Opinion of the Panel on Animal Feed of the Norwegian Scientific Committee for Food Safety

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Criteria for safe use of plant ingredients in diets for aquacultured fish

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ASSESSED BY

The report from the *ad hoc* group has been evaluated and approved by the Panel on Animal Feed of VKM.

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SUMMARY

The Norwegian Food Safety Authority (Mattilsynet) asked the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet) to assess if the criteria for safe use of plant ingredients in diets for aquacultured fish fulfil the Feed regulative §7 to "not induce negative health effects in the animal", and in this context aquacultured fish. The use of feed ingredients of both plant and animal origin is set by the regulation "Forskrift 7. November 2002 nr 1290", and amendments. The objective of the regulation is to protect animals, consumers and the environment. For animals, the feed shall not pose a risk, or danger, to their health.

Aspects to be assessed were whether the changes in fish diet ingredient composition seen in recent years with high levels of plant ingredients, plus additions of immunostimulants, would in any manner challenge fish health and if any ingredient should be limited due to its negative effect, or induce any long-term negative effect. "Long-term" here extends beyond normal production time for consumption, e.g. when substances that might affect fish health are included in broodstock diets. Atlantic salmon (*Salmo salar*), rainbow trout (*Onchorhyncus mykiss*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) should especially be addressed. However, since all life stages should be included, especially broodstock, and also possible long-term effects, and literature on these for the requested species is scarce, the assessment mentions studies on other species when relevant.

With the exception of full-fat and extracted soybean meal for salmonids, substituting at least part of the fishmeal fraction of aquafeeds with individual plant ingredients is promising, at least in the short to medium term. Indeed in some cases, diets containing up to 20% inclusion level of high-quality plant protein sources have resulted in better nutrient digestibility and growth parameters than the fishmeal-based control diets. When substituting fishmeal with plant ingredients, however, it is necessary to balance the diets regarding limiting amino acids and minerals. Adding plant proteins to fish diets result in the introduction of anti-nutritional factors. There is an urgent need to investigate consequences of various anti-nutritional factors, individually and in combinations, to nutrient digestibility, utilization and metabolism as well as to intestinal function, structure, defence mechanisms and microbiota. Long-term effects also merit investigation. This will aid in the ability to predict how a newly introduced plant ingredient as well as combinations of plant ingredients may affect the fish and identify steps needed to avoid adverse health effects.

As many of the potential disadvantages of using plant oils in salmonid diets are related to either very high levels of n-6 PUFA (most available oils) or very high levels of linseed oil, it would be recommended that mixtures of plant oils should be used as feed inclusions. By adjusting the ratio of n-6 and n-3 the level of eicosanoids can be controlled. By including palm oil, potential problems in lipid digestibility and transport can be controlled. A standard inclusion of soybean lecithin may also be advisory. These and other variants of mixtures of oil sources have been explored in recent years with some success in salmonid fish. Such mixtures do not seem to be necessary for marine fish.

Modern finfish aquaculture faces problems such as bone and skeletal deformities, cataracts, heart disorders, unspecific ulceration and various digestive disorders including intestinal colic in Atlantic cod, gastric dilatation (bloat) in rainbow trout, and intestinal tumours, at low

incidence, in Atlantic salmon broodstock. Most of the mentioned problems have been related to malnutrition, feed, intensive growth and/or unfavourable environmental conditions. The disorders are often not lethal, but may imply a fish welfare problem and increase the susceptibility to secondary disorders and infectious diseases. Major changes in feed composition and feed ingredients may increase the risk for such production-related disorders in intensive fish farming. Care should be taken when choosing plant alternatives, both types and qualities, to prevent nutrition-related diseases such as skeletal deformities, cataracts, heart conditions, and other, unspecific symptoms.

The change from marine- to plant-based diet ingredients, results in changed profile and content of undesirable substances. The list of undesirable substances included in the feed legislation is, in general, sufficient, but it should be considered to include pesticides in use today and more of the mycotoxins. Currently only aflatoxin B_1 is included, while only recommendations exist for other mycotoxins. Studies of dietary exposure to undesirable substances, e.g. pesticides and mycotoxins, and their toxic effects and toxicokinetics in fish are scarce.

To date, the application of pre- and probiotics for the improvement of aquatic environmental quality and for disease control in aquaculture seems promising; however, the information is limited and sometimes contradictory. Currently there are numerous gaps in existing knowledge about exogenous nucleotide application to fish including various aspects of digestion, absorption, metabolism, and influences on various physiological responses, especially expression of immunogenes and modulation of immunoglobulin production. As limited information is available about the effect of immunostimulants, prebiotics and nucleotides on gut morphology, this topic should be given high priority in future studies.

Heat processing of raw materials and of the complete fish diets may potentially alter nutritional properties of plant materials. However, the negative effects appear to be modest under practical conditions.

KEY WORDS

Plant ingredients, fish feed, plant proteins, fibres, anti-nutritional factors, plant oils, immunostimulatory agents, undesirable substances, genetically modified plants, processing methods, fish growth, health, intestinal function, feed utilization, salmon, rainbow trout, cod, halibut.

1. BACKGROUND

Marine products in feed for fish, such as fish oil and fishmeal, are limited resources globally. Additionally, low market availability impacts on cost. On this basis, for several years the farming and breeding industries have had considerable interest in finding a good replacement for marine-based fodder for farmed fish.

Fish feed has already contained a significant plant component for many years. Amongst the first plant protein feed ingredients which were tested was soybean (full-fat). However, after a while it was shown that when a large amount of full-fat soybean was used in fish feed, unwanted consequences resulted, including reduced fish growth and inflammatory reactions in the intestine. Therefore other plant-derived protein ingredients for feed have been sought as well as alternative processing methods.

Today various plant-derived ingredients are used in fish feed. Wheat is the most widely used ingredient, and is used as a carbohydrate source with the currently most utilised feed technology (extrusion). As a lipid source, both rapeseed oil and some palm oil, among others, are used. As a protein ingredient, among others, the following have been used: soybean (with varying degrees of processing), maize, peas, lupine, and broad beans.

Fish feed also includes ingredients which contain a high quantity of components which are intended to have a positive effect on fish health (preferably an immunostimulatory effect). The regulations, however, prohibit an association being made between claims regarding feed containing this type of component and health, when the effect from the feed is due to feed ingredients and not from approved additives with documented effect. Actual ingredients with such effects include extracts from yeast and from grape pips.

In regulation No. 1290 (7 November 2002) on feed commodities (the Feed Commodity Regulation) §7 *General Requirements for Feed Products,* it is stated that “feed commodities shall be clean, fresh, unadulterated, of satisfactory hygienic quality, and not resulting in a risk (danger) of injury or harm to animals or people, or harm to the environment.” There is no process for approval of feed ingredients (with the exception of genetically modified feed ingredients). However, there is a list of commonly used feed ingredients, with name and description (appendix 2 D of the Feed Commodity Regulation), and additionally in appendix 2 B a list of prohibited ingredients is provided. Furthermore, use of animal proteins is regulated by regulation No. 511 (29 March 2007) regarding the prohibition of use of animal proteins in feed for livestock.

Therefore some ingredients which are not a natural part of the diet of wild fish may have been used in corresponding species of farmed fish without adequate knowledge of the ingredients’ potential for negative health effects on the farmed fish. Amongst others, one might consider that the occurrence of intestinal tumours in breeding fish from three installations from 2005 could have been associated with intake of plant-derived feed ingredients.

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) was requested by the Norwegian Food Safety Authority (Mattilsynet) in the autumn 2007 to assess what should be used as a basis so that plant-derived ingredients in feed can fulfil the requirement in the Feed Commodity Regulation §7 of “not resulting in injury or harm to animals” (in this instance, farmed fish). Specific regulatory requirements that are in place to ensure animal health and public health must in any case be fulfilled; for example, the limits of undesirable substances and feed additives. Furthermore, VKM was particularly requested to identify feed ingredients of plant origin for which long-term use should be limited due to containing components with negative effects with respect to fish health.

The VKM, Panel on Animal Feed appointed an *ad hoc*-group consisting of both VKM members and external experts to perform this task. The *ad hoc*-group has written the background report “Criteria for safe use of plant ingredients in diets for aquacultured fish”. The *ad hoc* group has incorporated comments and suggestions from the Panel on Animal Feed during their work. The report has been evaluated and approved by the Panel of Animal Feed.

Based on the background report the Panel on Animal Feed has performed this risk assessment. All the documentation and references in this risk assessment can be found in the background report as well as a more thorough review of the different topics.

