ARRAINA
Advanced Research Initiatives for
Nutrition & Aquaculture

UNDERSTANDING VITAMINS, MINERALS AND OTHER NUTRIENTS IN FISH FEED DIETS BASED ON PLANT DERIVED INGREDIENTS

TECHNICAL BOOKLET 2016

AQUACULTURE NUTRIENT REQUIREMENTS

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INTRODUCTION

About This Booklet

This booklet is part of a series of three technical booklets on the nutrition and feed of fish farmed in Europe and is produced under the framework of the European Commission funded Seventh Framework Programme (FP7) project **ARRAINA (Advanced Research Initiatives for Nutrition & Aquaculture)**. These booklets try to address a wide range of stakeholders and society in general, in order to raise awareness on the science based knowledge supporting the development of high-quality, safe and environmentally sustainable aquaculture feeds.

This booklet is specifically aimed at **aquaculture feed producers**, but also targets other industrial segments (e.g. fish farmers, feed additive companies, retailers) and individuals interested in gaining further knowledge on the use of raw materials that are currently used in the feeds of farmed fish. The species in focus in the **ARRAINA** project are Atlantic salmon, rainbow trout, common carp, European sea bass and gilthead sea bream.

The rationale behind the development of this booklet is to provide a user-friendly resource for aquaculture feed producers, which will allow them to change premix additions, and the balance of vitamins, minerals and other specific nutrients, to fulfil the requirements of fish for growth and health.

The information provided in this booklet could serve as common ground for clearer and more transparent communication across the aquaculture production chain.

The first **ARRAINA** technical booklet was published in 2015, with a focus on feed ingredients in aquaculture, and is available on **www.arraina.eu**.

About Aquaculture Feeds in Europe

Today's fish feeds are produced from a large variety of ingredients. These have different nutritional properties. During feed production, the ingredient characteristics can cause considerable variation in the quality of finished feeds, e.g. affecting the bioavailability of the nutrients present. Sustainable fish feeds, manufactured based on solid scientific knowledge and reliable raw materials, can contribute to ensuring aquaculture as an efficient, environmentally sustainable and fish welfare friendly industry that produces highly nutritious quality food for humans.

The nutritional value of the feed will be affected by the ingredients used and the balance of micronutrients in the premix added. This refers to changes in the nutrient profile in terms of amino acids, fatty acids, vitamins and minerals and in nutrient availability, which impact on feed efficiency, growth and fish welfare. While information on the nutritional value of a given ingredient is available, and nutrient recommendations supporting maximum growth exist, these are developed based on purified and semi-purified diets and determined in fish during early life-stages.

The **ARRAINA** project has focused on critical windows in the production cycle to secure a sufficient and well-balanced diet during the total production period. One of the aims of the **ARRAINA** project is to establish new recommendations for Atlantic salmon, rainbow trout, common carp, gilthead sea bream and European seabass at critical windows during the production cycle.

About the ARRAINA Project

Fish feeds for cultured fish are typically based to some extent on fishmeal and fish oil derived from capture fisheries. There is increasing pressure on these raw materials due to growing demands from a variety of users, including the expanding aquaculture sector and human health market (e.g. fish oil food supplements). Hence, it is recognised that the sustainability, competitiveness and further development of aquaculture strongly depends on the replacement of fishmeal and fish oil with alternative ingredients such as plant-based feeds. **ARRAINA** has been responding to this need by measuring the long-term effects these changes in diet will have on the full life cycle of fish for which little is presently known.

By developing applied tools and solutions of technological interest in collaboration with SMEs, **ARRAINA** also intends to further strengthen the links between the scientific community and the European fish feed industry, contributing towards increasing the productivity and performance of the aquaculture sector.

For general information on the project, the project factsheet can be downloaded from the **ARRAINA** website, **www.arraina.eu** as a PDF. For more information, please contact the **ARRAINA** Project Coordinator, Dr Sadasivam Kaushik (**kaushik@st-pee.inra.fr**).

