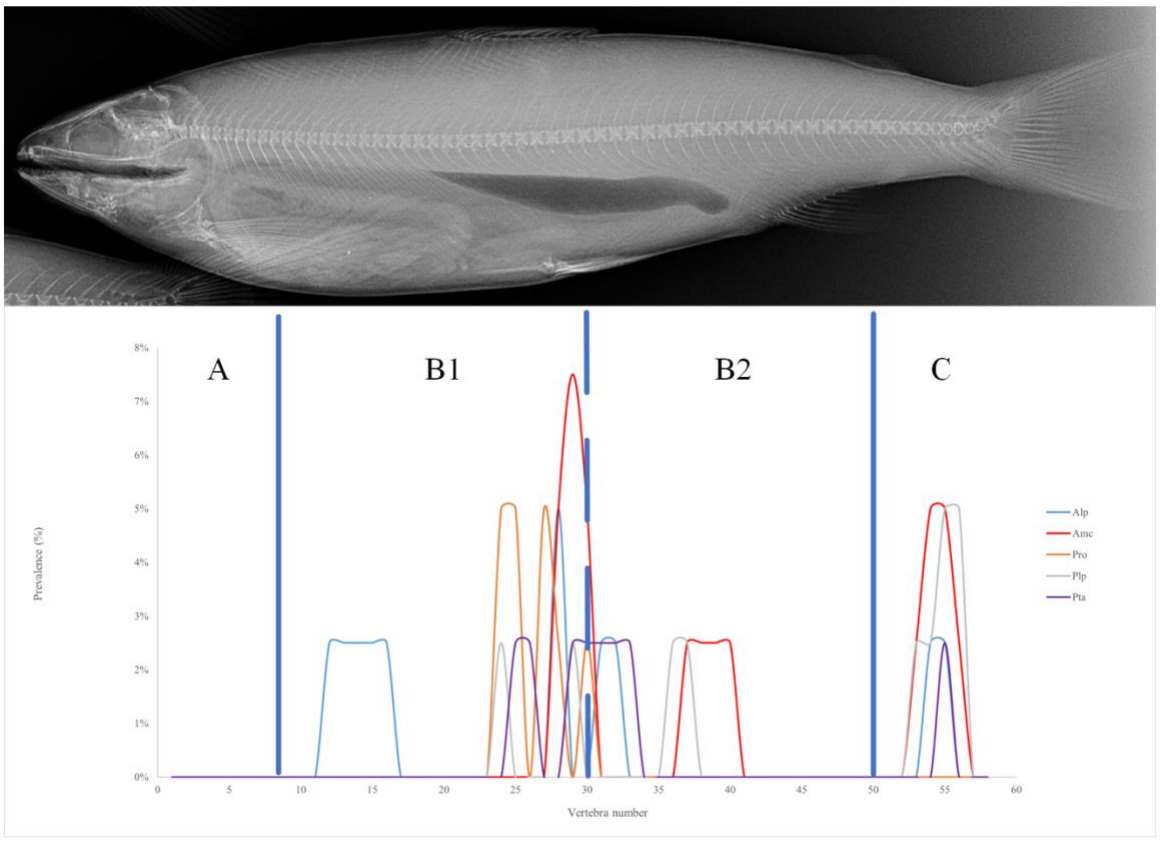


Figure 22 shows an un-deformed fish and the localisation and prevalence of vertebral deformities between the five experimental diets. The x-axis represents the vertebra number (1-58), and the y-axis represents the prevalence of deformities (%). As described in Kacem, Meunier and Baglinière. (1998), the Atlantic salmon vertebrae are divided into four regions A. post-cranial region (V1→8), B. middle region (B1-posterior truncal vertebrae (V9→30) and B2-anterior caudal vertebrae (V31→49)), and C. ural region (V50→58).

Types of deformities

The different types of vertebral deformities observed in this trial are presented in Figure 23. The most common deformity observed in this trial was type 19 (H), which changes the symmetry of the vertebral end plates. Complete fusion of two vertebral bodies was observed in diets Alp and Amc. This deformity leads to remodelling the fused vertebral bodies into one with multiple neural and haemal arches (Witten *et al.*, 2006), thus easily observed in x-ray images. According to Witten *et al.* (2006), successful fusions do not necessarily lead to fish with deformed vertebral columns.