2. INTRODUCTION

On a worldwide scale, fisheries landings remain constant at about 90 million tons of fish whereas aquaculture supplies about 50 million tons and is increasing at a rate around 8% per annum. This growth has resulted in an increased need for specialised compound feedstuffs, estimated at 20 to 25 million tons. Although this is only a small portion of the global animal feed production of around 620 million tons, the specific nature of aquafeeds (e.g. higher protein levels and lower carbohydrate levels than all other animal feeds) will result in special challenges if traditional fishmeal and oil are replaced with plants (e.g. completely different nutrient profiles, and contents of molecules unknown to carnivorous fish in nature). The four species in focus in this risk assessment, Atlantic salmon, rainbow trout, Atlantic halibut and Atlantic cod, are all intensively farmed fish species in Norway, recognised to have high dietary protein requirements, and especially the two salmonids are reared with energy-dense, lipid-rich diets.

Worldwide annual production of fishmeal (about 6 million tons) and fish oil (less than 1 million ton) has remained fairly stable for the last 20 years. Fishmeal and fish oil are produced from designated pelagic fisheries, mainly from Chile, Peru and the Atlantic. Efforts are constantly underway to ensure that the marine fisheries on which fishmeal and fish oil depend remain sustainable and are not over-exploited. Fishmeal and oil are also produced from trimmings, offal and/or by-catch, although to a limited extent. Within the European Union, it is estimated that in 2002 about 33% of the fishmeal produced was manufactured from trimmings from food fish processing (Huntington et al., 2004). No comparable data is available at the global level (FAO 2005).

The change from fishmeal and oil to various plant ingredients or other marine alternatives might imply several metabolic and health challenges for the farmed fish. Although there might not be an immediate, violent reaction to a certain food there might still be slow cumulative adverse effects resulting in overt disease or less than optimal health. This poses a great challenge, since knowledge of these effects is gained slowly and with difficulty, particularly if the causative principles remain unidentified (Liener, 1980). Further, fishmeal and oil are well balanced with regard to protein content, amino acid and fatty acid profiles, essential minerals and some B-vitamins, while plant fatty acid and amino acid profiles deviate greatly from the traditional marine resources. This places plant-derived feedstuffs at a disadvantage to fish-based ingredients in terms of their suitability for use in aquafeeds.

In 2008 fish diets in Norway were based on approximately 55% marine ingredients, the remainder being plant oils, plant proteins, binders, vitamins and antioxidants (FHL, 2008) The change to novel feed ingredients with different nutrient profiles has reinforced the need for updated requirement data, assuming that requirements have changed along with the other advances made in the aquaculture industry.

Plant protein-based diets need to be adjusted for several limiting amino acids, minerals and vitamins, to mimic fishmeal and meet nutritional requirements for the various farmed fish species. Most likely, the requirement data established in the early 1990s with slow-growing fish (NRC, 1993) does not cover the nutrient requirements for optimal fish health today. The improvements over the years in e.g. fish growth rates due to selective breeding and other optimized production practices will most probably not cover the requirements for health, and will not be adjusted to changes in environmental factors, such as climate. As an example, fish health was challenged when plant oils substituted the marine oil fraction in salmon diets (Ruyter et al. 2006; Jordal et al. 2007), despite the fact that the diets contained essential fatty acids from the fishmeal fraction, and the plant-based diets did, according to established requirements in 1993 and earlier, contain all essential nutrients.

In addition to the marked differences in growth rates within a species compared to when requirement data was established (NRC, 1993), large species differences exist and data from studies on one species cannot be implemented with success to another. For example, there exist very different plant protein utilization capacity and tolerance to components present in protein rich plants for the marine cod and halibut, as compared to salmon (Krogdahl et al. 2005; Hansen et al. 2006). This illustrates the need for species-specific data on health implications when introducing new plant-based diets.

Furthermore, with the increased inclusion of plant-based feedstuffs in diets, the intake of anti-nutritional factors (ANFs), including fibre, will increase. The various effects different ANFs have on digestive physiology and ultimately on metabolism will change the utilization of specific nutrients (Francis et al., 2001). This will change the dietary levels of specific nutrients needed to meet nutritional requirements. Research on the effects of ANFs is required in addition to the research needed to adjust recommended nutrient requirements for today's farmed fish.

From the official statistics on production, a total of 736,168 tons of salmon was sold in 2007. Rainbow trout sales were 77,578 tons and Atlantic cod sales were 9,611 tons. Cod production showed a small reduction compared to 2006. However, expectations for substantial production growth exist especially for the Atlantic cod. Only small volumes of other marine species were reported, among these Atlantic halibut, Arctic charr and plaice. The statistics show an increase in volume which in turn will lead to an increased need for fish feed in the years to come. Already marine oil volumes are too low to cover the needs in aquaculture, and fishmeal volumes are becoming more compatible with high fluctuations in prices, resulting in commercial diets containing plant ingredient types and volumes before thorough investigations on their health implications are known (Statistikk for Akvakultur, Fiskeridirektoratet 2008).

3. Terms Of Reference

The Norwegian Food Authority asked the Norwegian Scientific Committee for Food Safety to assess criteria to be used when evaluating plant ingredients in fish feed, so that these fulfil the Feed regulative §7 to "not induce negative health effects in the animal", and in this context aquacultured fish.

Further, plant ingredients should be identified which might induce long-term negative effects affecting fish health, and should therefore be recommended limited. "Long-term" refers here to extending in time significantly beyond that of standard production of farmed fish for consumption (for example, in the production and maintenance of breeding fish).

In particular the risk assessment should address the following issues:

- Whether plant ingredients contain specific protein types or protein fractions that should be limited in fish diets, and identify these.
- Identify anti-nutrients in plants that are already in use or are planned to be used, and assess to what extent the various plant ingredients can be tolerated by the fish.
- Assess interactions between anti-nutrients, and how such interactions should be considered when plant ingredients are to be used in diets for various aquacultured fish species.
- Whether the use of plant ingredients with high fibre contents should be limited in fish diets.
- Assess if plant lipids should be limited in fish diets.
- Assess whether feed ingredients containing glucans, nucleotides or other potent molecules, added due to their immunostimulatory effects, should be limited in diets for aquacultured fish species.
- Assess if processing methods, including the use of processing aids, could influence the ingredient to such an extent that the processing aid might be a risk factor for the aquacultured fish species.

The risk assessment should identify if various aquacultured fish species have different tolerance to the various plant ingredients, and in which way. In particular Atlantic salmon, rainbow trout, Atlantic cod and Atlantic halibut should be included.

4. Risk assessment

4.1. Utilization of plant protein resources by fish

As the use of fishmeal in the aquaculture industry decreases for various reasons, alternative, more cost-effective feedstuffs are being increasingly used as protein sources in formulated feeds for farmed fish. Various sources have been attempted from plant, microbial, and other animal sources. However, at least some of these alternative feed ingredients have been reported to have negative consequences on growth and feed utilization of farmed fish, depending on fish species and inclusion level in their diets. The causes, to name some possibilities studied and documented, may lie in low levels of protein and ω-3 fatty acids, unfavourable amino acid and mineral profiles, high levels of fibre and starch, and – perhaps most consequential – the presence of anti-nutritional factors (ANFs) and/or antigens. Specific effects of alternative protein sources on the digestive physiology of fish have been most closely studied in the case of soybean products in feeds for farmed salmonids. The causal agent(s) in soybeans that lead to reduced nutrient digestibility and the inflammatory response in the distal intestine are still largely unknown, but affect many specific digestive processes, from feed intake to enzyme activities to nutrient transport to gut histology. However, such details are often lacking in studies focusing on other alternative protein sources that are already in use in formulated feeds for various fish species. Thus the long-term implications they may have on fish production, health, and product quality are questionable, and this topic deserves continued, substantial investments in further research to preserve and enhance the sustainability of the aquaculture industry.

Losses due to disease represent a major cost in the aquaculture industry internationally, and diet composition is among several factors that may influence disease susceptibility. Optimal health and disease resistance is dependent not only on an optimal balance of nutrients available for all systemic needs, but also on optimal function of the gastrointestinal tract (GIT) and associated organs. The GIT is constantly exposed to a conglomeration of nutrients, ANFs and non-nutrients also comprising food antigens and micro-organisms. The digestive apparatus adjusts to changing diet composition, and the mucosal defence system provided by the GIT must protect the body from injurious agents and at the same time develop oral tolerance to antigens from the diet and commensal microbiota (see reviews by Chehade and Mayer, 2005; Sansonetti, 2004). The microbiota's species composition, which may be influenced by various dietary nutrients, non-nutrients and anti-nutrients, is also of importance for the host's gut and general health (Bauer et al., 2006).

The study of effects that various alternative protein-rich ingredients have on fish has largely been restricted to fish growth, feed conversion efficiency, and digestibility of the feed's nutrients. Due to this number of unknowns that can influence results of a particular study, little specific data is presented and the results of studies are discussed in more general terms.

The main challenges in using plant protein sources in diets for carnivorous fish lie in their often lower levels of protein and high levels of starch, unfavourable amino acid and mineral profiles, high levels of fibre and the presence of anti-nutritional factors (ANFs) and/or antigens. Especially the long-term implications they may have on fish production, health, and product quality are largely unknown.