MICRONUTRIENTS IN PLANT BASED DIETS

Vitamins and minerals are nutrients which are essential for normal fish growth, reproduction and general health status, even when required only in small amounts. Fish cannot synthesise vitamins themselves and therefore need an adequate amount in their diet to secure robust growth. Similarly, mineral requirement must be met through diet, although some minerals can (in part) be directly absorbed from the aquatic environment through the gills and intestine. Knowledge on the micronutrient requirements of fish is still fragmented. When dietary protein and lipid sources are changed from fish based to plant based ingredients, there is an associated change in the supply of a range of dietary nutrients, especially micronutrients. Large variations are found in vitamin and mineral contents when we compare fishmeal with plant-protein sources. The **ARRAINA** project has a strong focus on identifying minerals, vitamins, amino acids, lipid components (e.g. phospholipids, cholesterol) and other possible fishmeal and fish oil specific nutrients that need to be added to feeds containing high levels of plant products to avoid reduced performance and health status in farmed fish.

The profile of nutrients in the plant-based diet will mirror the nutrient composition of the ingredients used, and be significantly different from a feed based on fishmeal and fish oil. Normally, the micronutrients are added as a premix, based on existing recommendations such as those by United States National Research Council (NRC, 2011). One of the aims of **ARRAINA** was to investigate whether these recommendations had to be amended given the current inclusion level of plant based ingredients in fish feed (Figure 1). For instance, previous projects have shown that Atlantic salmon diets with a high inclusion of plant ingredients have a lower growth rate and increased body lipid compared to fish fed with marine based feeds, and that these effects are partly ameliorated by the addition of extra amino acids and micronutrients. **ARRAINA** had a dedicated section which aimed to identify which nutrients, and in which form, need to be added to plant based diets and also to study the metabolic consequences of changes in nutrient profiles and availability.

Short duration studies were performed during critical windows in the production cycle using practical plant based diets and applying regression design analysis. The design had at least seven graded levels of a nutrient package; from no addition to several times beyond the assumed required levels. Single nutrients and/or groups of nutrients have provided information on new recommendations based on growth, feed utilisation, metabolic responses and early biomarker identification. Further in focus in **ARRAINA** are lipid soluble vitamins, minerals, phospholipids, methionine, taurine and histidine.

Fish species in focus:

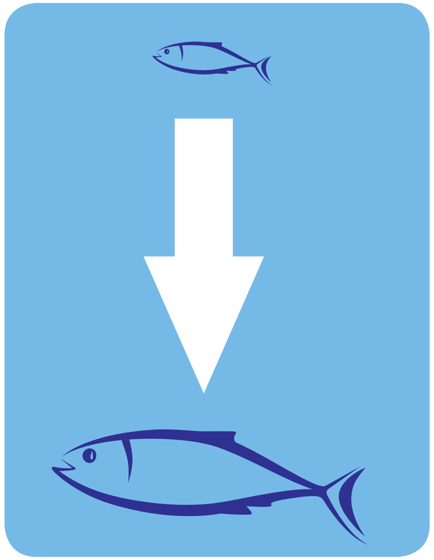
Atlantic salmon (*Salmo salar*)

Common carp (*Cyprinus carpio*)

European seabass (*Dicentrarchus labrax*)

Gilthead sea bream (*Sparus aurata*)

Rainbow trout (*Oncorhynchus mykiss*)



NRC FISH FEED DIETARY RECOMMENDATIONS (2011)

Traditional diet	Plant based diet
Fed FM and FO	Fed PP and VO
Fulfil requirement	Nutrient composition/availability unknown
Firm recommendations	No available recommendations

MOST STUDIES

Based on purified and semi-purified diets with the micronutrient in focus close to 100% available.

Studies are mostly dose-response with one single nutrient varying to establish requirement.

ARRAINA

Looking at critical windows in the production cycle may demand new nutrient profiles for optimum growth and health under practical conditions.

E.g. Phospholipids, sulphur amino acids, minerals, vitamins.

Figure 1. When feed ingredients change from fishmeal (FM) and fish oil (FO), to plant protein (PP) and vegetable oil (VO), **ARRAINA** has proven that there is need for new nutrient additions to maintain growth and fish health.

SULPHUR AMINO ACIDS (METHIONINE)

Methionine is an indispensable amino acid in fish as in other animals. According to NRC (2011), the requirement of juvenile Atlantic salmon for methoionine is about 7 g/kg dry diet. Methionine requirement is often given as total sulphur amino acids (cystine plus methionine). Thus, a 10 to 12.8 g/kg diet depending on the life stages as cysteine may spare methionine but not vice versa (NRC, 2011). Methionine is the first limiting amino acid in most oil seeds. Deficiency of methionine reduces protein growth and may result in development of fatty liver, especially if the animal is also deficient in choline.