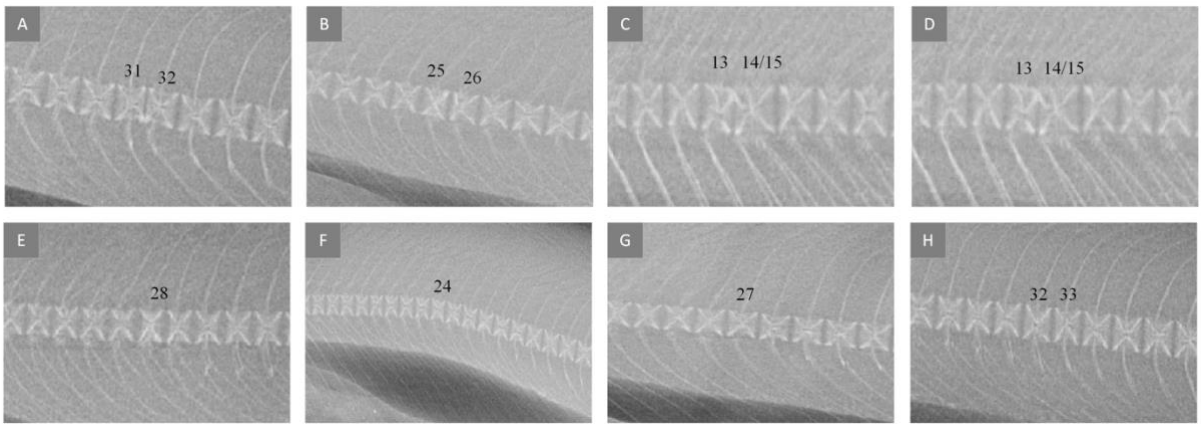


Figure 23: Types of vertebral deformities observed with radiography at the end of the freshwater phase. Examples of deformities include (A) type 3, (B) type 5, (C) type 6, (D) type 7, (E) type 12, (F) type 15, (G) type 17, and (H) type 19. A number above the vertebra indicates the localisation of the deformity.

3.9. Vertebrae measurements and mechanical strength data

3.9.1 Morphological measurements

Measurements of the vertebra for the determination of morphological differences are presented in Table 12. Among the different measurements, no significant effects were observed ($p > 0.05$, ANOVA).

Table 12

Morphological measurements of vertebra.

	Alp	Amc	Pro	P-value	Pro	Plp	Pta	P-value
Cranial caudal height (VH)	3.40 ± 0.21	3.33 ± 0.31	3.24 ± 0.32	n.s.	3.24 ± 0.32	3.39 ± 0.26	3.37 ± 0.30	n.s.
Dorsal ventral diameter (VD1)	3.29 ± 0.19	3.28 ± 0.27	3.23 ± 0.26	n.s.	3.23 ± 0.26	3.32 ± 0.29	3.38 ± 0.25	n.s.
Lateral diameter (VD2)	3.17 ± 0.22	3.10 ± 0.35	3.15 ± 0.28	n.s.	3.15 ± 0.28	3.01 ± 0.29	3.15 ± 0.29	n.s.
Average diameter (VD)	3.23 ± 0.20	3.19 ± 0.31	3.24 ± 0.28	n.s.	3.24 ± 0.28	3.12 ± 0.26	3.27 ± 0.25	n.s.
Vertebrae area (VA)	8.23 ± 1.01	8.06 ± 1.57	8.30 ± 1.49	n.s.	8.30 ± 1.49	7.70 ± 1.30	8.42 ± 1.31	n.s.

Notes: Data represents measurements from 48 vertebrae per diet group, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found (n.s.).

3.9.2 Mechanical strength curves

A load-displacement curve based on a mechanical compression test of Atlantic salmon vertebrae at the end of the freshwater phase is presented in Figure 24.

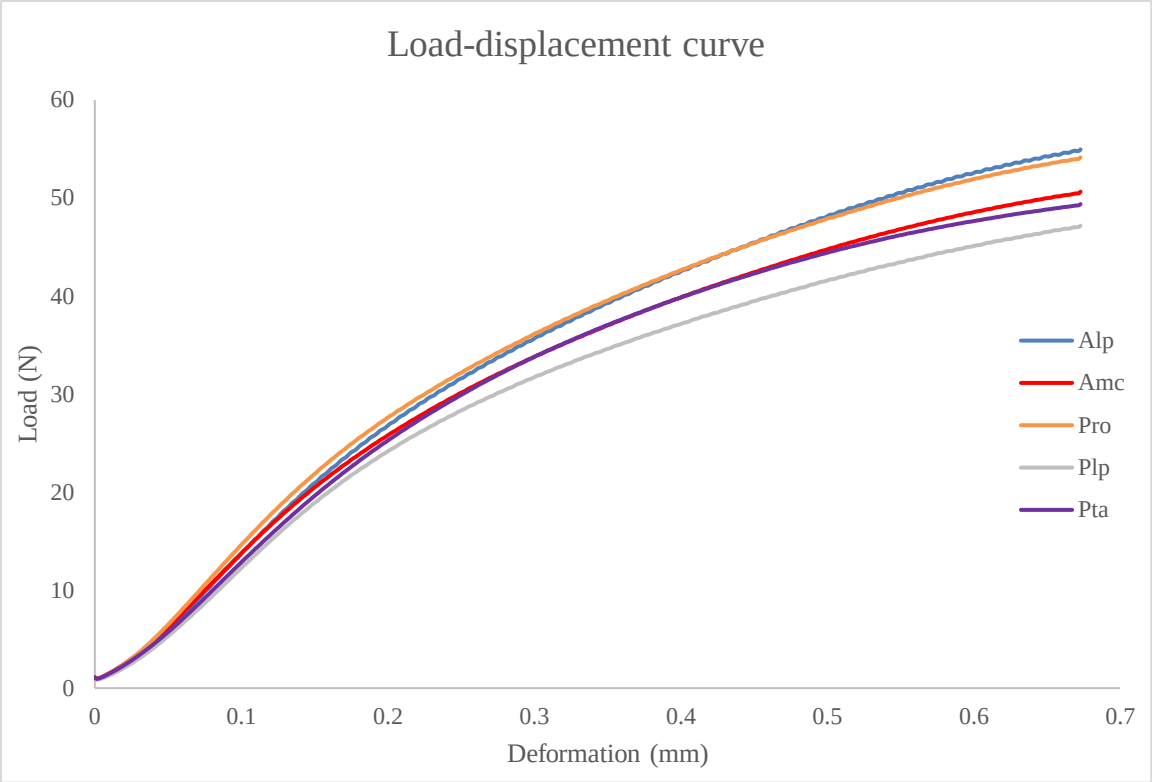


Figure 24: Load-displacement curve for mechanical compression of Atlantic salmon vertebra centra. Graphs represent pooled mean values of 16 fish and 48 compression tests per diet group.

