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Ås, March 2011
Jon Øvrum Hansen

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List of Abbreviations

CCAMLR = Convention for the Conservation of Antarctic Marine Living Resources

F^- = fluoride

FCR = feed conversion ratio

PDKM = partly deshelled krill meal

PPC = pea protein concentrate

SGR = specific growth rate

WKM = Whole krill meal

List of Papers

I. Hansen, J.Ø., Penn, M., Øverland, M., Shearer, K.D., Krogdahl, Å., Mydland, L.T., Storebakken, T., 2010. High inclusion of partially deshelled and whole krill meals in diets for Atlantic salmon (*Salmo salar*). Aquaculture. 310, 164-172.

II. Hansen, J.Ø., Shearer, K.D., Øverland, M., Penn, M., Krogdahl, Å., Mydland, L.T., Storebakken, T., 2011. Replacement of LT fish meal with a mixture of partially deshelled krill meal and pea protein concentrates in diets for Atlantic salmon (*Salmo salar*). Aquaculture. In Press.

III. Hansen, J.Ø., Penn, M.H., Shearer, K.D., Storebakken, T., Øverland, M. Tissue fluoride accumulation and kidney lesions in freshwater reared Atlantic salmon (*Salmo salar*) fed high dietary fluoride concentrations. Submitted to Aquaculture Nutrition

IV. Hansen, J.Ø., Shearer, K.D., Øverland, M., Storebakken, T. Dietary calcium supplementation reduces the bioavailability of fluoride from krill shell and NaF in rainbow trout (*Oncorhynchus mykiss*) reared in fresh water. Aquaculture. Accepted manuscript.

Abstract

Antarctic krill (*Euphausia superba*) may be the largest biomass of a single species worldwide and is a promising ingredient for use in fish feeds. Krill have a balanced amino acid profile, and krill lipids are rich in phospholipids with a high concentration of n-3 polyunsaturated fatty acids. In addition, krill are highly palatable to fish. The limitations in availability of fish meal worldwide, and the increasing decline in pelagic fish populations increase fish meal prices. Use of krill in fish feed must be funded on science-based, sustainable krill harvest. The main objective of this thesis was to enhance understanding of Antarctic krill as a feed ingredient for salmonids. The sub objectives were to increase knowledge regarding: 1) nutritional effects of krill shell in fish feeds; 2) response to different dietary levels of krill meal in Atlantic salmon (*Salmo salar*); and 3) uptake and accumulation of fluoride in salmonids reared in fresh or salt water.

Atlantic salmon reared in salt water, fed whole krill meal (WKM) at the expense of fish meal, grew at a lower rate during the first feeding period compared to fish fed fish meal or partly deshelled krill meal (PDKM). In a second experiment, increasing levels of a PDKM:pea protein concentrate (PPC) mixture (3.5:1) in diets for Atlantic salmon showed an optimum growth rate at a PDKM/PPC level of 400 g kg^{-1}. In both experiments there were larger differences in growth rates and/or weight gain between the krill fed fish and the fish meal control group during the early part of the experiment compared to the total feeding period.

Complete replacement of fish meal with WKM in diets for Atlantic salmon, reared in salt water, tended to reduce lipid digestibility compared to a replacement with PDKM. Increasing dietary level of a PDKM/PPC mixture, on the other hand, gave a linear increase in lipid digestibility. Salmon fed WKM or an increased level of PDKM/PPC had lower digestibility of several amino acids compared to those fed fish meal as the sole protein source. The high dietary copper level in the WKM and PDKM were excreted through the salmon faeces. There was no increase in copper accumulation in liver or whole body of salmon fed these diets.