Methionine is the main methyl donor in the body. Before methionine can donate its methyl group it needs to be activated by ATP forming S-adenosylmethionine (SAM), which is able to transfer its methyl group to different methyl acceptors requiring methylation. Examples are synthesis of carnitine, creatinine and phosphatidylethanolamine among others. Upon donation of the methyl group, SAM is converted to S-adenosylhomocysteine (SAH), which is rapidly transformed to homocysteine. Homocysteine may be transmethylated to methionine accepting methyl group from betaine, remethylated accepting methyl group from folic acid or trans-sulphurated to cystathionine and further metabolised to cysteine. Cysteine is one of the amino acids of the tripeptide glutathione. Glutathione is an abundant and important endogenous antioxidant in fish and other animals. If not used for synthesis of glutathione, cysteine is metabolised to taurine which is excreted in urine. As well as methionine, taurine may also be low in diets high in plant protein ingredients. Although Atlantic salmon has not lost the ability to transulphurate and produce taurine, the amount produced depends on the availability of dietary methionine. An interaction between the B-vitamins and sulphur amino acid metabolism is evident, since trans-sulphuration requires vitamin B6, while B12 is needed for methionine synthase and remethylation along with the methyl donor folic acid. Diets low in methionine increase liver triacylglycerol (TAG) in fish and as such may have an impact on metabolic health in line with the development of non-alcoholic fatty liver disease (NAFLD) described in rodents and humans.

ARRAINA used soy protein concentrate and pea protein as protein sources and produced diets increasing in methionine from 7 to 8.5 g/kg and taurine from 0.7 to 5.92 g/kg diet in a nutrient package along with vitamins and minerals. Results on growth and deposition revealed an increase in protein retention and lowered liver lipid at a dietary level around 1.5 times the NRC recommendations (2011). This could, in part, be attributed to the increased methionine, from 7 g/kg to 8 g/kg, as **ARRAINA** previously found that salmon fed diets low in methionine had reduced growth due to reduced protein growth (Espe *et al.* 2014) where methionine ranged from 7 to 11.4 g/kg diet.

PHOSPHOLIPIDS

It is well established that dietary intact phospholipids (PL) can improve growth performance of several freshwater and marine fish species (Coutteau *et al.*, 1997). The PL requirement appears to be restricted to early life stages, and the specific evidence supporting a requirement for dietary intact PL in fish is improved growth in both larvae and early juveniles, increased survival rates and decreased incidence of malformation in larvae, and perhaps increased stress resistance (Tocher *et al.,* 2008). In Atlantic salmon, early studies suggested there was a requirement for dietary PL based on growth performance and survival in parr up to 1.7 g initial weight, whereas in fish of 7.5 g initial weight, no requirement was observed (Poston, 1990). However, it was unclear from these studies what the precise level of requirement was or if different PL classes had differential effects in satisfying the requirement. Furthermore, the biochemical mechanism underpinning PL requirements in early life stages of fish is unknown.

ARRAINA performed a study that aimed to better define the effects of dietary PL in Atlantic salmon including quantitative and qualitative requirements, period of requirement and biochemical/molecular mechanisms of requirement (Taylor *et al.,* 2015). Fry were fed diets supplemented with krill oil or soybean lecithin at five levels (non-supplemented, 2.6, 3.2, 3.6 and 4.2 % total PL) and fish were sampled at various times up to smolt. There was a positive correlation (r^2 = 0.59 to 0.72) between survival and dietary PL supplementation. Growth was improved by increased dietary supply of PL with highest growth achieved in fish fed krill PL at 2.6 % and soy lecithin at 3.6 % that reflected phosphatidylcholine (PC) contents of the PL preparations. Dietary PL only improved growth in fish up to 2.5 g, and had no growth-promoting effect from 2.5 g to smolt. Intestinal steatosis was observed in 2.5 g fish fed the non-supplemented diet and lower levels of soy lecithin, whereas it was absent from 2.5 g fish fed krill oil and higher levels of soy lecithin, and fish at all later stages. Prevalence of vertebral deformities was low but was reduced by increasing dietary PL with krill oil generally being more effective. Thus, the study confirmed the requirement for dietary PL in Atlantic salmon and better defined the level and period of requirement.