A stress-strain curve of Atlantic salmon vertebrae at the end of the freshwater phase is presented in Figure 25.

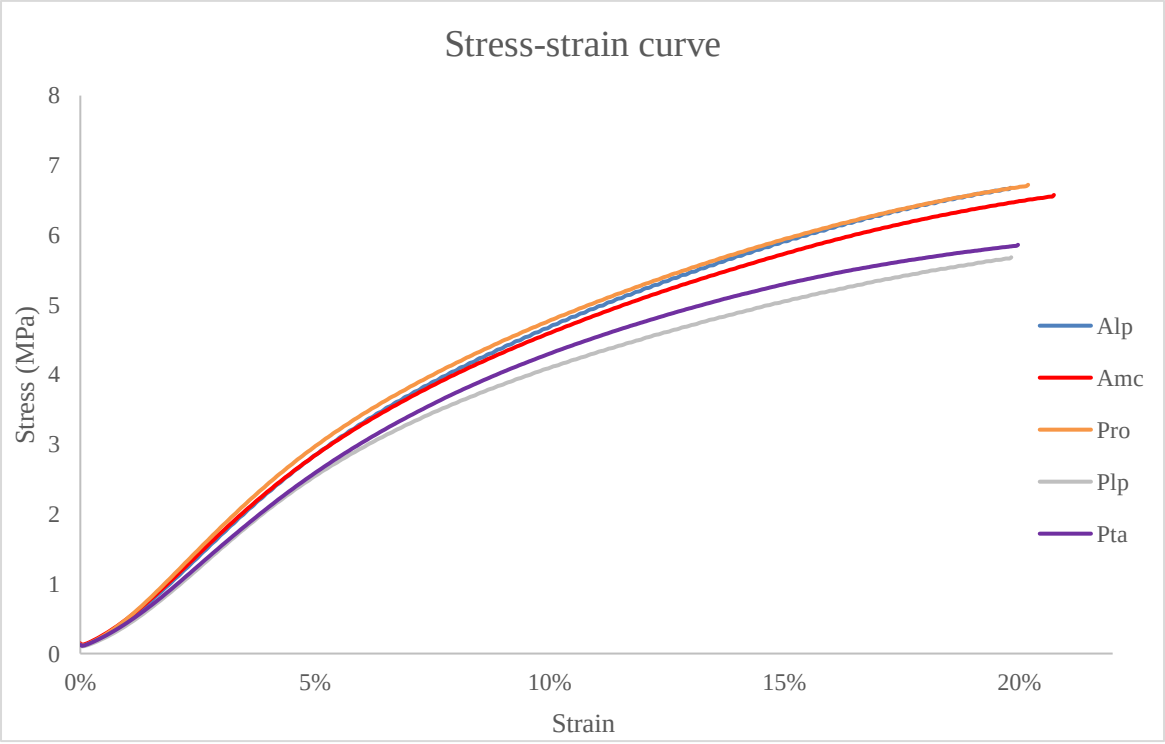


Figure 25: Stress-strain curve of Atlantic salmon vertebra fed different diets (Alp, Amc, Pro, Plp, and Pta). Stress (external force) and strain (structural deformation) were calculated based on load-deformation data, with each diet's mean area and cranial-caudal height of vertebrae, respectively.

3.9.3 Macroanalysis of compression test

The comparison of mechanical data between feed P source (Alp, Amc, and Pro) and inclusion of phytase (Pro, Plp, and Pta), both adjusted and not adjusted for vertebrae size, is presented in Table x. No significant effects were observed when comparing Alp, Amc, and Pro groups. When comparing the groups Pro, Plp, and Pta, the only significant effect was observed when calculating the modulus of elasticity. The modulus of elasticity was significantly higher in Pro compared to Pta ($p < 0.05$, ANOVA) but not when compared to Plp ($p > 0.05$, ANOVA).

Table 13

Mechanical properties of vertebra.

	Alp	Amc	Pro	P-value	Pro	Plp	Pta	P-value
Mechanical properties (corrected for size of the vertebral centra)								
Modulus of elasticity (MPa/%)	0.65 ± 0.07	0.65 ± 0.09	0.69 ± 0.08	n.s.	0.69 ± 0.08^{x}	0.61 ± 0.08^{xy}	0.59 ± 0.08^{y}	0.03
Yield-point(MPa) (0.2% offset)	2.8 ± 0.7	2.2 ± 0.5	2.4 ± 0.5	n.s.	2.4 ± 0.5	2.4 ± 0.6	2.2 ± 0.5	n.s.
Failure point (MPa)	17.0 ± 2.5	15.7 ± 1.7	16.1 ± 2.7	n.s.	16.1 ± 2.7	15.2 ± 1.9	15.4 ± 1.9	n.s.
Resilience (MPa x %)	51.0 ± 11.1	43.2 ± 7.7	44.4 ± 7.3	n.s.	44.4 ± 7.3	42.9 ± 6.7	45.0 ± 10.0	n.s.
Mechanical properties (not corrected for size of the vertebral centra)								
Stiffness N/mm	160 ± 17	156 ± 34	171 ± 23	n.s.	171 ± 23	148 ± 22	148 ± 22	n.s.
Yield load (N, 0.4% offset)	49 ± 8	44 ± 9	47 ± 8	n.s.	47 ± 8	41 ± 6	45 ± 7	n.s.
Resilience (N x mm)	16 ± 4	13 ± 3	14 ± 3	n.s.	14 ± 3	13 ± 2	14 ± 3	n.s.