Histological changes in the kidney where observed in salmon fed diets with WKM or PDKM. These changes were described as mild to moderate degeneration, apoptosis and/or necrosis of renal tubule cells. Similarly, feeding increasing levels of a PDKM/PPC mixture to salmon produced dose dependent histological changes in the kidney. Despite the low dietary

bioavailability of fluoride in salt water reared fish, we hypothized that the high dietary fluoride level in the krill meals caused the observed kidney changes. This was based on findings in other animals. High dietary fluoride fed to salmon reared in freshwater did, however, not induce similar kidney changes as seen in the krill fed salmon reared in salt water. Freshwater reared salmon fed diets with a fluoride level at 3,500 mg kg^{-1} from sodium fluoride (NaF), however, had crystalline material within distal tubules and/or collecting ducts in the kidney, which probably are small ureterolithiasis (commonly called kidney stones).

Dietary fluoride in the form of NaF was quickly absorbed and reached a peak in plasma fluoride at three hours in rainbow trout (*Oncorhynchus mykiss*). Trout fed fluoride from krill shell did not show the same clear peak in plasma concentration, and the estimated cumulative absorption of fluoride was reduced by nearly that half compared to the NaF fed fish. Supplementation of dietary calcium nearly prevented the fluoride uptake in fish fed the NaF diet, while adding calcium to the diets with krill shell reduced the uptake to nearly the half.

Atlantic salmon reared in freshwater and fed a low-fluoride control diet or two diets with fluoride levels of 150 or 350 mg kg^{-1} with krill shells as fluoride source had higher faecal excretion of fluoride compared to salmon fed fluoride levels of 1,500 and 3,500 mg kg^{-1} from NaF. Salmon fed the two krill shell diets showed no difference in fluoride accumulation in whole body, bone, muscle, liver, or kidney compared to the low-fluoride control fed fish. Fish fed the NaF diets showed increased fluoride accumulation in both liver and kidney, and especially in the bones.

The major implications of this research for practical feed production are that when using a high inclusion of kill in diets for Atlantic salmon, a partly deshelled krill meal is preferred to avoid possible negative effects of the shell fraction on growth rates and nutrient digestibility. Diets containing 150 and 350 mg fluoride kg^{-1} from krill did not cause any histological changes in gills, liver or kidneys of Atlantic salmon reared in freshwater. The high concentration of calcium and magnesium in salt water is probably the main cause for the strong limitation in fluoride uptake in salmon.

Sammendrag

Den totale biomassen av Antarktisk krill (*Euphausia superba*) er trolig verdens største av en enkelt art og er en lovende ingrediens for bruk i fiskefôr. Krill har en balansert aminosyreprofil og fettet i krill er rik på fosfolipider som inneholder en høy andel flerumettede n-3 fettsyrer. Krill er også en god smaksattraktant i fiskefôr. Den begrensende tilgangen av fiskemel og den tiltagende reduksjonen i pelagiske fiskepopulasjoner har ført til en økning i prisen på fiskemel. Bruk av krill i fiskefôr forutsetter at ressurser blir høstet på en bærekraftig måte med forankring i forskning. Hovedmålet med denne doktorgraden var å øke kunnskapen om krill som fôringrediens til laksefisk. Undermålene var å bidra til økt forståelse av: 1) ernæringsmessige effekter av krillskall i fiskefôr, 2) laksens (*Salmo salar*) respons til ulike mengder krillmel i fôret, og 3) opptak og akkumulering av fluor hos laksefisk i fersk- og sjøvann.

En fullstendig erstatning av LT fiskemel med krillmel med skall (WKM) i fôr til laks i saltvann gav lavere vekst i første del av fôringsperioden sammenlignet med laks fôret et fiskemelbasert fôr eller fôr der fiskemelet ble erstattet med et delvis avskallet krillmel (PDKM). En økende erstatning av fiskemel med en blanding av PDKM og erteprotein-konsentrat (PPC) (3.5:1) i fôr til laks førte til raskest tilvekst ved et inklusjonsnivå på 400 g av blandingen per kg i det andre fôringsforsøket. I begge disse forsøkene var det større forskjell i veksthastighet og/eller vektøkning mellom fisk fôret med krill og fiskemel i første del av fôringsforsøket sammenlignet med hele foringsperioden.