The molecular mechanism underlying PL requirement in Atlantic salmon was also investigated in **ARRAINA** with a dedicated qPCR study targeting PL biosynthetic genes in specific tissues (Carmona *et al.,* 2015). Liver and intestine were collected from fry (required PL) and parr (no requirement for dietary PL) fed the non-supplemented PL diet. In-silico analysis and cloning demonstrated that Atlantic salmon possess a full set of enzymes for the endogenous production of PL. The major genes of PC, phosphatidylethanolamine (PE) and phosphatidylinositol (PI) biosynthesis displayed lower expression in the intestine during the early developmental stage (fry). The data were consistent with the hypothesis that the intestine of salmon is immature at the early developmental stage with limited capacity for endogenous PL biosynthesis, and that intact PC, PE and PI were required in the diet at this stage. PC and sphingomyelin (SM) constitute the predominant PL in chylomicrons, involved in the transport of dietary lipids from the intestine to the rest of the body. As SM can be produced from PC in the intestine of fry, this suggests that supplementation of dietary PC alone during early developmental stages would be sufficient to promote chylomicron formation. This would support effective transport of dietary lipids, including PL precursors, from intestine to liver. There, biosynthesis of PE, PI and phosphatidylserine was not compromised as in the intestine facilitating efficient utilisation of dietary energy and the endogenous production of membrane PL for rapid growth and development.

B-VITAMINS

A shift in dietary ingredients may also cause changes in levels and bioavailability of B-vitamins. Many feed ingredients of plant origin replacing fishmeal contain lower levels of B-vitamins or chemical forms with lower bioavailability, potentially causing deficiencies (Hansen *et al.* 2015). All B-vitamin deficiencies result in reduced growth and feed intake (anorexia), and most B-vitamin deficiency symptoms include anaemia (except thiamine) and changes in body colouration (Figure 2). Even though some primary deficiency signs may indicate which B-vitamins are deficient, such deficiencies are not easily diagnosed (Figure 2).

Thiamine (B1) acts as a coenzyme in energy metabolism. Organs using glucose as an energy source are sensitive to thiamine deficiency: the nervous system, gonads, eye lens and red blood cells. Typical deficiency signs are loss of appetite, anorexia, reduced growth, and abnormal swimming behaviour. Thiamine is a coenzyme for the enzyme transketolase, an enzyme in the pentose phosphate pathway and tissue. Thiamine pyrophosphate concentration together with thiamine level, has been shown to be a reliable biomarker for thiamine status (Masumoto *et al.,* 1987).

Riboflavin (B2) is one of the coenzymes essential for energy metabolism, such as oxidases and reductases, and acts in the metabolism of protein, lipid and carbohydrates. Specific symptoms of deficiency involve cataract and photophobia. In riboflavin requirement studies, besides tissue riboflavin status, the activity of hepatic D-amino acid oxidase (D-AAO) appears to be a good marker of adequacy or deficiency (Woodward, 1994).