4.1.1 SOYBEAN MEAL (*GLYCINE MAX*)

Soybean meal (SBM) is one of the most commonly used protein sources in animal feeds due to its high protein content and favourable amino acid profile. However, even when heat-treated and supplemented with limiting amino acids, full-fat as well as defatted (standard; hexane-extracted) SBM-containing feeds lead to decreased growth, lower feed intake, lower energy and fat digestibilities, and fecal dry matter in all salmonid species studied.

Soybean meal (quality dehulled) as the sole protein source is shown to lead to growth arrest and increased mortality in rainbow trout. These SBM products also cause an inflammatory response in the distal intestine (enteritis) of salmonids, which may at least partially explain the effects on growth parameters and feed utilization. Further processing of the SBM with alcohol extraction to produce soybean protein concentrate appears to remove the as-yet unknown causal agent(s) of the enteritis and supports very acceptable growth parameters and feed utilization.

Atlantic cod and halibut appear to tolerate full-fat and standard SBM better than Atlantic salmon and rainbow trout. Levels up to 24% of the total diet for cod and 36% of the total diet for halibut did not markedly affect growth or feed utilization, nor did it cause inflammatory responses in their intestines.

4.1.2 LUPIN (*LUPINUS* SP.)

As demonstrated in studies on several fish species, the proteins in kernel meals of dehulled white (*Lupinus albus*), sweet (*L. angustifolius*) and yellow (*L. luteus*) lupines are highly digestible. It has been estimated that sweet lupine kernel meal may be included in diets for rainbow trout up to 30 or 40% of total diet without significantly influencing growth and nutrient utilization. No histological changes were observed in the pyloric caeca or distal intestine of rainbow trout fed up to 50% yellow lupine kernel meal (of total diet), although hepatocytes appeared to have a lower level of lipid droplets in the fish fed the 50% inclusion level. Nor were histological changes observed in the distal intestine of Atlantic salmon fed 24% of total diet of dehulled, low-alkaloid white lupine meal, or 30% of total diet of kernel meals or protein concentrates from yellow lupine or sweet lupine. In the stomachs of these fish, however, higher severity of ulcer-like lesions connected with the lupine-containing diets was reported.

4.1.3 PEA MEAL

Pea meal has potential as an alternative protein source although the use of low-processed pea meal in feeds for carnivorous fish is limited by its high starch content (ca. 50% of pea meal is starch). Pea protein concentrate or isolate is therefore shown to be more suitable.

For Atlantic salmon, pea protein concentrate at inclusion levels of up to 28% of total diet led to apparent digestibility coefficients for dry matter, nitrogen, and energy similar to those of a commercial diet and higher than a control diet containing fishmeal as the sole protein source. In another study, Atlantic salmon fed 20% pea protein concentrate (of total diet) was found to lower digestibility of energy, but no significant differences in digestibility of other macronutrients or amino acids and significant effects on growth performance, body composition, intestinal brush border maltase activities, faecal trypsin activities, or intestinal histology were found compared to the fishmeal control diet. However, Atlantic salmon in yet another feeding trial fed 30% pea protein concentrate (of total diet) exhibited lower growth rates, lipid and starch digestibility, and distal intestinal weight, as well as histological changes in the distal intestine characteristic of an inflammatory response. No other intestinal regions showed signs of inflammation.

Rainbow trout also digest pea protein well at lower inclusion levels, although 30% pea meal in total diets led to lower dry matter, protein, energy and particularly phosphorus digestibility compared to fishmeal-based diets.

4.1.4 Canola meal

Canola, developed from rapeseed to contain lower levels of glucosinolates, and heat-treated rapeseed meal appear to have promise as alternative protein sources for fish. In Atlantic salmon, 18.3% (of total diet) inclusion of low-glucosinolate, extracted and heat-treated rapeseed meal resulted in higher lipid digestibility but lower crude protein digestibility than a fishmeal control diet. Histological changes were not observed in the stomach, mid or distal intestine. In rainbow trout, inclusion levels in total diet of canola protein concentrate of 19% to 38% led to reduced growth and feed intake compared to fishmeal controls. In Atlantic cod, apparent protein and energy digestibility for canola meal has been estimated to be relatively low at 76.0 and 60.6% (ADCs), but higher for canola protein concentrate at 88.8 and 83.3% (ADCs), respectively.

4.1.5 Sunflower meal

Sunflower meal (partially dehulled) in diets for Atlantic salmon had ADC of 88% for protein and could be included in post-smolt diets up to 27% of total diet without adverse effects on growth performance, feed utilization or body composition. At a 22.9% inclusion level (of total diet) of extracted and mildly heat-treated sunflower meal, higher lipid digestibility but lower crude protein digestibility than a fishmeal control diet was reported. Histological changes were not detected in the stomach, mid or distal intestine. Sunflower meal at an inclusion level of 41% in a total diet for rainbow trout resulted in improved crude protein digestibility but reduced nitrogen-free extract and dry matter digestibilities compared to a fishmeal-based control diet.

4.1.6 Cottonseed meal

Solvent-extracted cottonseed meal at inclusion levels of more than 50% in total diets for various fish species causes growth depression, but levels of up to 30% of total diet (replacing 50% of fishmeal protein) appeared to be well tolerated by rainbow trout juveniles and did not significantly infringe on growth parameters, feed conversion, nutrient digestibility or mineral availability. A long-term feeding trial (35 months) showed that even inclusion levels as high as 59% of total diet, which was a complete replacement of fishmeal, did not impact fish growth negatively. However, female rainbow trout fertility was negatively affected by complete replacement of fishmeal with cottonseed meal.

4.1.7 Wheat gluten

Wheat gluten is a highly digestible protein source for rainbow trout, Atlantic salmon and Atlantic cod. It does not cause morphological changes in the intestinal tissues of salmon. Wheat gluten up to a level of 50% of dietary protein (29% of total diet) can be added to salmon diets without reducing protein, amino acid, fat, and energy digestibility, although α-amylase inhibitors in wheat appear to reduce starch digestibility. Corn gluten protein as a protein source is also highly digestible for Atlantic salmon and Atlantic cod. In Atlantic salmon, no morphological changes in the stomach, mid and distal intestinal tissues were observed when using whole corn meal as a carbohydrate source in fishmeal-based diets for parr, nor 20% corn gluten of total diet, added as a protein source in diets for post-smolt.

4.1.8 Potato protein concentrate

Potato protein concentrate in diets for rainbow trout resulted in severe appetite loss, even at dietary inclusion levels as low as 5% of total diet. However, if processed to remove inherent solanidine glycoalkaloids and protease inhibitors, up to 20% potato protein concentrate of total diet may be included in diets for salmonids without causing adverse effects on appetite, growth, nutrient digestibility, or nutrient retention.

4.1.9 Other protein sources

Rice protein concentrate can be included at levels up to 20% in total diets for trout without negatively affecting growth performance. Faba beans have limited application in diets for carnivorous fish due to the high starch content. Peanut meal has not been tested in cold-water carnivorous species.

Mixing various plant protein feedstuffs in formulated feeds has been attempted in recent years with varying results. It appears that the proportion of feedstuffs from various sources and the degree of fishmeal substitution that is acceptable will vary depending on fish species and their dietary requirements and preferences. Possible additive/synergistic effects among ANFs, antigens, and/or toxins present in the various feedstuffs that comprise a mixture should also be taken into consideration (see below).

4.1.10 Conclusion – plant proteins

With the exception of full-fat and extracted soybean meal for salmonids, substituting at least part of the fishmeal fraction of aquafeeds with individual plant ingredients seems promising, at least in the short to medium term. Indeed in some cases, diets containing up to 20% of high quality plant protein sources resulted in better nutrient digestibility and growth parameters than the fishmeal-based control diets. This reveals that not all fishmeals are of optimal nutritional quality. When substituting fishmeal with plant ingredients, however, it may be necessary to adjust the diets regarding limiting amino acids and minerals.

Information regarding ANFs in the plant ingredients and feeds containing them is rarely given in publications reporting data from feeding trials. Combining various plant ingredients in feeds, thereby limiting the concentration of anti-nutritional factors/antigens inherent in single ingredients, has promise regarding complete substitution of fishmeal.

However, each blend of plant ingredients needs to be thoroughly tested in order to rule out adverse effects caused by combinations of ANFs/antigens. Long term studies of metabolic and health aspects of individual plant ingredients as well as various mixes in aquafeeds are needed for all species. Also, little knowledge exists regarding the consequences of adding various plant ingredients to diets of juveniles for the various fish species and effects on broodstock and subsequent reproductive parameters.

4.2 Anti-nutritional factors

Anti-nutritional factors (ANFs) are defined as innate components of a food/feed ingredient that have a limiting effect on the food/feed intake, digestion, and/or nutrient absorption. Possibly the most limiting factor for the use of plant feed ingredients as nutrient sources for fish are ANFs inherent to them. ANFs will most likely cause changes in nutrient availability and/or utilization, physiological responses, and ultimately, metabolism and will therefore necessitate changes in recommended dietary levels of various nutrients in aquafeeds.