Når laks i saltvann ble gitt fôr der fiskemel var fullstendig erstattet med WKM, var det en tendens til redusert fordøyeligheten av fett sammenlignet med laks som fikk PDKM. En lineær økning i fettfordøyelighet ble påvist i laks fôret med økende andel av PDKM/PPC. Laks fôret WKM eller økende nivå av PDKM/PPC hadde lavere fordøyelighet av flere aminosyrer sammenlignet med dem som hadde fiskemel som eneste proteinkilde. Det høye innholdet av kobber i WKM og PDKM ble effektivt skilt ut i laksens avføring. Det var ingen økt akkumulering av kobber i verken lever eller helkropp i laks som fikk krill i fôret.

Både laks fôret med WKM og PDKM hadde histologiske forandringer i nyrene. Forandringene kan beskrives som mild til moderat degenerering, apoptose og, eller nekrose av tubulicellene i nyrene. Økende innblanding av PDKM/PPC i fôret gav en doseavhengig forandring i nyrevevet. Selv om biotilgjengligheten av fluor for fisk i saltvann er lav testet vi

hypotesen at disse histologiske forandringene ble forårsaket av det høye fluornivået i krillmelene. Dette var basert på resultater fra andre dyreslag. Høye fluornivåer i fôr til laks i ferskvann forårsaket imidlertid ikke de samme nyreforandringer som ble oppdaget hos laks fôret krill i sjøvann. En fluormengde på 3500 mg fluor per kg fôr (NaF) førte imidlertid til krystalliserte strukturer, antageligvis små nyresteiner, i de distale tubuli og/eller samlerørene i nyrene hos laks i ferskvann.

Fluor i form av NaF i fôret ble hurtig tatt opp i blodet hos regnbueørret (*Oncorhynchus mykiss*), og nådde en topp i blodplasma etter tre timer. Ørret fôret med krillskall som fluorkilde oppnådde ikke denne samme tydelige toppen i plasmafluor og den estimerte kumulative absorpsjonen var halvert sammenlignet med fisk fôret med NaF. Tilsetting av ekstra kalsium i fôret forhindret opptak av fluor i fisk fôret med NaF og halverte opptaket hos fisk fôret med krillskall som fluorkilde.

Laks i ferskvann som fikk en lav-fluorkontroll og to fôr med 150 og 350 mg fluor kg fra krillskall hadde høyere utskillelse av fluor i avføringen sammenlignet med laks fôret med 1500 og 3500 mg fluor kg med NaF som fluorkilde. Laks som fikk de to fôrene med krillskall akkumulerte ikke mer fluor i muskel, bein, lever, nyre, eller totalt for hele kroppen enn fisk fôret lav-kontrolldietten. Laks som fikk høy konsentrasjon av NaF i fôret akkumulerte fluor både i lever og nyre, og spesielt i bein.

De viktigste implikasjonene av dette arbeidet for praktisk fôrproduksjon er at hvis store mengder krill skal inngå i fôr til laks, bør delvis eller helt avskallet krill benyttes for å unngå eventuelle negative effekter fra krillskallfraksjonen på tilveksthastighet og fordøyelighet av næringsstoffer. Fôr som inneholdt 150 eller 350 mg fluor kg^{-1} fra krillskall forårsaket ingen histologiske forandringer i verken gjeller, lever eller nyre hos laks i ferskvann. Det høye innholdet av kalsium og magnesium i sjøvann er sannsynligvis årsaken til redusert opptak av fluor fra krill hos laks.

1. General introduction

The Norwegian salmon (*Salmo salar*) farming industry began in the early 1960`s with simple wooden sea cages, but developed into Norway's second largest export industry after oil. In 2010, salmon production was estimated at 925,000 tons, of which 784,000 tons, with a value of 31.4 billion NOK, was exported. As in Norway, the global farming of carnivorous species has increased. Together with the increased farming of warm-water fish such as different species of catfish and tilapia, the demand for marine protein sources are increased (FAO, 2010).