Notes: mechanical properties of vertebral centra, both adjusted and not adjusted for size, presented as mean ± S.D. Significant differences between diets Alp, Amc, and Pro are denoted by the letters a-c. The differences between the diets Pro, Plp, and Pta are denoted by x-z, following ANOVA and Tukey's HSD post hoc test. The absence of letters means no significant effect was found (n.s.).

Stress-strain curve (adapted from Figure 25) comparing Pro with low P diets Plp and Pta is presented in Figure 26.

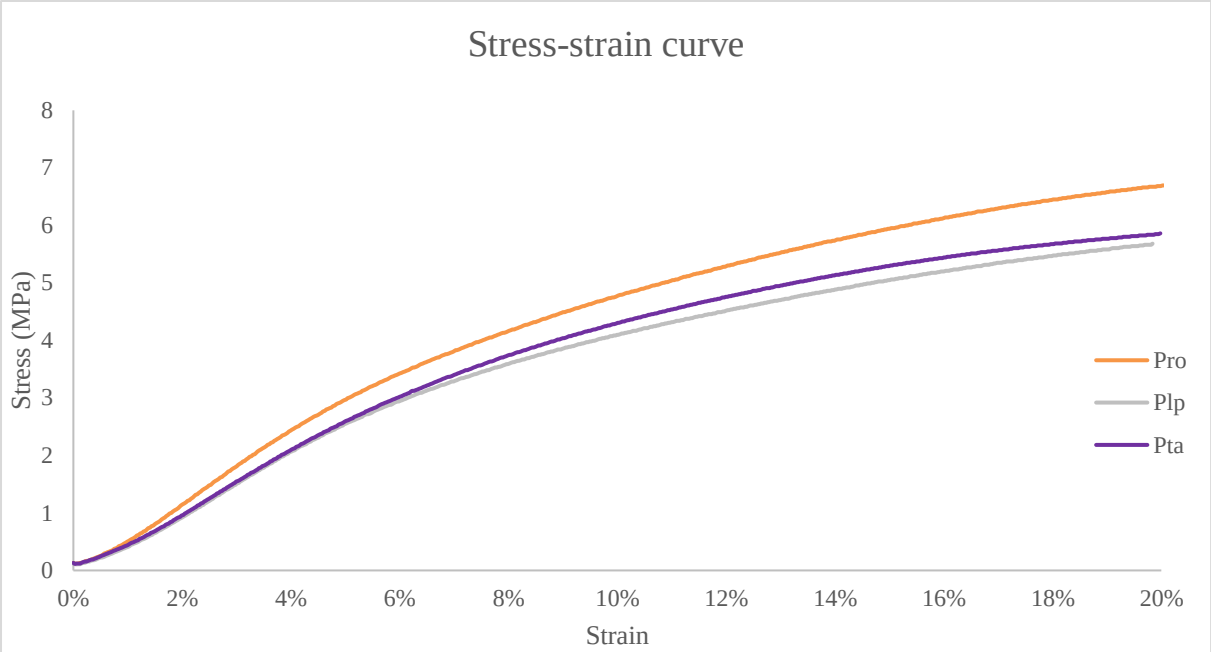


Figure 26: Stress-strain curve of Atlantic salmon vertebra fed different diets (Pro, Plp, and Pta). Stress and strain were calculated based on load-deformation data, with each diet's mean area and cranial-caudal height of vertebrae, respectively.

Summary of results

A summary of the stepwise evaluation of mineralisation and bone health in Atlantic salmon is presented in Figure 27.