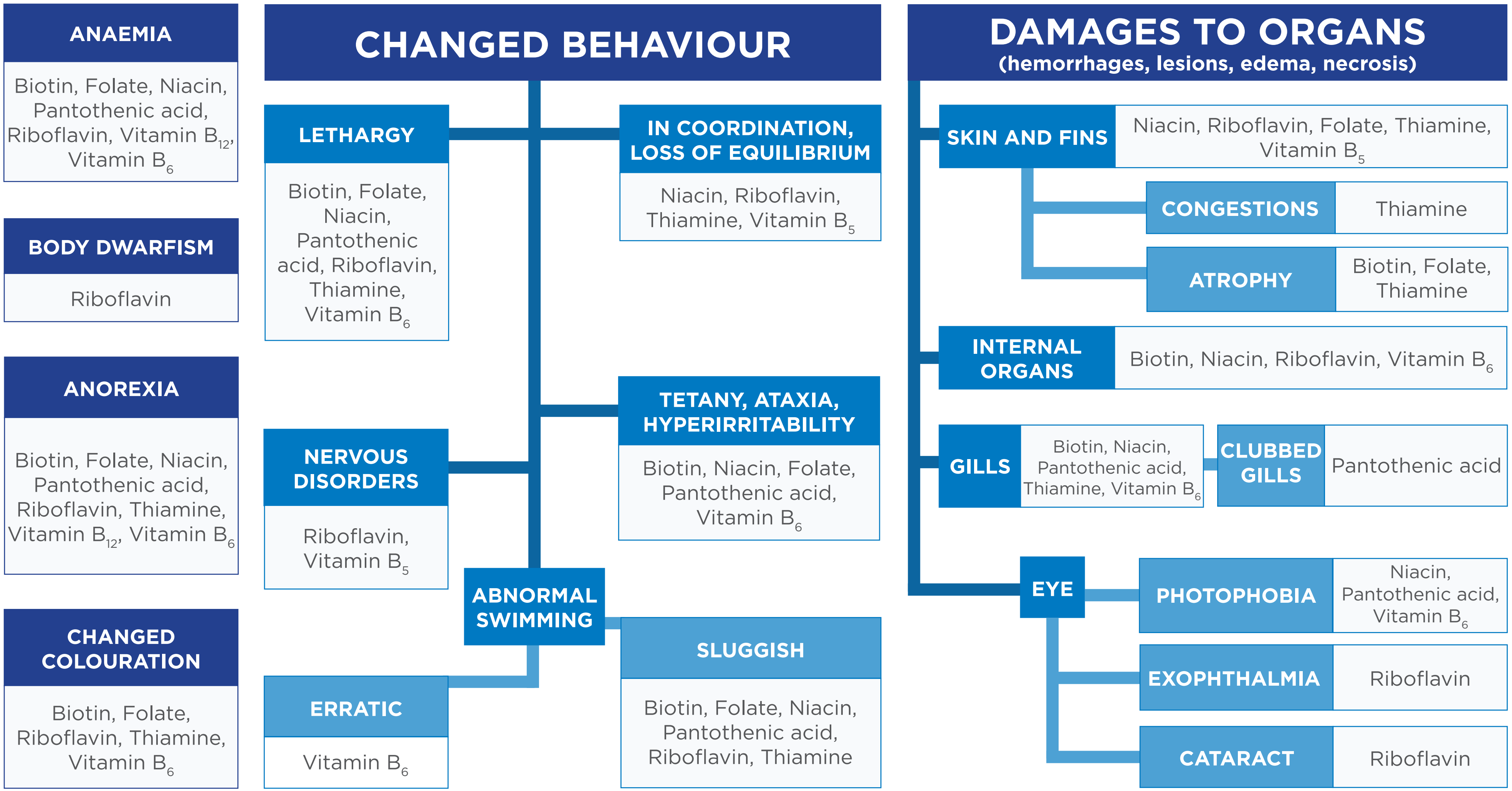


Figure 2. Clinical deficiency symptoms of B-vitamin deficiency.

Niacin (B3) is part of NAD and NADPH and transfers H+ and e- in redox reactions in the metabolism of carbohydrates, lipids and amino acids. Whether fish are capable of synthesising NAD from tryptophan is not clear (Woodward, 1994). Deficiency results in damages to skin and fins (haemorrhages, lesions, erosion and dermopathy). Hepatic tissue level has been used together with growth to determine requirement.

Pantothenic acid (B5) transfers acetyl and acyl groups in energy metabolism. Tissues rich in mitochondria (rapid cell division tissues) are sensitive to deficiency (e.g. gills). Hyperplasia of the epithelial cells in gills, causing clubbed gills, is a typical B5 deficiency sign (Wilson *et al.,* 1983; Hansen *et al.,* 2015).

Pyridoxin (B6) functions as a phosphorylated coenzyme in transaminases (amino acid metabolism), as part of transferases in lipid metabolism, and is needed for the production of neurotransmitters. Symptoms of deficiency are many, diverse and severe. **ARRAINA** have used muscle aspartate amino acid transferase (ASAT) as an early biomarker for arriving at recommendations on B6 levels (Albrektsen et al., 1993).

Biotin (B7) is a coenzyme and an intermediate carrier of CO_2 in carboxylation-decarboxylation reactions (fatty acid and amino acid metabolism). NRC (2011) concludes that due to very low identified requirement of this vitamin for most fish, the ingredients are normally sufficient in biotin. If biotin deficiency is identified in fish, it is most often coupled to lowered digestibility function and reduced growth. **ARRAINA** results confirmed that there was no need to add biotin as a part of the premix; the dietary ingredients supplied the required levels.

Folate (B9) acts in the sulphur amino acid metabolism as a general methyl donor in remethylation of homocysteine to methionine, and in the biosynthesis of purines and pyrimidines. Deficiency results in loss of appetite, anorexia and reduced growth. Folate deficiency, in close interaction with lack of sufficient vitamin B12, leads to megaloblastic anaemia.

Cobalamin (B12) is a coenzyme in the remethylation of homocystein to methionine, and the production of succinyl-CoA. Deficiency results in anorexia and anaemia.