In 2008, the aquaculture industry used 68 and 88% of the world's production of fish meal and fish oil, respectively (Tacon and Metian, 2008). For carnivorous species such as Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and cod (*Gadus morhua*), the feed cost represents approximately 50% of the production cost. Fish meal production has been relatively constant for several decades. However, an increase in the size of the global aquaculture industry and the fact that fish meal production is limited has resulted in raising prices, reaching as much as $2,000 per tonne.

Efforts to replace fish meal have been focused primarily on plant ingredients. Considerable progress has been made in replacing fish meal with a variety of plant protein ingredients such as soybeans, lupins, peas, and canola (Gatlin et al., 2007). Although these fishmeal alternatives are highly available at a low cost, they represent several challenges including a lower content of protein and energy, an unbalanced amino acid composition, a higher content of fiber and non-starch polysacarides, and the presence of a wide range of anti nutritional factors. This may lead to reduced feed intake, growth and affect health (Gatlin et al., 2007; Naylor et al., 2009). Great focus on sustainability has also reached the aquaculture industry. With declining wild fish stocks worldwide, the aquaculture industry has focused on replacing fish meal with plant ingredients to achieve a more sustainable feed. The question that should be addressed and kept in mind is, how sustainable is world plant production, with limited top soil, freshwater and fertilizers.

Marine ingredients such as krill, with high protein content, favorable amino acid and fatty acid profiles and palatability enhancement properties, may counteract some of the adverse effects associated with plant ingredients. There is, however, also challenging properties with the krill (Storebakken, 1988) and the purpose of this thesis work was to increased knowledge of Antarctic

krill (*Euphausia superba)* as a feed ingredient for salmonids. Of specific interest were the krill shell fraction and the high fluoride content.

2. Background

2.1 Antarctic krill, *Euphausia superb*a

2.1.1 Biology and harvesting

The Antarctic krill is a shrimp-like crustacean in the order Euphausiacea. The name krill originates from Norwegian and means "young fish" and is now used as a term for members of the family euphausiids. *E. superba* are located in the Antarctic Ocean and are mainly found below 50° S, with the highest concentrations in the Atlantic sector. For the sake of simplicity, Antarctic krill will be referred to as krill in this thesis. Krill are filter-feeders and feed on phytoplankton. Generally, during spring and summer when the phytoplankton bloom, krill are distributed in the oceanic regions, but they move toward the coastal regions during autumn (Kawaguchi and Nicol, 2007). Krill can be 6-7 cm long and live for up to seven years. Krill have been studied both in controlled laboratory experiments and in field studies (Nicol, 2000). Krill have been observed to shrink after excessive starvation for (211 days), and this reduction in body size is believed to be an adaptation to facilitate over-wintering (Ikeda and Dixon, 1982). This shows that a traditional length-based aging method of the krill has limited relevance.

Krill form the basis of the eco pyramid of consumers in the Antarctic ecosystem and serve as food for the birds, fish, whales and seals. One of the biggest consumers of krill is the crabeater seal (*Lobodon carcinophagus*) (Mori and Butterwoth, 2006), which have an estimated population of approximately 15 million. The baleen whale population in the south Antarctica was heavily harvested early in the 20th century, which has led to the concept of the great whale krill surplus. This theory is just a part of the discussion of the size of the biomass of what may be the largest population of a single invertebrate species on earth. The krill biomass is estimated to be between 67 and 297 million tons (Siegel, 2005). Knox (2007) describes the challenges of obtaining a reliable estimate of the standing biomass of Atlantic krill.