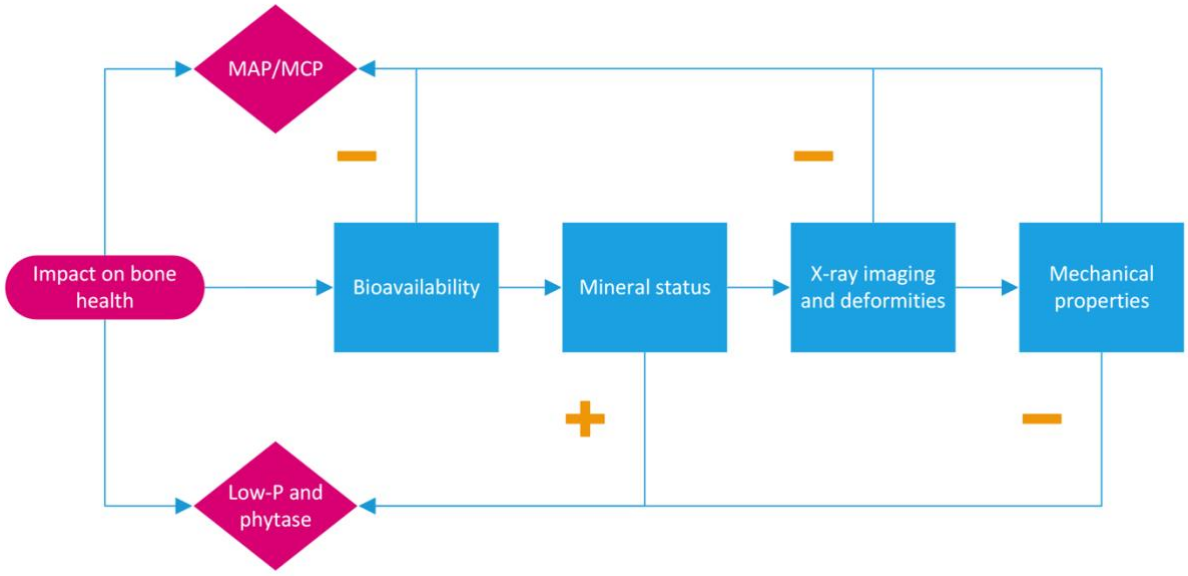


Figure 27 summarises the results and how the different diets impacted bone health. First, we looked at the bioavailability of nutrients, mainly focusing on P. The bioavailability of P was negatively impacted when replacing 50% of MAP with MCP, and an increase in vertebral deformities was also observed. Including phytase improved the mineralisation of scales (Zn, by 15%); however, it could not improve bone health in low P diets.

4. Discussion

Modern feed formulations have featured a reduction in the amount of fishmeal, an increase in the levels of plant protein ingredients, phytase application to improve the availability of phytate-P, and inorganic feed P to meet the nutrient requirements of the fish. This experiment aimed to determine the effects of different feed P regimes and the inclusion of a novel low-temperature E. coli phytase in the feed for Atlantic salmon pre- and post-smolt and compare mineral status and bone health parameters between experimental diets. In summary, replacing a portion of MAP with MCP reduced available P and increased vertebral deformities, and the low-P diets, irrespective of phytase inclusion, resulting in softer vertebrae. In both research objectives, bone health was negatively impacted by suboptimal phosphorus nutrition.

4.1. Phosphorus nutrition and requirement

The phosphorus requirement of Atlantic salmon is recommended to be 8 g/kg available P (NRC, 2011). In our study, the feeds were above 8 g/kg, except for Amc, which was marginally low at 7.3 g/kg, which is approx. 10% lower than required. Phosphorus deficiency leads to poor growth and low mineralisation in the whole body and bones (Baeverfjord, Åsgård and Shearer, 1998; Witten *et al.,* 2019). However, it depends on the magnitude and duration of exposure to P-deficient diets. In the present study, Amc-fed fish had similar growth, whole body and tissue P status compared to other groups.

The mineral content in the whole body, scales, and vertebrae was comparable to the literature on optimal mineral nutrition for Atlantic salmon (Table 2). Optimal plasma P levels are considered 3.5 ± 1.7 mmol/L; however, in this trial, plasma levels were 18.5-19.2 mmol/L. Previous studies have measured P as phosphate with other analytical methods, such as a calorimetric kit (Vielma and Lall, 1998; Antony Jesu Prabhu *et al.*, 2014). In contrast, in this study, P is measured as ionic P. By using the UltraWave for sample digestion, all organic compounds are broken down, releasing both inorganic P and P bound to organic compounds. The differences in plasma P levels in the present study and previous studies are likely related to qualitative analysis; however, they remain an open question. Thus, the growth and body mineral status indicated that the available P supply in Amc fed fish was not highly deficient. Having said that, it has been shown that even marginal P deficiency during critical life stages can predispose the fish to deformities or poor mineralisation. Fjelldal, Lock, *et al.* (2012) showed that low soluble P fed salmon smolts developed softer and more deformed vertebrae at later stages. When comparing low, medium, and high soluble P, the low P diet caused reduced

bone mineralisation, stiffness, and increased vertebral deformities compared to both the medium and high P diets (Fjelldal, Hansen and Albrektsen, 2012). However, it has been shown that low-mineralised salmon bone can re-mineralise and restore the mechanical properties of vertebral bodies (Fraser *et al.*, 2019). Following the findings by Fjelldal, Hansen and Albrektsen (2012), Fraser *et al.* (2019) found that vertebral pathologies are more frequent when fed a low P diet in freshwater. In addition, the authors found a correlation between low to medium/high P in freshwater and seawater, respectively, but not the opposite. Optimal P nutrition in freshwater prevents vertebral pathologies at later stages. Therefore, to accommodate the nutritional P needs of Atlantic salmon, it is crucial to provide bioavailable sources while maintaining low P effluents.

4.2. Feed P sources, mineralisation, and bone health

Inorganic P is a dietary supplement used in fish feeds that increases fish development and feed utilisation, but its high inclusion level can lead to eutrophication in the environment. One objective of this trial was to evaluate the availability of P from mono ammonium phosphate (MAP) and a combination of MAP and monocalcium phosphate (MCP) as feed phosphorus sources. If the diet Alp is a reference for supplemental MAP (100%), Amc and Pro were supplemented with 50 and 76% MAP, respectively. To meet the total P requirement in Amc, 0.77% of MCP was added, and in Pro, the requirement was met by increasing fishmeal inclusion by 7% points (30 to 37%). All three diets had the same level of total P (1.5%); however, fish fed Amc had lower P availability, leading to 0.73% available P compared to Alp (0.85%) and Pro (0.83%). The reduced availability of P when given MCP compared to MAP is in line with previous findings (Morales *et al.*, 2018). In that study, the aim was to evaluate the effect of different sources of inorganic phosphate, including MAP and MCP, in a plant-based diet for rainbow trout. They found that MAP resulted in higher levels of P digestibility (90%) compared to MCP (70%). In practical terms, this does not directly translate for Atlantic salmon, as these two species have shown a clear difference in their capacity to digest P (Glencross *et al.*, 2004). Morales *et al.* (2018) also found that fish fed the diet supplemented with MAP released a higher amount of non-protein N fraction to the water, probably as undigested ammonium through the faeces released by fish. Increased ammonia levels may affect the biofilter in RAS and increase the system's nitrogen load. Further, dissolved phosphate would increase in the system water compared to the source water, in line with Prabhu (2015), who found up to 70-fold higher levels of dissolved P in a near-zero RAS. Several studies have shown a positive effect on growth or body P balance with low water exchange RAS (Van Bussel *et al.*, 2013; Antony Jesu Prabhu *et*

al., 2017); however, not in salmonids. In the present study, the trial was not conducted in individual RAS systems, and dissolved phosphate in the rearing water was not measured as it could not be linked to the specific diets.