Listed below are the known ANFs and the plant feedstuff in which they are found. It is possible that still unidentified compounds in plants may be important in fish nutrition and health. Little is available in the literature concerning effects of specific ANFs on fish or levels of various ANFs in feeds containing plant ingredients. Thus it is difficult to assess health effects, especially long-term effects, on fish. No data has been found regarding effects of isolated ANFs on Atlantic cod and halibut.

Table 1 Important anti-nutrients present in some commonly used alternative fish feed ingredients (Francis et al., 2001a).

Plant feedstuff	*Anti-nutrient present*
Soybean meal	Proteinase inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, phytosterols, allergens
Kidney beans	Proteinase, amylase and lipase inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, phytosterols, allergens
Rapeseed meal	Proteinase inhibitors, glucosinolates, phytic acid, tannins
Lupin seed meal	Proteinase inhibitors, saponins, phytoestrogens, alkaloids
Pea seed meal	Proteinase inhibitors, lectins, tannins, cyanogens, phytic acid, saponins, antivitamins
Sunflower oil cake	Proteinase inhibitors, saponins, arginase inhibitor
Cotton seed meal	Phytic acid, phytoestrogens, gossypol, antivitamins, cyclopropenoid acid
Alfalfa leaf meal	Proteinase inhibitors, saponins, phytoestrogens, antivitamins
Mustard oil cake	Glucosinolates, tannins
Sesame meal	Phytic acid, proteinase inhibitors

Enzyme inhibitors, i.e. proteinase, amylase and lipase inhibitors, are proteins that inhibit the activity of the respective enzymes, often by forming stable complexes with the enzymes. By doing so, they decrease protein, starch and lipid digestibility. They may also often increase endogenous losses of the enzymes, and may cause feedback signals to the pancreas that result in increased secretion of the enzymes. This may have long-term implications on pancreas function so that even relatively low levels of enzyme inhibitors can have a significant impact on dietary nutrient levels needed to meet requirements caused by the endogenous losses over time.

4.2.1 PROTEINASE INHIBITORS

In the intestine, proteinase inhibitors first form a rather stable complex with trypsin, thereby reducing trypsin activity. This in turn stimulates secretion of PZ-CCK (Pancreozymin-Cholecystokinin) from the gut wall. This hormone stimulates secretion of trypsin from pancreatic tissue and stimulates the gall bladder to empty its content into the intestine. In some animals, proteinase inhibitors cause pancreatic hypertrophy. Whether this also takes place in fish is not clear.

In studies with salmonids, proteinase inhibitors have been found to reduce apparent digestibility not only of protein but also of lipid (Berg-Lea et al., 1989; Krogdahl et al., 1994; Olli et al., 1994). The effects on digestibility correspond to a decrease in trypsin activity and presumably chymotrypsin, which is also inhibited by soybean proteinase inhibitors. Saturated fatty acids appear more severely affected than unsaturated.
The results indicate that the proteinase inhibitors stimulate pancreatic enzyme secretion, causing the enzyme level of the intestinal content (trypsin protein) to increase. However, the activity in the intestinal content is not increased. The enzyme activity seems unaffected when

fed diets with the lower inhibitor levels and short-term feeding, but higher levels decrease the activity. After longer-term feeding it may seem that the pancreas no longer manages to compensate for decreased enzyme activity by increasing secretion. Thus enzyme production does not appear to keep up with the increased demand.

4.2.2 AMYLASE INHIBITORS

Effects of amylase inhibitors in diets for Atlantic salmon may be of limited practical significance, since the amylase of this species seems to have a defective substrate anchor, reducing its catalytic ability and the ability of salmon to utilize starch. Proteinase and lipase inhibitor activities may account for the impaired protein and lipid digestibility in Atlantic salmon fed on diets with an inclusion of solvent extracted SBM. However, no feeding trials for Atlantic salmon, rainbow trout, Atlantic cod or Atlantic halibut have been reported that investigated dietary effects of isolated plant lipase inhibitors.

4.2.3 LECTINS

Lectins (previously known as agglutinins or hemagglutinins) are a group of heterogeneous (glyco)proteins which bind reversibly to specific mono- or oligosaccharides. The mono- or oligosaccharides that lectins bind to may be an intrinsic part of many biologically important substances, so-called glycosubstances or glycoconjugates. These are present on cell surfaces, for example. By binding to cell surfaces, lectins may change cell functions and responses. Thus they can agglutinate cells, modulate the functioning of enzymes, transport proteins, receptors etc., act as growth promoters and immunostimulants, and mimic or block endogenous signalling substances. Thus they may have varied effects on digestive and absorptive processes.

Furthermore, glycation of cells varies depending on many factors: animal species, its age, genetic make-up, blood group specificity, health status, diet, and bacterial flora in the intestine, as well as intestinal region and mucosal cell type. Therefore, there is a wide range of potential binding sites for lectins in the gut. This variability in glycation patterns may help explain the variability in biological effects of different lectins in different species of animals.

The potency of the lectin's effect on cell metabolism appears to be correlated with the strength of its binding, which in turn is dependent not only on its defined sugar/carbohydrate specificity, but also on other structural intricacies of the ligand. Binding of soybean lectin (agglutinin; SBA) to the intestinal brush border membrane of Atlantic salmon and rainbow trout has been demonstrated. Higher maximum binding and lower dissociation constants were observed in the distal intestine relative to the more proximal areas. This could indicate that the distal intestine would be more sensitive to a potentially toxic effect of soybean lectin or other anti-nutritional factors or antigens. However, soybean lectin has been largely ruled out as the sole cause of reduced digestive function and the inflammation caused by soybean meal in salmonids.

4.2.4 SAPONINS

Saponins are amphipathic glycosides that disrupt cell membranes and can have antimicrobial, immunostimulatory, glucocorticoid and antioxidant activities. They inhibit protein and lipid digestion, vitamin absorption, and cholesterol metabolism. The involvement of saponins from soybeans in an inflammatory response in the distal intestine of salmon has recently been indicated, albeit not alone but in combination with unidentified components found in lupin meal.

4.2.5 PHYTOOESTROGENS/-STEROLS

The research on effects of phytooestrogens/-sterols in farmed fish has focused on reproductive parameters. Little is known about other physiological responses following dietary intake of phytooestrogens in any fish. Glucosinolates/goitrogens are a group of substances that disrupt thyroid hormone production, partially by interfering with iodine availability. They also cause reduced palatability and thus reduced growth, as well as affect liver and kidney functions.

4.2.6 FIBRES

Fibres are polymers of monosaccharides. They vary in their solubility in water, size and molecular structure. Dietary fibres alter flow, impair interactions, affect intestinal receptors, restrict nutrient diffusion, change microbial diversity and activities, and change absorptive surfaces. The variability of the compounds belonging to the fibre complex and the varying degrees that they affect these parameters make it difficult to conclude on a general basis regarding maximum inclusion level. Little is known on their specific effects in fish.

4.2.7 TANNINS

Tannins are phenols that may decrease feed digestibility by binding digestive enzymes or nutrients as well as reduce feed palatability due to their astringent flavour. Little is known on effects in fish.

4.2.8 PHYTIC ACIDS AND PHYTATE

Phytic acids and phytate are found in significant amounts in plant feedstuffs since they are the primary storage form of phosphate and inositol in seeds. They complex with mineral ions and possibly with protein, thus reducing their digestion/absorption. Dietary inclusion of phytic acid has been shown to reduce growth in salmonids as well as reduce digestibility and retention of protein and various minerals in Atlantic salmon. Effects in cod or halibut have apparently not been studied. Involvement of phytic acids in the development of skeletal deformities has not been fully resolved. However, diets for fish should probably not exceed 5 g per kg feed.

4.2.9 OLIGOSACCHARIDES

Oligosaccharides, i.e. α-galactosyl homologues of sucrose, appear to interfere with nutrient digestion and may have osmotic activity in salmonids. They also appear to be fermentable by gut microbiota in Atlantic salmon. Effects in cod and halibut have apparently not been studied. An upper limit of 50 g per kg feed has been suggested.

4.2.10 ALLERGENS

Whether fish react to allergens or feed antigens in general is not known. It has not been conclusively demonstrated in fish that antigens/allergens can elicit hypersensitivity reactions, including allergic reactions, as they may in susceptible mammals. Nor has any conclusive data been presented in the literature that antigens are involved in feed-induced pathologies in fish, including soybean meal-induced inflammation in the distal intestine of salmonids.

4.2.11 GOSSYPOL

The toxic, systemic effects of gossypol – reduced haematocrit, hemoglobin, reproductive capacity as well as lesions in liver, kidney, spleen and gonads – in various fish species have been documented but the mechanisms behind the reduced growth and nutrient digestibilities are unknown. These responses were observed in rainbow trout fed cottonseed meal-containing diets with free gossypol levels of between 30 and 40 mg kg^{-1} feed. Gossypol levels less than 20 mg kg^{-1} feed, the maximum level allowed by the EU Commission, did not cause these reductions in growth and nutrient digestibility. Long-term effects and tolerance levels for salmon, cod and halibut are not known.