The CCAMLR (Convention for the Conservation of Antarctic Marine Living Resources) is the organization that manages the krill harvest in the Southern Ocean. During the CCAMLR-2000 Survey, CCAMLR estimated the krill biomass to be 37 million tons for the southwest Atlantic which formed the basis of an estimation of a precautionary yield of 3.47 million tons in this area. The estimated total krill catch is believed to be 150,000-180,000 tons for 2010, which

is approximately a 40% increase since 2009 (Schiermeier, 2010). The total catch for 2010 will also be higher compared to years since 2000, but less than in the late 1980's when the total catch was up to 400,000 tons (Fig. 1).

The krill fishing fleet has developed new and improved techniques to harvest and process the krill onboard. This has improved the potential use of krill for both food and feed production, and has led to an increased interest in krill worldwide. In addition to Norway's three krill harvesting vessels, China expects to increase its harvesting of krill and Russia is building two large krill harvesting vessels equipped with on-board processing equipment and is expected to be fishing in 2011. This increased interest in krill harvesting, together with the concern of climate change and its possible negative effect on krill populations (reviewed by Nicole, 2006; Siegel, 2010) shows the need for increased research on management of the Antarctic krill resources to ensure sustainable population in the future.

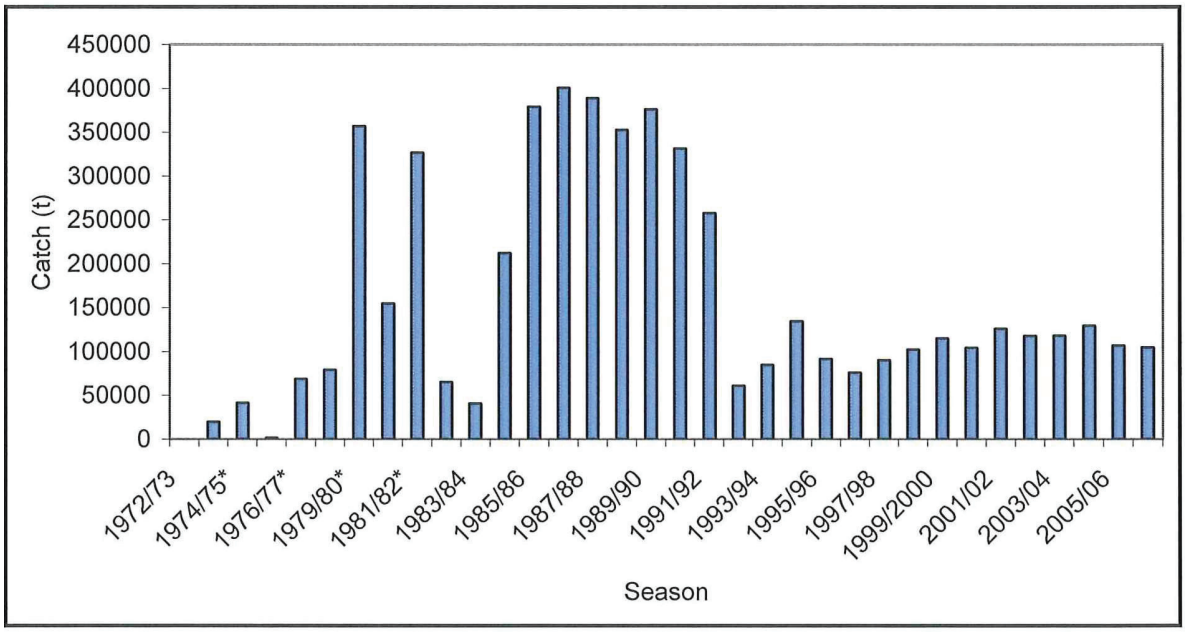

Figure 1. Annual Antarctic krill catches in 1972-2006 in Area 48 (Medley et al., 2009), the predominant area for krill harvesting and is described in Thomson et al. (2000).