Based on growth, nutrient retention and metabolic biomarkers used in studies undertaken as part of **ARRAINA**, NRC (2011) recommendations existing for rainbow trout (*O. mykiss*) for **folate**, **thiamine** and **biotin** were identified to be valid also for Atlantic salmon (*S. salar*), while **pantothenic acid, niacin, cobalamin** and **pyridoxine** needed to be increased above the NRC (2011) recommendations. Detailed information on these studies will be made available as publications after peer-review.

VITAMIN C

Vitamin C / ascorbic acid (Figure 3) acts as a co-factor in at least eight reactions, many of them involved in collagen synthesis. The main symptom of vitamin C deficiency in fish is deformity of the vertebra (scoliosis and lordosis) caused by inhibition of collagen synthesis which can be measured as reduced concentration of hydroxyl-proline. The vitamin also acts as a water-soluble antioxidant, scavenging reactive oxygen species, and interacts with vitamin E in the protection of the organism against lipid peroxidation. Vitamin C is unstable and its free form is rapidly lost if present in feed or feed ingredients. Therefore, ascorbate for use in feed is most often esterified to phosphate in mono- or poly phosphates and thereby protected against oxidation.

In studies with Atlantic salmon, no gross symptoms of vitamin C deficiency were identified, even though vitamin C levels in the diets without addition of micronutrients were below the recommended levels for salmon (20 mg/kg, NRC, 2011). Based on whole body concentrations and retention, and responses in the antioxidant system, vitamin C should be added to Atlantic salmon diets at >100 mg/kg.

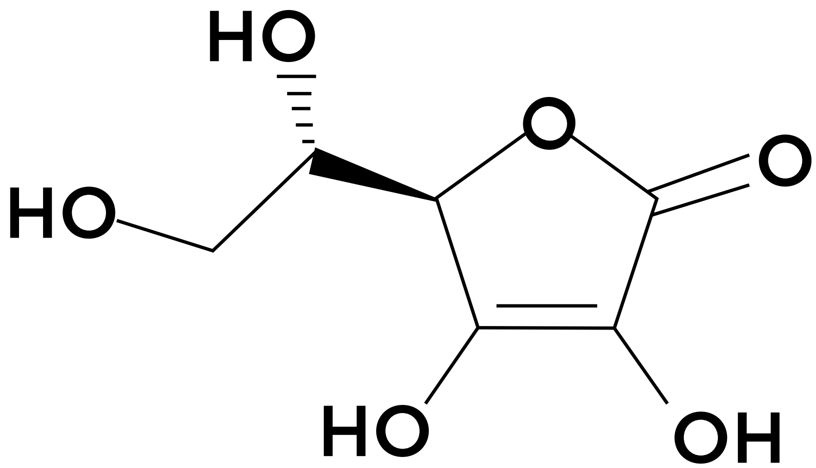


Figure 3. Ascorbic acid (vitamin C)

LIPID SOLUBLE VITAMINS

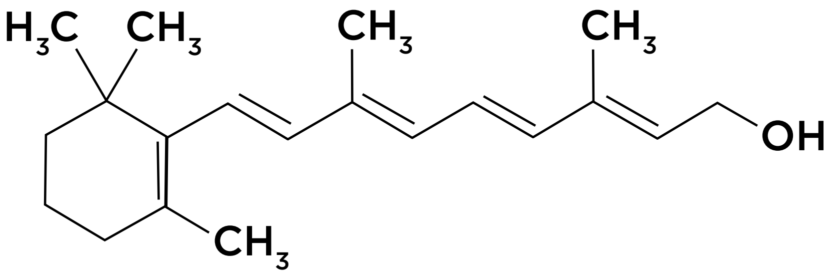
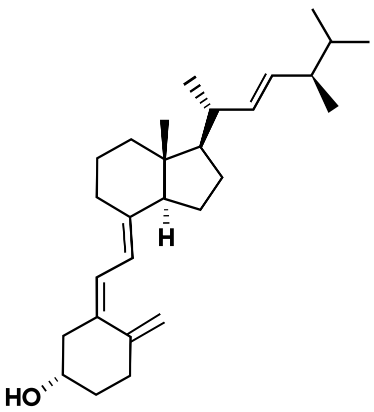


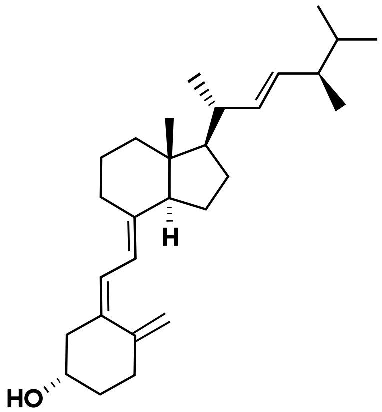
Figure 4. Retinol

Vitamin A (VA) (Figure 4) plays an important role in visual processes, stimulating new cell growth and aiding in maintaining resistance to infection. VA in diets high in marine ingredients is mainly derived from preformed retinyl esters or from xanthophylls (e.g. astaxanthin). Plant based ingredients contain carotenoids which may be converted into VA by the fish. Atlantic salmon and many other fish species utilise astaxanthin and a variety of carotenoids as a provitamin A source, however the efficiency of this conversion is unclear.