4.2.12 GLYCOALKALOIDS

Among the various glycoalkaloids, those found in potatoes, α-solanine and α-chaconine, are of the most practical importance. These potent toxins, which permeabolize cell membranes and inhibit cholinesterase, limit the use of potato protein concentrate, unless measures are taken during processing to remove them. The main anti-nutritional activity is in reduced feed intake due to their bitter taste. Tolerance levels are not known for any fish species but due to their toxicity they should not be present in feeds.

4.2.13 ARGINASE INHIBITORS

Arginase inhibitors, such as chlorogenic acid in sunflower seeds, may be associated with reduced protein digestibility of sunflower meal in salmon but the mechanisms are unknown. Tolerance levels and long-term effects are unknown.

4.2.14 QUINOLIZIDINE ALKALOIDS

Quinolizidine alkaloids, such as lupinine, gramine and sparteine in lupin meals, are toxins that inhibit motor coordination and muscular control in mammals. Data regarding this or other anti-nutritional activities are lacking in fish, but the bitter taste may be responsible for reduced feed intake in rainbow trout and Atlantic salmon. Tolerance levels for these fish may be between 100 and 500 mg kg^{-1}. But tolerance levels for cod and halibut are unknown as are long term-effects for any fish species.

4.2.15 COMBINED EFFECTS OF ANFS

Combined effects of ANFs have not been extensively studied in any animals. Tannins in combination with lectins, cyanogenic glycosides and saponins appear to reduce the deleterious effects of the individual ANFs. However, additive interactions with deleterious effects on intestinal function or structure have been reported for saponin and lectin on rabbit tissue *in vitro*, soybean lectin and protease inhibitor on Atlantic salmon intestinal tissue *in vitro*, and saponin and unidentified component(s) of lupin meal in Atlantic salmon distal intestine *in vivo*. Thus there is a need to test combinations of plant ingredients and ANFs on a case-by-case basis to assess any potential consequences to fish health.

5.2.16 CONCLUSION – ANFS

Knowledge regarding responses to various qualities/processing methods of soybean meal in feeds for salmonids as well as to various extracts of and/or purified ANFs in soybeans has resulted in some insights in ANF effects and their possible role in the SBM-induced enteritis.

Saponins, non-starch poly-/oligosaccharides, phytoestrogens, phytosterols and/or antigenic peptides may potentially have a role in inducing the inflammation. However, it cannot be ruled out that as-yet unidentified components as well as the gut microbiota may be involved.

Various processing measures presently employed in feed manufacturing may decrease the activity or concentration of individual ANFs. However, recent findings suggest combinations of various ANFs, including the specific ANFs mentioned above, may have particular significance in causing adverse effects to intestinal structure, function and defense mechanisms at lower levels than what the individual ANFs would elicit.

More research is needed to investigate the effects of various ANFs, individually and in combinations, on nutrient digestibility, utilization and metabolism as well as to intestinal function, structure, defense mechanisms and microbiota. Long-term effects also merit investigation. This will aid in the ability to predict how a newly introduced plant ingredient as well as combinations of plant ingredients may affect the fish and identify steps needed to reduce/prevent adverse health effects.

4.3 Plant oils

All plant oils lack the long chain highly unsaturated fatty acids (HUFA) typical for marine oils (eicosapentaenoic acid, EPA, docosahexaenoic acid, DHA and arachidonic acid (ARA). The predominant oils such as soybean and canola oils are rich in 18:2n-6 or saturated fatty acids as 16:0 in palm oil. Other oils are also available, but are produced in smaller amounts. These include canola oil, linseed oil (rich in 18:3n-3) and certain safflower species (rich in 18:1n-9) but may be even more important in future aquaculture feeds. The long chain polyunsaturated fatty acids from plants are often, and also in this report, categorized as PUFAs from plants.

In general, plant oils provide good energy sources. Medium chain triacylglycerols (MCT), such as those found in coconut oil, appear to be particularly good substrates, increasing performance at relatively low inclusion levels. At higher levels poor performance and high mortality has been observed. The results differ with species and size of fish, and there is relatively little data available, particularly in cod and halibut. In general, adding 1-3% of total diet as MCT to fish larvae appears safe. Larger fish appear to tolerate higher levels of MCT. In salmonids up to 10% of total diet and in cod 4% of total diet would unlikely pose any risk as judged from available literature. The maximum level of inclusion may also depend on type of MCT. Shorter chain MCT (C_6 or C_8) appears to cause more problems than longer chain MCT, such as coconut oil (C_{10}).

As marine fish lack the ability to elongate and desaturate plant PUFAs to the longer chain HUFAs (EPA, DHA and ARA) which are needed for normal homeostasis, these must be supplied through using marine raw materials in the diets. Thus, the highest level of plant oil inclusion is the point where essential fatty acid deficiency will develop. This depends on which marine lipids are used in the inclusion.

For juvenile and adult marine fish the requirement of EPA and DHA is probably in the range of 0.5-1.5% each in the total diet, and somewhat higher for larvae. It is also possible that the requirement should be estimated at around 10-20% of the dietary lipid. The requirement of

ARA may be in the range of 0.3-0.5% of total diet. Existing data are, however, from other species than cod and halibut.

Anadromous fish such as Atlantic salmon and rainbow trout have some capacity to elongate and desaturate plant PUFA to EPA, DHA and ARA. Plant oils can thus to a large extent replace marine-type oils in the diets for these fish species. For rainbow trout, the essential fatty acid requirement is around 1-2% of total diet if supplied as C_{18}, and half of that if given as HUFAs. It has also been suggested that the level should be 10-20% of dietary lipid depending on the form of delivery used. In rainbow trout, it has been suggested that DHA should be regarded as an essential fatty acid due to a too low conversion rate from 18:3n-3. Similar mechanisms seem likely in Atlantic salmon.

If the essential fatty acid requirements are covered, plant oils do not seem to cause significant adverse effects in marine fish. Some elongase activity of C_{18} PUFA to their C_{20} HUFA counterparts has been seen. The activity appears very low in Atlantic cod and remains unknown in halibut. If produced, C_{20} HUFA may compete with ARA and EPA for active sites for eicosanoid production. The significance of this is unknown.

Fish probably have a high requirement for phospholipids (PL). If fishmeal contains relatively large amounts of marine HUFAs used as protein source, this is only a problem in larval fish where addition of soy lecithin (in the range 2-6% of total diet) is advised. However, as other protein sources are now introduced, addition of some soybean lecithin (or marine PL) should be considered for larger fish as well (1-3% of total diet).

In freshwater fish, the effect of adding plant oils in feed on fish immunity is inconclusive. Cases of increased disease resistance or immuncompetence in fish given feed with added high n-6 PUFA oils (mostly 18:2n-6) have been attributed to production of more eicosanoids from ARA with more potent activity in inflammatory processes than those from EPA. There are also several studies suggesting that n-6 PUFA-rich oils will reduce fish immunocompetence. This may be related to altered eicosanoid cascade in addition to changes in membrane fluidity. The reasons for these discrepancies remains unknown and may be caused by several factors including type of study, environmental effects, strain, species and interference of other dietary components. At present however, exchanging up to 50% of the fish oil does not seem to pose any risk to the fish.

Increased level of ARA (from 18:2n-6 rich oils) and its related eicosanoids may also enhance the stress-response and may increase the level of subclinical stress that may affect the responsiveness to environmental stress or noxious substances affecting fish health in the long term.

Oxidative stress does not seem to be a major problem related to plant oils, provided that they are of good quality, as most plant oil products have lower oxidative potential than most fish oils and therefore lower the potential oxidative burden. The only potential problem is found with high levels of linseed oil (high content of 18:3n-3), which may contribute to oxidative stress. Although no concrete figures are available for salmonids, cod and halibut, around 25% addition of linseed oil (of dietary lipid) appears to be relatively safe.

The requirement for essential fatty acids in broodstock nutrition seems to be in the same range as that for fish in the grow-out stages. For sparids, 1-2% of total diet has been suggested, for salmonids 1% of total diet, and for turbot 20% n-3 HUFAs of dietary lipid. There are also reports suggesting that even higher levels of long chain polyunsaturated fatty acids should be used. At very high levels of n-3 HUFAs, however, negative effects are sometimes observed. It has also been argued for both cod and some salmonids that the level of ARA in broodstock diets should be increased compared to normal standard diets. Cases of increased egg quality following this recommendation have been published. In rainbow trout no effect on fecundity or egg viability was found, despite being fed corn oil as supplemental oil source.