Concordant with the significantly lower availability of P, the prevalence of vertebral deformities was higher in fish fed Amc. In this trial, analysis of x-ray images revealed a higher prevalence of deformities in fish fed Amc (25%) compared to those fed the other diets (12.5-15%). Fjelldal, Hansen and Berg. (2007) found that fish showing vertebral deformities on radiographs had equal growth compared to fish without deformities on radiographs. Despite all groups showing some deformities, the overall number was significantly higher in fish fed Amc, with no difference in growth between groups. Fish fed the diet Amc had 20 deformed vertebrae compared to 8-11 in the other groups.

The most common deformity observed in the Amc group was an internal dorsal or ventral shift (type 19), accounting for 45% of the total deformed vertebra. The development of such an asymmetrical deformity remains unknown. However, such a deformity can result from remodelling changes or disruption of normal deposition of new vertebra bone, leading to excessive deposition in some regions of the vertebra and depressed deposition in others (Jawad *et al.*, 2018).

Compared to our findings, this might be a connection between the slightly reduced available P in Amc and the prevalence of type 19 deformities in the fish. Fish groups with the second highest prevalence of type 19 were both the low-P diets (Plp and Pta). The total number of deformed vertebrae in these diets was approximately half of what was observed in Amc. However, type 19 accounted for 40 and 63% of the total malformed vertebrae in Plp and Pta, respectively. In contrast to Amc, these diets were sufficient in available P; however, they had a much lower Ca:P ratio in the feeds. On the other hand, a study by Vielma and Lall (1998) found that an increase in the Ca:P ratio from 0.2 to 2.1 did not affect bone mineralisation when the requirement of dietary P was met. Similarly, we also did not find any difference in bone mineral concentrations. However, further evaluation of vertebral deformities and mechanical properties of the vertebrae indicated bone health issues. In addition, the indication of a higher occurrence of NC could be associated with a low Ca:P ratio in Pta and Plp.

4.3. Mechanical properties of the vertebra

Morphological measurements of the vertebra were performed before the mechanical compression test. Comparable results were found between the experimental diets. After the compression tests, macro analysis of the data revealed a significantly lower modulus of elasticity (MPa/%) in Pta (0.59 ± 0.08) compared to Pro (0.69 ± 0.08). Additionally, a tendency was observed for Plp (0.61 ± 0.08), but Alp and Amc had comparable mechanical strength to Pro. This indicates a higher softness in the vertebra of low P diets. The increased softness of the vertebral body may have detrimental effects on the intervertebral tissues, inhibiting normal vertebral growth. Specifically, a vertebral body with reduced stiffness may undergo slight anterior-posterior compression when the lateral musculature contracts and return to its original shape when the muscle relaxes. Although this non-deformational change may seem benign, it can potentially damage the notochordal sheet or cause other harm to the notochord. Ultimately, this could disrupt the normal longitudinal growth of the vertebral bodies (Baeverfjord *et al.*, 2019). Drábiková *et al.* (2022) found that fish fed high P in the freshwater phase had indications of more brittle bones. Deformed fish in the high P group had a significantly lower Ca:P ratio of the vertebrae than non-deformed fish in the regular P group (Drábiková *et al.*, 2022). In contrast, our findings of an increased Ca:P ratio in the vertebrae of fish fed Pta suggest that tilting the Ca:P ratio in the feed led to a lower mechanical strength. Further studies are needed to investigate the influence of dietary Ca:P ratio on bone mechanical properties.

Poor mechanical strength of vertebrae can be an early indicator of pronounced vertebral deformities at later life stages (Drábiková *et al.*, 2022). Bone deformities are known to cause the downgrading of salmon at harvest (Fjelldal, Hansen, *et al.*, 2012; Baeverfjord *et al.*, 2019). This is not merely an issue of welfare; it also considerably impacts the economic aspects. Even if the deformity is not severe, it can lead to many downgrades. Even small deformations in the vertebrae can affect the muscle texture around the bone. This does not necessarily mean machine-related issues with filets; however, it could still affect the muscle tissue leading to a lower-quality product. In that way, bone deformities act as a silent killer for the industry, eating and growing comparably to non-deformed fish; however, it does not provide the same economic benefit. If the deformities become severe, it can impact the growth of the fish (Hansen *et al.*, 2010) and the machine fileting process (Sullivan *et al.*, 2007). Fjelldal, Hansen, *et al.* (2012) noted in a review that in a group of harvest-size Atlantic salmon, 70% were diagnosed as deformed by X-ray imaging and 26% by palpation; however, only 4% were downgraded. Nevertheless, this review was done a long time ago, and according to the annual fish health report, deformities are one of the major issues in salmon production to date (Sommerset *et al.*,

2022; Wiik-Nielsen, 2023). Therefore, it is essential to address further issues related to deformities, to reduce downgrading and improve the welfare of the fish.