2.1.2 Chemical composition

Fresh krill contain approximately 20% dry matter, moisture free the approximately chemical composition is 60 to 78% crude protein, 7 to 26% crude lipid and 12 to 17% ash (reviewed by Martin, 1979; Storebakken, 1988; Tou et al., 2007). The high variation in chemical content is a result of complex interaction between sex, age-classes, season and area of harvest, but the primary cause of the high variation in lipid content is due to the reproductive investment of female krill (Pond et al., 1995). The high variation in lipid content will then also affect the proposition of the other nutrients when values are expressed on a dry basis. The mineral content in meals from krill and amphipods is described by Moren et al. (2006). In krill meal, both fluoride and copper exceeded the EU upper limit of feedstuffs, whereas arsenic, cadmium, lead and mercury were all within the EU limit.

CRUDE PROTEIN AND AMINO ACIDS

Based on traditional methods for feed analysis, crude protein is determined by multiplying the total nitrogen content in the sample by a factor of 6.25. Krill exoskeleton contains chitin, a glucosamine polymer. The nitrogen content in this molecule will result in bias in estimates of protein in krill when protein is based on total nitrogen content only. In addition, other non-amino nitrogen compounds such as nucleotides, volatile bases, and trimethylamine will contribute to the crude protein estimate. The amino acid composition of krill meets the amino acids requirements for both Pacific salmon and Atlantic salmon (Table 1). The free amino acid fraction in freshly caught krill is 7-8% of dry weight (Ellingsen and Mohr, 1979). This, together with the high protein solubility of krill, explains the high nitrogen content found in the water soluble fraction during krill processing (Kolakowski, 1989).

The content of non-protein compounds, especially free amino acids and trimethylamine oxide can be high in krill (Ellingsen and Mohr, 1979). One reason for this is the highly reactive hydrolytic enzymes, including proteases, nucleases and phospholipases found in the digestive tract of krill which start to break down the tissue *post mortem* (Anheller et al., 1989). The enzymes present in krill are adapted to the low temperature Antarctic environment, and even at storage temperatures below freezing the enzymatic break down is ongoing (Ellingsen and Mohr,

1987). Due to this, krill easily spoil and a rapid processing of krill after harvest is necessary to inhibit these enzymes. Heat/boiling is used to stop hydrolysis. A fairly new fishing method, which uses a pump that continuously extracts krill from the cod end of the trawl, is a more gentle way of landing krill (CCAMLR, 2004). This inhibits crushing of the krill in the trawl which reduces spoilage of krill post mortem (Ellingsen and Mohr, 1987).

Table 1. Recommended dietary amino acid compositions for Pacific salmon (*Onchorynchus* spp.) and Atlantic salmon compared to amino acid levels in krill meal and LT fish meal (based on Nicol et al., 2000)

	Requirement		Composition		
% of crude protein	Pacific salmon[1]	Atlantic salmon	Krill meal[2]	Krill meal[3]	LT fish meal[4]
Arginine	5.4	5.1[5]	5.8	4.8	5.6
Histidine	1.6		1.9	1.6	1.9
Isoleucine	2.0		7.5	4.1	3.9
Leucine	3.5		4.9	6.3	6.9
Lysine	4.5	5.0 [6]	6.5	6.2	6.9
Methionine + cysteine	3.6	2.4 [7]	3.8	3.4	3.6
Phenylalanine + tyrosine	4.6		8.9	6.3	5.9
Threonine	2.0	2.7 [8]	4.0	4.0	4.3
Tryptophan	0.4		1.2-1.5	ND[9]	ND
Valine	2.9		5.3	4.2	4.5

[1]NRC (1993).
[2]Rehbein (1981).
[3]Analyzed values from partly deshelled krill meal (Paper I).
[4]Analyzed values from Paper I.
[5] Post-smolt, Berge et al. (1998).
[6] Fish size: 600-1,000 g, Espe et al. (2007).
[7] Fingerling, only methionine, Rollin et al. (1994).
[8] Fingerling, Bodin et al. (2008).
[9] Not determined.