4.3.1 CONCLUSION – PLANT OILS

As many of the potential disadvantages of using plant oils in salmonid diets are related to either very high levels of n-6 PUFA (most available oils) or very high levels of linseed oil, it would be recommended that mixtures of plant oils should be used as feed inclusions. By adjusting the ratio of n-6 and n-3, the level of eicosanoids can be controlled. By including palm oil, potential problems in lipid digestibility and transport can be controlled. A standard inclusion of soybean lecithin may also be advisable. These and other variants of mixtures of oil sources have been explored in recent years with some success in salmonid fish. Such mixtures do not seem to be necessary for marine fish.

Care should be taken in choosing plant alternatives, both types and qualities, to prevent nutrition-related diseases such as skeletal deformities, cataracts, heart conditions, and other, unspecific symptoms.

4.4 Undesirable substances

Feed ingredients of plant and animal origin may contain compounds not inherent to the plant or animal, for example pesticides, mycotoxins, environmental pollutants or substances that occur during processing of the feed ingredients. Further, feed material of plant origin may contain elevated levels of heavy metals due to geological characteristics of the soil. To ensure that animal feed does not pose any threat to animal and human health or to the environment, statutory limits for maximum content have been set for a number of undesirable substances in feed materials and complete feedingstuffs (Legislation No. 7, 2002/32/EC).

Plant ingredients are different from marine ingredients with regard to the presence of undesirable substances, e.g. the levels of dioxins and PCBs are lower in plant oil than in marine oils. On the other hand, plant ingredients and feed may contain elevated levels of undesirable substances such as pesticides, mycotoxins and phytotoxins.

4.4.1 PESTICIDES

The European feed legislation currently includes 10 different pesticides which have all recently been assessed as undesirable substances in animal feed by the European Food Safety Authority (EFSA). Of the 10 pesticides only endosulfan is still in use, while the use of the rest has been banned in the European Economic Area (EEA area).

Feed ingredients are, however, traded on the global market. Hence, one may find elevated levels of pesticides which are banned in the EEA area in ingredients originating from countries were the pesticides are still in use or have only recently been banned.

There is little or limited data available on the occurrence of pesticides in feed materials and feeds. In general, organic chlorinated pesticides are seldom found in plant ingredients. When pesticides are found in plant ingredients it is generally in low levels and within the current limits for maximum content. However, there is still a risk that some plant ingredients may be accidentally polluted. The generally low levels of pesticides and other chlorinated compounds in plant ingredients is in contrast to the higher contents of these compounds reported in ingredients of animal origin, and especially in fish oil and fishmeal.

The adverse effects of pesticides included in the current legislation on fish have been evaluated by EFSA. The number of studies of the dietary toxicity of pesticides in fish is limited. Fish are generally highly sensitive to waterborne exposure to organochlorine pesticides, and some studies indicate that fish may also be sensitive to oral exposure. It is, however, important to bear in mind that waterborne exposure cannot be used to conclude on oral exposure.

In addition to endosulfan, a wide range of pesticides are in use in today's agriculture. Feed materials of plant origin can contain residues of these pesticides. They are, however, not included in the current feed legislation, and hence not monitored. There is a need for a thorough survey of their presence in feed ingredients of plant origin. If present, studies of their toxic effects in fish should be done. Further, if potentially hazardous levels of certain pesticides are identified, these pesticides should be included in the feed legislation.

4.4.2 MYCOTOXINS

Mycotoxins are toxic compounds produced by fungus, e.g. *Aspergillus* spp., *Penicillum* spp. and *Fusarium* spp. Relevant mycotoxins with regard to feed ingredients and feed are aflatoxins B_1, deoxynivalenol and other trichothecenes, fumonisins, ochratoxin A and zearalenone. They have all been recently assessed by EFSA.

Mycotoxins are more commonly found in feed materials of plant origin, especially in maize and small grains, and products thereof, than in feed materials of animal origin. Aflatoxin B_1 is the only mycotoxin included in the European feed legislation, but recommendations with guidance values for some other mycotoxins in feed are given.

Occurrence data for mycotoxins in feed and feed materials are limited. For aflatoxin B_1, products coming from regions with subtropical or tropical climate have been identified as being of high risk with regard to contamination (EFSA, 2004a) and the following products are presumed to have a potential for contamination with aflatoxin B_1; groundnut, copra, palm-kernel, cotton seed, babassu, maize and their products (2002/32/EC). Deoxynivalenol and ochratoxin A are usually found in cereals, such as wheat, rye, barley and maize and their products (EFSA, 2004b; EFSA, 2004c), while fumonisins and zearalenone are common in maize and maize products (EFSA, 2005f; EFSA, 2004d).

In Norway in 2006 various mycotoxins were detected in samples (only 21 samples were analysed) of feed materials of plant origin (Maage et al., 2007). Toxicological studies of various mycotoxins in animals have shown a broad range of effects and some may be carcinogenic, mutagenic or immunotoxic. The knowledge of the toxic effects and toxicokinetics of mycotoxins in fish is limited, and studies of dietary exposure to mycotoxins are scarce.

4.4.3 Phytotoxins

Phytotoxins are a group of toxic substances produced by plants. The current feed legislation includes botanical impurities and natural plant toxins (hydrocyanic acid, free gossypol, theobromine and glucosinolates). However, SCAN (the Scientific Committee on Animal Nutrition) concluded in a scientific opinion that the list of plant species and products included in the legislation "… does not represent the present taxonomic status of potential botanical contaminants or the real risk represented by such impurities in modern animal feeding practice" and recommends that the list be re-evaluated (SCAN, 2003). With the increased use of plant ingredients in fish feed, a re-evaluation is still relevant. Some of the natural substances in feed materials of plant origin (plant protein) are anti-nutritional factors which should be re-evaluated.

4.4.4 Polyaromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds containing two or more fused aromatic rings. PAHs are not included in the feed legislation, only in the food legislation. Many of the PAHs are carcinogenic and immunotoxic, and benzo[*a*]pyrene (BaP) has been selected as an indicator for the occurrence and effect of PAHs in food (SCF, 2002). A range of PAHs, including BaP, are included in the Norwegian surveillance program on feed for fish and other aquatic animals, and results from 2006 show that the level of PAHs, contrary to other organic pollutants, is higher in plant oils than in marine oils, with mean concentrations of BaP of 3.1 (n = 5) and 0.6 (n = 9) µg/kg feed, respectively (Maage et al. 2007).

However, only a few studies have investigated the toxicity of dietary PAHs to fish when fed contaminated feed over a longer period. One study reported a reduction in weight in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) fed a mixture of 21 PAHs (22 µg/g fish ww/day) for 54 days (Meador et al., 2006), while no effect on growth was seen in juvenile grouper (*Epinephelus aerolatus*) fed BaP (12.5 µg/g fish ww/day) for 4 weeks (Wu et al., 2003).

4.4.4 Nitrosamines

Nitrosamines, or N-nitroso compounds (NOC), are carcinogenic, and toxic effects have been reported for fish. There is a limited number of dietary studies, but the development of liver tumours has been reported for rainbow trout after dietary exposure to dimethylnitosamine (Ashley and Halver, 1968; Grieco et al., 1978). In rainbow trout exposed to graded levels of dimethylnitosamine (0, 3, 50, 200, 400 and 800 mg/kg feed) no hepatic changes were observed after 26 weeks of exposure, while after 52 weeks tumours had developed in the liver in rainbow trout in the three highest exposure groups (Grieco et al., 1978).

Nitrosamines may be formed during the processing of feeds. However, the formation of nitrosamines from processing of plant ingredients has not been identified.

4.4.5 Conclusions

Available data on the occurrence and levels of undesirable substances in feed material of plant origin is limited. It is important to continue the monitoring of contaminants in feed materials and feed, with special focus on pesticides, mycotoxins and PAHs in feed materials of plant origin. Many pesticides in use in today's agriculture are not included in the current feed legislation. Their presence in feed material of plant origin should be investigated, and their potential toxicity to fish should be studied.

The list of undesirable substances included in the feed legislation is, in general, sufficient, but it should be considered to include pesticides in use today and more of the mycotoxins.

Studies of dietary exposure to undesirable substances, e.g. pesticides and mycotoxins, and their toxic effects in fish are scarce. More studies, and especially long-term feeding trials, are needed for improved risk assessments.

4.5 Genetically modified ingredients

Few studies are performed on the use of genetically modified (GM) plant proteins in fish diets. However, some publications exist on the use of Round-up Ready soybeans (RRS) and Bt-maize in salmon diets. These show varying results, the major trend being that no or very small effects on fish growth or feed utilization are found. Few alterations in organ sizes were found, except for the gastrointestinal tract, which seems to be affected by the plant ingredients especially, but regardless whether the plant is GM or not. These latter results are not consistent. Some data show that parts of the immune system can be affected by GM. These results are not consistent, however. The glucose transporter system in the pyloric region of the salmon intestine seems to be altered by Bt-maize, and the stress resistance, measured as changes in mRNA expression of heat-shock proteins 27 and 70, was in some studies altered, but not in other studies. No clear conclusion is drawn on the effect on fish health and the use of RRS or Bt-maize in salmon diets.