4.4. Nephrocalcinosis and Ca:P ratio

Like bone deformities, nephrocalcinosis (NC) is a mineralisation problem; however, more in terms of an ion regulatory imbalance leading to the deposition of minerals in the kidney. In contrast to deformities, NC is one of the main culprits of increased mortality in juvenile salmonid production (Wiik-Nielsen, 2023). The annual fish health report also states that NC is the second highest factor leading to reduced growth and poor welfare and tops the list for conditions with an increased incidence (Wiik-Nielsen, 2023).

The Ca:P ratio of Atlantic salmon varies according to life stage and environment. Smolts migrating downstream to the ocean were found to have a significantly higher concentration of phosphorus than adults migrating upstream to spawn (Baeverfjord *et al.*, 2019). This suggests that proper mineralisation is essential for successful seawater transfer, and the phosphorus status of fish at transfer may affect their performance in the seawater phase. Investigating the phosphorus levels in salmon before seawater transfer and the amount of phosphorus associated with the structural bone matrix (as indicated by the Ca:P ratio) is thus critical (Baeverfjord *et al.*, 2019). In this trial, nephrocalcinosis was a co-occurring observation when sampling with qualitative or semi-quantitative scoring. Nevertheless, the low P diets differed from the other diets. The prevalence of NC (score 1) was highest in the low P diets Plp (56%) and Pta (63%) compared to the other diets (25-31%). This finding might be related to the Ca:P ratio in the feeds, where Plp (0.85) and Pta (0.77) were much lower than the other diets (1.0-1.1) and suggest a sub-optimal balance of the essential structural elements. As reviewed in Lall and Lewis-McCrea. (2007), increasing or decreasing the Ca:P ratio interferes with the absorption and excretion of P and Ca.

The concentration of Ca in the low P feeds (1.0-1.1%) was significantly lower than in the high P feeds (1.5-1.7%). The importance of dietary Ca in salmonid nutrition has been largely overlooked despite Ca being essential in forming bones and body ion homeostasis. Studies have indicated that low levels of Ca can affect tissue mineral concentrations in fast-growing salmonids (Antony Jesu Prabhu, Schrama and Kaushik, 2016). Oxalates are antinutritional factors abundantly found in plant and animal sources and are formed naturally in the body (Prakash Sharma *et al.*, 2013). Increased dietary ingestion can lead to NC, as seen in human studies (Bhasin, 2015). Oxalates are the primary salts that form NC crystals in fish, and thus excretion of oxalate via the GI tract is important to minimise the risk of NC in fish (Whittamore,

2020). Accordingly, an imbalanced Ca:P ratio can promote NC by forming calcium oxalate complexes in the kidney.

In addition to lower Ca levels, Plp and Pta had a lower total P content of 1.3% as opposed to the other experimental diets with 1.5%. However, apart from Amc, available P were comparable between the experimental diets. The Ca:P ratio in the different tissues did not differ much, which is expected since this is mainly for the homeostasis in the body in terms of how the Ca and P are utilised and excreted. Nevertheless, the vertebral Ca:P ratio in Pta (2.0) was higher than all other diets (1.94-1.95); however, only significantly different from Plp. Interestingly, the molar Ca:P ratio of vertebrae in fish fed Pta (1.54) was higher than in all other diets (1.50). Nevertheless, all ratios were within the reported range (1.25-1.62) for Atlantic salmon, as reviewed in (Baeverfjord *et al.,* 2019). Despite P requirements being met, the ratio seems insufficient due to Ca levels which may impact the fish's ability to mineralise appropriately. This might be the reason for the lower mechanical strength in the low P diets (Pta and Plp), suggesting that a higher Ca:P ratio might negatively impact bone health. Concordantly, it could provide information on the necessity of Ca supplementation, despite NRC (2011) only recommending supplementation when reared in Ca-free water. However, to prove that Ca is the reason for the increased prevalence of NC, you would need to do a study with Ca-free water by precipitating all the Ca in the water. However, in practical terms, this is very experimental and cannot be replicated on a bigger scale. These findings suggest that an imbalance between Ca and P may lead to a higher prevalence of NC.

4.5. Adding phytase: does it improve P nutrition and bone health in low-P feeds?

Regarding P availability due to phytase, no significant effect was found in Pta compared to Plp. Fish were fed diets containing P at relatively high levels, which would provide a reasonable amount of available P to meet requirements, thereby limiting the response to phytase. Available P was higher than 0.8% in both diets, which were excessive in terms of dissolved P waste output (Dalsgaard *et al.,* 2009). The observation made in the present study is in line with remarks made by Sajjadi and Carter (2004) and Greiling *et al.* (2019), where a phytase supplement did not increase P digestibility when inorganic P was also supplemented. This does not mean that the phytase did not work in this study; however, it was less effective in Atlantic salmon under these settings of ingredients. On the contrary, a study by Adeshina *et al.* (2023) on Nile tilapia found that a microbial phytase could completely replace feed P with no negative impacts. The authors of this study did not look at bone health parameters and mineralisation but found no adverse effects on growth, gut morphometry, blood profile, antioxidant activities, and immunological

response. Whether the same result would be found in Atlantic salmon fed more extreme diets is an open question. An earlier study on dietary phytase for rainbow trout at 11°C demonstrated the potential to improve the nutritive value of canola protein concentrate and availability of phytate-P with the highest dose of 4500 FTU/kg (Forster *et al.*, 1999). However, a recent study on rainbow trout fed dietary phytase found no effects on growth and nutrient digestibility at 11°C (Yigit *et al.*, 2018). The study by Lee *et al.* (2020) found significant improvements in the utilisation and excretion of P and N when rainbow trout were given a supplemental low-temperature (11°C) E. coli phytase. There is evidence that the digestibility of P differs between rainbow trout and Atlantic salmon when fed the same diet (Glencross *et al.*, 2004; Greiling *et al.*, 2019); however, the objective of this study was to evaluate the effect of the same phytase in Atlantic salmon.