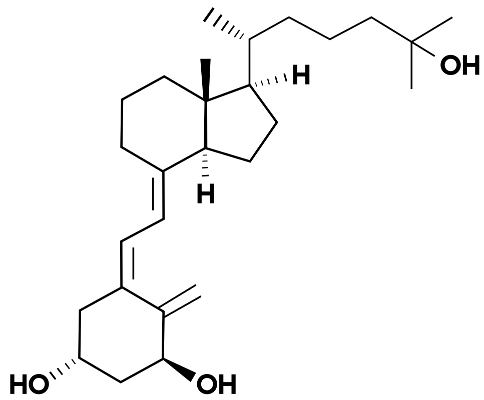
Vitamin D (Figure 5) in the form of the active metabolite 1,25-dihydroxycholecalciferol [1,25(OH)2D3] is involved in intestinal Ca2+ and phosphorus absorption, renal Ca2+ reabsorption and bone remodelling. Vitamin D is also believed to play an important role in the immune response. Fish do not synthesise vitamin D and are fully dependent on dietary sources to meet their requirement. In all fish species vitamin D3 is the primary form. It has been suggested that this form has a higher bioavailability for fish, resulting in a higher uptake from the diet of vitamin D3 compared with vitamin D2. Also, the binding of vitamin D2 to transport proteins in plasma appears to be lower than vitamin D3, which could result in an increased clearance of vitamin D2 from the plasma. Vitamin D in plant ingredients is always in the form of vitamin D2. No new recommendations can be made yet on the best level of vitamin D from the **ARRAINA** project.



Vitamin D2 (Ergocalciferol)



Vitamin D3 (Cholecalciferol)



1,25-dihydroxycholecalciferol (Calcitriol)

Figure 5. Vitamin D

Vitamin E (Figure 6) denotes a group of compounds, the tocopherols and tocotrienols, where α-tocopherol (α-TOH) has the highest biological activity. Plant oils contain high levels and different compositions of tocopherols and tocotrienols, therefore plant-based diets often contain appreciable amounts of vitamin E. However, natural vitamin E is unstable and is degraded in the feed production process, while added vitamin E most often is in the form of α-tocopheryl acetate (α-TOAc), protected against oxidation. Vitamin E inhibits lipid peroxidation in the body, transferring radicals to vitamin C, which pass them further so that they can be neutralised by energy metabolism. Anaemia is the first apparent symptom of vitamin E deficiency in salmon, secondary to oxidation of red blood cell membrane lipids.

In the studies with Atlantic salmon, the basal diet had α-TOH concentrations of 50 and 76 mg/kg respectively and also contained other tocopherols, while the minimum requirement is given as 60 mg/kg α-TOH (NRC, 2011). Although no symptoms of vitamin E deficiency were detected in the fish, dietary α-TOAc levels of >100 mg/kg are recommended, based on growth, body stores and markers of redox system in the fish body.