4.6 Immunostimulants, prebiotics and nucleotides

The immune response can be modulated by β-glucans and high-M-alginate. β-glucans are glucose polymers that are major structural components of the cell wall of yeast, fungi, and bacteria, but also of cereals such as oat and barley. There is broad structural variation in the β-glucans from these different sources, which may influence their physiological functions. Alginate is a polysaccharide composed of β-1,4-D-mannuronic acid (M) and α-L-glucuronic acid (G). *In vitro* studies as well as *in vivo* studies in fish show that especially β-glucans derived from fungi, yeast, and alginate have immune modulating properties. Most frequently evaluated are effects on macrophage activation and the activity of lysozyme, respiratory bursts and leukocytes, all of which have been suggested to contribute to the increased resistance against infections, observed after immunostimulant exposure.

Any reduction of both diversity and quantity of the gut microbiota is likely to reduce the effective barrier mechanism normally provided by the commensal microbiota; this leads to a reduction of competition against secondary potential pathogens from the surrounding environment.

To date, the application of pre- and probiotics for the improvement of aquatic environmental quality and disease control in aquaculture seems promising; unfortunately, the information is limited and sometimes contrasting. Owing to these uncertain and incomplete results, there are still no standardized protocols for testing the effects of these products and their impact on farmed fish welfare, growth and health status.

Currently there are numerous gaps in existing knowledge about exogenous nucleotide application to fish, including various aspects of digestion, absorption, metabolism, and influences on various physiological responses, especially expression of immunogenes and modulation of immunoglobulin production. Additional information is also needed in regard to age/size-related responses, appropriate doses and timing of administration.

4.7 PROCESSING

Plant ingredients are subjected to processing before being used as feed. In the extrusion process, plant materials mixed with other ingredients are heated to above 100 °C with water present. This process alters the tri-dimensional structure of proteins and starch, and can induce new covalent bonds which will produce substances that may be harmful to fish health. Maillard products may be formed due to reactions between an amino acid and a reducing sugar, and these may be further modified to produce toxic substances. The extent to which toxic compounds are formed in common feed production processes have not been extensively studied, but it is likely that any adverse effects will mainly be associated with reduced protein digestibility.

Some plant ingredients are processed as raw materials, for example heated in conjunction with the extraction of the oil fraction or due to mechanical processes such as dehulling. These processes may improve nutritive value of the ingredient through elimination of anti-nutritive properties of proteins and through removal of harmful substances through removal of parts of the plant.

In conclusion, heat processing of raw materials and of the complete fish diets may alter nutritional properties of plant materials. However, the negative effects of processing appear to be modest under practical conditions.

4.8 PATHOLOGIES LINKED TO FEED

Modern finfish aquaculture faces problems such as bone and skeletal deformities, cataracts, heart disorders, unspecific ulceration and various digestive disorders including intestinal colic in Atlantic cod and gastric dilatation (bloat) in rainbow trout. Focus has also been put on a seldom, still significant, occurrence of intestinal tumours in Atlantic salmon broodstock. Most of the mentioned problems show some kind of relation to malnutrition, feed composition, intensive growth and/or unfavourable environmental conditions. The disorders are often non-lethal, but may increase the susceptibility to secondary disorders and infectious diseases. Major changes in feed composition and feed ingredients may increase the risk for such production-related disorders in intensive fish farming.

Several nutrition related cataracts, such as spleen and liver cataracts, white fishmeal cataract, or rancid low quality feed cataracts, have been seen as a consequence of introducing novel feed ingredients in fish feeds, reflecting nutrient deficiencies, reduced nutrient availability and oxidative challenges.

Bone deformities in juvenile and adult fish are periodically observed with high prevalence in intensive aquaculture, and are also regarded as disorders of multidisciplinary origin. Several nutrients in deficiency and/or excess cause bone disorders. From a nutritional point of view, both development and maintenance of the bone tissue can be affected, and may include impairments in bone cell differentiation and function, matrix composition and bone tissue mineralization.

It is well documented that both wild and farmed fish may develop various heart disorders, including lesions and arteriosclerosis. The dietary correlation to the progression of heart disorders is not well documented, and there is too little information available to be able to link these conditions to feeding high levels of plant oils.

5. Risk characterization / conclusions

Limited information exists on the interactive effects when exchanging large parts of the marine feed ingredients, both the fishmeal and the fish oil with plant ingredients, in diets for Atlantic salmon, rainbow trout, cod and halibut. Very few, if any, results from long-term experiments exist.

Most studies report production and health results with single ingredients, immunostimulants, or single nutrients in focus, and are mostly based on studies of short duration, from weeks to a few months. Studies where several plant ingredients are utilized in the diet to maximize plant ingredients without compromising nutrient needs are, however, in progress. Preliminary data show that unexpected results may occur on e.g. changes in lipid metabolism due to simultaneous changes in both the lipid and protein part of the diet. The use of plant ingredients in aquafeeds will expose the fish to various anti-nutritional factors. With their effects on feed intake, digestive processes and possibly metabolism, this will change the recommended levels of various nutrients that need to be present in aquafeeds.

Combinations of various plant ingredients may also give rise to combinations of anti-nutritional factors/plant components, which can have adverse health effects. Whether or not mixtures affect fish health in a different manner than when components are tested individually remains to be clarified. Some plant ingredients have, however, been used for several decades due to their starch contents (energy) and binding properties, especially ground (and extruded) wheat and maize. So far, no adverse effects from these additions have been reported. However, new data are needed when new ingredients or higher levels of plant proteins are added, as these also add to the starch level in the diets. Studies on Atlantic salmon, rainbow trout, cod and halibut have all shown that there are limits to how much dietary starch can be utilized.

It is a prerequisite that the use of feed ingredients of plant origin complies with Norwegian and European feed legislation, which aims to ensure good fish health and consumer safety. The feed ingredients must comply with current maximum levels set for a range of undesirable substances. The list of undesirable substances is, in general, sufficient. However, as feed production is a rapidly developing area with new feed ingredients being introduced frequently, the legislation must develop accordingly to ensure fish health and consumer safety. With the increased use of feed material of plant origin in feed for fish, the list of pesticides and mycotoxins included in the current legislation ought to be revised.

Of importance when considering the nutritional quality of proteins in plant ingredients are the amino acid balance of the protein fraction and whether the amount of any amino acids may be limiting relative to the nutritional needs of the fish. Most plant proteins are limited in certain essential amino acids, most commonly lysine and methionine. Therefore, purified amino acids may need to be added to feeds containing high levels of proteins from plants. On the other hand, other amino acids may be over-abundant in plant ingredients and may lead to imbalances in amino acid availability or even toxic effects.

Many plant ingredients may contain proteins/peptides (protein fractions) that may elicit a hypersensitivity reaction in sensitized mammalian individuals. Generally, antigens are short peptides that may be inherent in the food/feed ingredient or they may be a product of protein digestion. For salmonids, components of soybeans appear to cause an inflammatory response in the distal intestine in all individuals that consume soybean meal-containing diets.

Although T cells appear to be involved in the reaction, it is not clear whether the inflammation is caused by proteins/peptides, anti-nutrients, pathogens (bacteria, virus etc.), or combinations of these. Nor is it clear whether it can be classified as a hypersensitivity reaction since many characteristics of fishes' immune system and its responses are as yet unknown. For example, granules of mast cells in most fish species do not contain histamine and it is therefore questionable whether fish can react allergically to allergens.

Anti-nutrients are defined as innate components of a food/feed ingredient that have a limiting effect on the food/feed intake, digestion, and/or nutrient absorption. The ad hoc group report "Criteria for safe use of plant ingredients in diets for aquacultured fish" lists and describes anti-nutrients present in plant ingredients in detail that may be considered appropriate for use in formulated feeds for farmed fish. The Panel on Animal Feed considers saponins, proteinase inhibitors, and lectins to be the most potentially deleterious to fish health. Tolerance levels for the various known anti-nutrients and thus for inclusion levels for the plant ingredients have been identified in the report when sufficient data is available. Many studies indicate that inclusion levels of a specific plant ingredient of more than 20% of the total diet can have consequences to fish growth and nutrient digestibility and therefore potentially to fish health. Generally, an upper limit of about 20% inclusion level of total diet for most plant ingredients in feeds for carnivorous fish species may appear to be applicable. An exception is full-fat and extracted soybean meal for both Atlantic salmon and rainbow trout, which should be limited to approximately 10 and 5% inclusion levels, respectively, of total diet. However, cod and halibut appear to tolerate these soybean products well, but the exact tolerance level is impossible to state.

Long-term effects of plant ingredients and/or their isolated anti-nutrients have generally not been conducted. Nor is there sufficient data on effects of combinations of anti-nutrients/plant ingredients. The Panel on Animal Feed concludes that further research is needed to identify long-term effects on both individual anti-nutrients and combinations thereof as well as to further characterize plant feedstuffs in order to identify other components that may be deleterious to fish health. Until more information is available, only highly processed plant ingredients should be used. Especially plant ingredients processed using heat in combination with enzyme/fermentation treatment and/or alcohol extraction appears to be well tolerated by carnivorous fish.