LIPIDS

As mention previously, the total lipid level in krill varies to a great extent (Pond et al., 1995). It differs from fish oil, in that the amount of phospholipids is higher in krill oil with levels ranging between 30 and 51% of total lipids (Clarke, 1980; Fricke et al., 1984; Gigliotti et al., 2010). The major group of phospholipids found in krill is phosphatidylcholine, 33.3 to 35.6% of total lipids (Fricke et al., 1984; Winther et al., 2010). A total of 69 different choline-containing phospholipids have been reported in krill, seven of these probably have an omega 3 fatty acid attached to both sn-1 and sn-2 position of the glycerol molecule (Winther et al., 2010). The fatty acids in krill are similar to those found in other crustaceans and marine fish and vary with the total lipid content (Fricke et al., 1984; Virtue et al., 1993).

CHITIN

The exoskeleton of all crusteances including krill contains chitin. Chitin is a long chain polymer, similar to cellulose, except that the chitin polymer has replaced a hydroxyl group at the C-2 position of each glucose unite with an acetylamino group, CH_3CONH- (Hart et al., 1999). This acetyl group forms a hydrogen bond with the OH group in C-5 position of the next glucose molecule in the sugar chain, which creates a more stable fiber structure than cellulose. The presence of these free acetylamino groups provides bindings sites for proteins, which further explains chitins ability to inhibit enzymes (Kilinç et al., 2006). The chitin content in krill is around 30 g kg^{-1} dry weight (Nicol and Hosie, 1993). Chitin has been shown to depress growth in juvenile rainbow trout at dietary levels up to 250 g kg^{-1} (Lindsay, 1984), and in juvenile carp (*Cyprinus carpio*) at of 10 g kg^{-1} (Gopalakannan and Arul, 2006). Furthermore, increased dietary chitin decreased lipid digestibility in juvenile tilapia (*Oreochromis niloticus* x *O. aureus*) (Shiau and Yu, 1999).

2.1.3 Krill in fish feed

Storebakken (1988) summarized several experiments where krill was tested as a protein source for rainbow trout where total replacement of fish meal with krill meal caused either reduced or similar growth as the fish meal control. Partial replacement of fish meal with krill showed similar, or better growth performance. Recent findings, however, showed reduced specific growth rate (SGR) of rainbow trout fed 30% replacement of fish meal with krill meal compared to a fish meal control (Yoshitomi et al., 2006). Further, they found no growth reduction in rainbow trout fed a diet with total replacement of fish meal with a deshelled krill meal (Yoshitomi et al., 2007). Olsen et al. (2006) also reported no reduction in specific SGR in Atlantic salmon fed a diet with a total replacement of fish meal with krill meal compared to a fish meal control. Fish fed diets were 20 or 40% of the fish meal was replaced with krill meal had better SGR compared to the fish meal control. Olsen et al. (2006) concluded that krill meal could fully replace fish meal in diets to Atlantic salmon without effecting growth, feed utilization or fish health. In addition, Suontama et al. (2007a) described no negative effects on Atlantic salmon reared in sea water when fed diets where fish meal was replaced with up to 60% Northern krill (*Thysanoessa inermis*), 40% Antarctic krill (*Euphausia superba*), or 40% Arctic amphipod (*Themisto libellula*), respectively. From the latter experiment, the Atlantic salmon fed a diet were 40% of the fish meal was replaced with Antarctic krill meal obtained better SGR than fish fed a fish meal control for the first 100 days of feeding. Additional studies have focused on the effects of dietary krill meal supplementation on sensory attributes and final product quality of Atlantic salmon and Atlantic cod (Karlsen et al., 2006; Suontama et al., 2007b).