A study by Dalsgaard *et al.* (2009) looked at the effect of supplemented fungal phytase on performance and P availability in rainbow trout at 11°C. The authors found that dissolved P waste output from fish fed a phytase-supplemented diet containing 0.71% available P was significantly higher and suggested that the P requirement was already reached. Additionally, they found an even higher increase in dissolved P waste output with diets containing 0.81% available P, suggesting that the P requirement was exceeded. Nevertheless, whether increased dissolved P impacts salmonid bone health in RAS remains an open question.

The phytase influenced the mineral status of Zn in freshwater. The concentration of Zn in the fish scales was significantly higher than in all other diet groups, indicating a positive effect of the phytase. This aligns with Bacchetta *et al.* (2021) findings, investigating the impact of phytase inclusion (4000 PU/kg) in juvenile pacú fed a plant-based diet. Phytase inclusion led to a higher Zn content in the whole body; however, no difference in growth, bone weight, ash, Ca, or P. The experimental feeds contained similar levels of Zn (160-170 mg/kg), which suggests a clear improvement of dietary Zn utilisation by phytase addition. However, we did not see the same effect on the AAC of Zn; nevertheless, the mean plasma Zn in Pta fed fish was numerically higher than Plp (286 vs 264 µmol/L). In a recent study by Philip *et al.* (2023), the authors found a preventive effect of supplementing high levels of Zn and Se on the incidence and severity of vertebral deformities at 12°C, providing evidence for the role in mineralisation and bone health in salmonids. The maximum current limit for Zn in the diet is 180 mg/kg for complete feeds for salmonids in the EU, and EFSA has proposed to lower it further to 150 mg/kg feed. If the proposed reduction to 150 mg Zn/kg feed by EFSA is implemented, findings

in the present study suggest that including phytase could improve Zn mineralisation. Similar to Zn, the concentration of scale Cu was higher in fish fed phytase (Pta) than in the diet without (Plp), indicating that phytase can improve micro-mineral status.

Selenium is an essential micromineral required for normal development and antioxidant protection. Fishmeal is considered an important source of Se, and replacing fishmeal with plant-derived ingredients reduces Se content in fish feeds (Antony Jesu Prabhu *et al.*, 2020). The present maximum limit for Se in the diet is 0.5 mg/kg for complete feeds for salmonids in the EU; however, Antony Jesu Prabhu *et al.* (2020) found that the requirement is approx. 0.7 mg/kg. In the feeds, the Se concentration of Alp, Amc, and Pro was 0.8, 0.9, and 0.9 mg/kg, respectively.

In contrast, the diet groups Plp and Pta, which contained the least amount of FM, had Se concentrations of 0.6 mg/kg. This difference in feed Se resulted in low Se status of the tissues analysed; however, no difference in the availability was observed. Interestingly, the effects persisted in seawater in both scales and the whole body. The micromineral content of the plasma in seawater was not analysed; however, the same result would likely be found.

5. Hypotheses

$H0_1$: Substituting half of MAP with MCP in the feed for Atlantic salmon pre-smolt reared in a RAS does not affect the mineral status and bone health, **is rejected**. **$H1_1$:** Substituting half of MAP with MCP in the feed for Atlantic salmon pre-smolt reared in a RAS affects mineral status and bone health, **is accepted**.

$H0_2$: Including an enhanced low-temperature phytase (*Escherichia coli*) in the feed for Atlantic salmon pre-smolt reared in a RAS does not affect the mineral status and bone health, **is rejected**. **$H1_2$:** Including an enhanced low-temperature phytase (*Escherichia coli*) in the feed for Atlantic salmon pre-smolt reared in a RAS affects the mineral status and bone health, **is accepted**.

6. Conclusions

All the experimental diets supported comparable growth and tissue P or Ca status in Atlantic salmon. The availability of P was reduced in fish fed the diet Amc, inducing twice as many vertebral deformities. Fish fed the diet with phytase inclusion did not improve P availability or status in low-P diets; however, it influenced the mineralisation of Zn and Cu in the scales. In the low-P diets, despite available P levels being met, the low Ca:P ratio in the feeds affected the occurrence of nephrocalcinosis and the mechanical properties of the vertebrae.

7. Future perspectives

Further research is warranted to evaluate the efficiency of phytase inclusion in salmonid feeds. The results of this experiment show that this phytase is effective in a low-temperature environment and Atlantic salmon and highlights the need for further studies on:

1. The effect of phytase on mineral nutrition in a phosphorus deficient diet, increasing the bioavailability of plant-derived ingredients, reducing the amount of supplemental inorganic feed phosphates, and lessening the environmental impact of P to create more sustainable freshwater feeds.
2. The effect of increased dissolved phosphate on bone health in a RAS, and the impact of tilting the balance between Ca and P in the feeds.

8. References

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