Recent data indicate that interactions between anti-nutrients may be of more practical importance to fish/animal health than formerly appreciated. Of particular interest are apparently deleterious interactions between saponins and other anti-nutrients, as well as between lectins and proteinase inhibitors. Such interactions appear to substantially lower the tolerance of even individual feed ingredients, as exemplified by soybean meal's effects in salmonids. Research is needed to study other possible interactions and the mechanisms involved in the potentiating effect such interactions may have. In this way it may also be possible to link practical fish health issues with anti-nutrient exposure over the longer term.

The use of *in vitro* methods may be helpful initially to screen for effects of such interactions on tissue/cell function and viability, especially for anti-nutrients and combinations of anti-nutrients that are difficult to isolate and therefore expensive. However, *in vivo* trials are needed to confirm the *in vitro* data as well as to assess the effects on the whole animal and its health, especially in the long term.

Dietary fibres alter flow, impair interactions, affect intestinal receptors, restrict nutrient diffusion, change microbial diversity and activities, and change absorptive surfaces. The variability of the compounds belonging to the fibre complex and the varying degrees to which they affect these parameters make it difficult to conclude on a general basis regarding maximum inclusion level. Little is known on their specific effects in fish since few experiments have been conducted with isolated fibre in the diets. No data from long-term studies exist, describing fibre effects on fish health.

The addition of medium chain triglycerides (MCT) should be performed with caution with maximum 1-3% of total diet in larval fish. Little data exist on cod, halibut and salmonid fish. In larger fish, both Atlantic salmon and rainbow trout, a cautionary limit of 10% of total diet would not pose any risk nor does 5% of total diet in cod. But this also depends on type of MCT used. Fish may have an absolute requirement for phospholipids (mainly phosphatidylcholine) but exact figures are lacking. The Panel on Animal Feed concludes that a dietary inclusion of 2-6% of total diet for larval fish and 1-3% of total diet for larger fish would not pose any health risk.

In marine fish addition of plant oils must not reduce the level of marine essential fatty acids below minimum requirement levels. The actual level of inclusion will thus depend on type of marine lipid used in the diets. Actual essential fatty acid levels for Atlantic cod and A. halibut is not known but rough estimates exist. Breeders may have higher requirements for some marine PUFAs including arachidonic acid (ARA).

In freshwater fish 100% replacement of fish oil with plant oil is possible as these fish produce their essential fatty acids (marine) from 18:2n-6/18:3n-3 with some question marks for DHA. Essential fatty acid requirements are assumed to be in the range 0.5-1.5% of total diet depending on type of fatty acid and size of fish. Breeders may have higher requirements for some marine HUFAs, including ARA.

Plant oils may alter eicosanoid cascade, immunology and stress responses in salmonid fish, particularly with high alterations in the n-6/n-3 ratio from the typical "marine" ratio. At present, the Panel on Animal Feed would not recommend an inclusion level exceeding 50% plant oils.

Using mixtures of different plant oils (e.g. soybean, linseed, palm oil) producing a “marine” ratio of n-6/n-3 may lower the problems and may increase inclusion levels above the recommended 50% of the dietary lipid without compromising fish health.

Oxidative stress is not a general problem, with the possible exception of linseed oil. An upper inclusion level of 25% of total dietary lipid is suggested, but there are few data available.

As limited knowledge is available about potentially negative effects of dietary β-glucans, alginate, nucleotides or other potent molecules, especially with respect to long-term effects on fish health and efficiency, more knowledge is needed on this topic before it is possible to recommend any inclusion level and/or recommend any limitations in fish feed.

More effort on challenge studies, both *in vivo* and *in vitro*, should be given high priority in the future studies.

As limited information is available about the effect of immunostimulants, prebiotics and nucleotides on gut morphology, these topics should be given priority in future studies. This is of substantial relevance as the gastrointestinal tract of fish is one of the major infection routes.

As only some information is available about the effect of nucleotides on growth, gut function structure and whether toxification is possible, the Panel on Animal Feed is not able to conclude on any limitations or recommended inclusions. Studies on this topic have to be carried out in future.

Interactive effects of immunostimulants and anti-nutritional factors in plants may exist but there is no knowledge available to make any recommendations on e.g. plant-based diets and additions of immunostimulants. However as given in the background for this risk assessment, several of the anti-nutritional factors possess, to a certain degree, some immunostimulatory effects.

Extrusion processing is the predominant method used for shaping fish feeds into particles with suitable shape and handling properties. Due to the heat and moisture applied, this process has a potential to affect chemical components in the feed. These changes may be beneficial in terms of inactivating substances which would otherwise be harmful, or they may be adverse in terms of forming new compounds with harmful effects. At temperatures above 55 °C, the tri-dimensional structure of proteins will be altered through breakage of non-covalent bonds. Heat may therefore affect the functional properties of the proteins. Hence, harmful proteins such as enzyme inhibitors may be eliminated through feed processing. Amino acid racemisation and protein cross-links may take place during processing. These heat-induced chemical changes may reduce protein digestibility, but will mainly be formed under conditions of high pH.

Fibre is a component strictly associated with plant material. The use of plant ingredients will therefore inevitably result in fibre being introduced into fish feeds. It has been shown that solubility of fibres may increase with processing. Thus, plant fibres may increase their anti-nutritive properties as a consequence of extrusion processing. Soluble fibres with anti-nutritive properties are mainly found in fibre-rich cereals such as rye, oats and barley, although wheat may also exhibit anti-nutritive properties caused by soluble fibres. Since small quantities of these cereals are used in fish feeds, soluble fibre does not appear to be a major problem.

Processing aids are components that are primarily added to the feed mix prior to processing to facilitate processing and/or enhance physical quality of the feed. Such aids are not very commonly used in extruded feeds for fish, but may include fibrous viscous components and enzymes. Fibrous components may have adverse effects as described for fibres above, while the enzymes added to aid flow are not likely to have any negative effects due to the fact that they do not hydrolyse feed components into harmful substances, e.g. amylase.

Processing of diets for fish may potentially alter nutritional properties of plant materials. Processing, particularly of raw materials, may remove or inactivate harmful substances. Extrusion processing has the potential to induce chemical changes which may result in appearance of harmful substances. However, the negative effects appear to be modest under practical conditions.

6. CHALLENGES (GAPS OF KNOWLEDGE)

As seen throughout the report of the *ad hoc*-group, most results have been obtained from short term studies, which have focused on single dietary ingredients or even single components specific for the ingredients. Very few studies have been performed to form a basis for evaluating the long-term effects, as was requested of this assessment. There is, accordingly, a need for studies investigating long-term effects and combined effects in fish when using feed plant ingredients (both plant proteins and plant lipids).The consequence not only for the fish itself, but for the final product quality, e.g. changes in nutrient composition, is also important.

For the plant proteins new data are needed when new ingredients or higher levels of plant proteins are added. Studies of the long-term effects of plant ingredients and/or their isolated anti-nutrients have generally not been conducted. Nor is there sufficient data on the effects of combinations of anti-nutrients/plant ingredients. Further research is needed to identify long-term effects of both individual anti-nutrients and combinations thereof as well as to further characterize plant feedstuffs to identify other possible components that may be deleterious to fish health.

The use of *in vitro* methods may be helpful to initially screen for effects of such interactions on tissue/cell functions and viability, especially for anti-nutrients and combinations of anti-nutrients that are difficult to isolate and therefore expensive. Ultimately, however, *in vivo* trials are needed to confirm the *in vitro* data as well as to assess the effects on the whole animal and its health, especially in the long-term studies.

Actual essential fatty acid requirements for Atlantic cod and Atlantic halibut are unknown and only rough estimates exist for practical diets to salmon and trout. There is only fragmentary knowledge on the long-term effects of the different plant lipids on fish health and welfare. There is also a general lack of knowledge on the effects of dietary interactions with other feed components, e.g. various oil sources, lipid-protein interactions, and lipid-vitamin interactions.

For the immunostimulants more efforts on challenge studies, both *in vivo* and *in vitro*, should be given priority in future studies. The use of short-time studies is insufficient as a basis to conclude on any limitations or recommended inclusions.

Interactive effects of immunostimulants and anti-nutritional factors in plants may exist but knowledge is lacking, and no recommendation is possible to give on e.g. plant-based diets and additions of immunostimulants.

New feed ingredients are being introduced frequently and the feed legislation must develop accordingly to ensure fish health and consumer safety. Revisions of the current legislations must be based on risk assessments which rely on scientific data. Today such data are scarce, and there is a need for studies on known and emerging undesirable substances in feed ingredients of plant origin. Such studies include a characterization of their occurrence, their effects in fish, and the possible transfer of the compounds from feed to fish fillet.