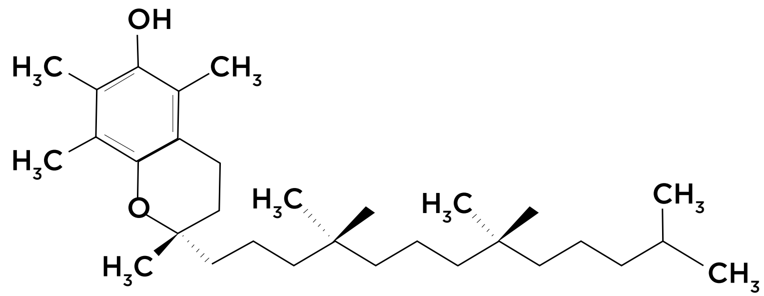


Figure 6. Vitamin E

Vitamin K plays vital roles in blood coagulation and aids bone mineralisation in fish, but the suggested minimum requirement varies considerably depending on the source of vitamin K used. Vitamin K occurs naturally as phylloquinone (vitamin K1) found in plant ingredients) and menaquinone (vitamin K2) found in bacteria and ingredients of animal origin. In addition, there is a synthetic provitamin menadione (vitamin K3), primarily used as a vitamin K source in animal feed. Menadione is unstable during feed processing and storage and the dietary content may reach critically low levels. Recent studies also question the availability of menadione in feed for salmonids. Some vegetable oils used as alternatives to fish oil, such as soybean oil (2.7 mg/kg) or rapeseed oil (1.1 mg/kg), may contain higher levels of natural vitamin K forms compared to marine ingredients (0.01 to 1.0 mg/kg). To reduce unnecessary vitamin supplementation and feed costs, information is needed on the concentration and bioavailability of naturally occurring vitamins in the feed ingredients used.

MINERALS

Selenium is necessary for redox regulation and for the protection of the body against oxidative stress, although very high levels can be toxic. Selenium is also integral to selenocysteine, where the sulphur atom in cysteine is replaced by selenium. Selenocysteine is built into selenoproteins, many of which participate in redox reactions in the body. Examples are the glutathione peroxidase and thioredoxine reductase protein families and SelenoproteinP, a transport protein for selenium. Studies undertaken as part of **ARRAINA** confirmed that there is a linear relationship between dietary and whole body selenium concentrations in Atlantic salmon. It is difficult to find good biomarkers for fulfilment of the selenium requirement in fish. There is not sufficient data to recommend changes in minimum dietary concentrations from NRC (2011) of 0.15 mg/kg for salmon, 0.35 mg/kg more generally for fish.

Manganese (Mn) functions as a cofactor, activator or integrated part of metalloenzymes and takes part in intermediary metabolism and antioxidation. Non-specific Mn-activated enzymes include kinases, transferases, hydrolases, and decarboxylases. Mn-containing metalloenzymes include arginase, pyruvate carboxylase and superoxide dismutase. Manganese levels in some plant ingredients can be high. In the **ARRAINA** experiments on Atlantic salmon, the manganese levels in the plant ingredients were sufficient to cover the manganese requirement (Figure 7).

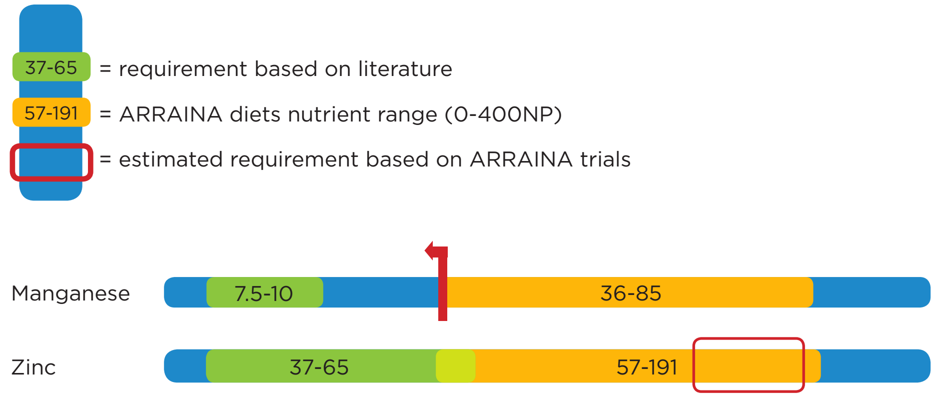


Figure 7. Change in requirement (mg/kg) for manganese and zinc based on **ARRAINA** regression trial for Atlantic salmon (*S. salar*) in seawater; recommended level for plant based diet estimated by red arrow (Mn), and red frame (Zn).

Zinc (Zn): The essential function of zinc is based on its role as an integral constituent of a number of metalloenzymes (enzyme proteins containing metal ions), e.g. carbonic anhydrase, alkaline phosphatase, carboxypeptidases, cytosolic superoxide dismutase, and as a catalyst for regulating the activity of specific Zn-dependent enzymes. As such, Zn is important for metabolic processes of carbohydrates, lipids and proteins. Fish have a dietary need for zinc, but are also able to take up zinc directly from the water through their gills.

In the **ARRAINA** experiments on Atlantic salmon there was a clear difference in zinc requirement of freshwater and seawater fish. Whilst the freshwater fish requirement was in line with the assumed requirement, the seawater fish had a much higher requirement for zinc.

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