Taste substances in fish usually have a molecular weight less than 10,000 Daltons. These are molecules such as free amino acids, betaines, nucleotides, nucleosides, amines, sugars and organic acids (reviewed by Kasumyan and Døving, 2003). Feeding stimulants have been shown to improve feed acceptability and growth performance of striped bass (*Morone saxatilis*) (Papatryphon and Soares, 2000), and Atlantic salmon (Toften et al., 1995; Toften and Jobling, 1997). Krill contain high levels of soluble proteins (Kolakowski, 1989) and free amino acids (Ellingsen and Mohr, 1979), and this is probably why krill meal has been shown as a good feeding stimulant for sea bream (*Pagrus major*) (Shimizu et al., 1990), largemouth bass (*Micropterus salmoides*) (Kubitza and Lovshin, 1997) and Nile tilapia (*Oreochromisniloticus,* L)

(Gaber, 2005). Krill hydrolisates have also been shown to be a good feed attractant for fish. Improved feed intake was obtained by adding hydrolisates through surface coating compared to adding the hydrolisate in the dry feed mixture prior to pelleting (Oikawa and March, 1997; Kolkovski et al., 2000). In addtion, freeze-dried krill, including the soluble protien fraction, stimulated feeding activity of juvenile Atlantic cod and Atlantic halibut (*Hippoglossus hippoglossus*) which resulted in increased growth performance and nutrient utilization (Tibbetts et al., 2010).

2.2 Fluoride

Fluoride is the anion (F^-) of fluorine, a pale yellowish gas categorized as a halogen in the Periodic system. It is one of the most electronegative elements. Due to its high ability to react with other elements, such as calcium and hydrogen, fluoride does not exist as a free element in nature. In nature, fluoride is found mainly in combination with calcium as fluorite (CaF_2) or as fluorapatite ($Ca_5(PO_4)_3F$) in the earth's crust. Fluoride is a highly toxic element, and the toxicity was discovered nearly 1000 year ago when cattle on Iceland died after grazing land exposed to volcanic ash with a high fluoride content (McDowell, 1992). The farmers also found out that the animals recovered after feeding hay harvested prior to the eruption.

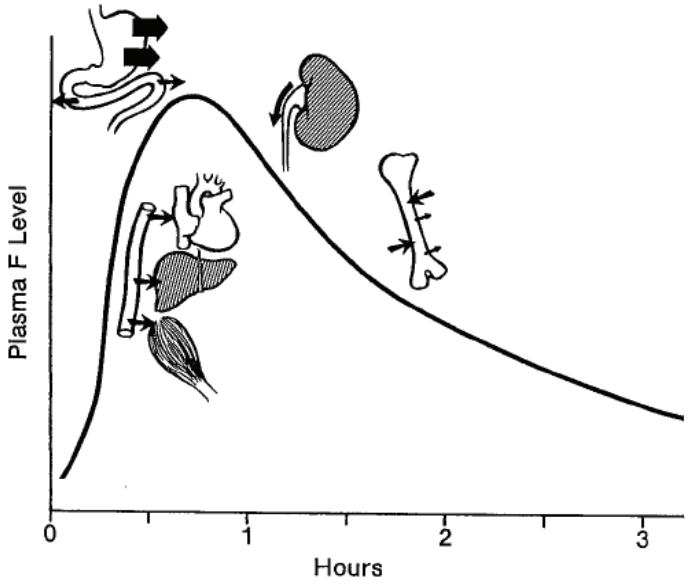

Figure 2. A typical plasma fluoride concentration curve after ingestion of a small dose of fluoride in humans (Whitford, 1996).

2.2.1 The metabolism of fluoride

Fluoride metabolism in fish has not been widely investigated; therefore, this section contains information from experiments with terrestrial animals as well as fish. Fluoride is taken up by the lungs if an animal is exposed to hydrogen fluoride (HF) gas, or through the oral cavity, stomach or intestine. In fish exposed to water-borne fluoride the gills are probably the main organ of absorption. Neuhold and Sigler (1962) found decreased sensitivity to fluoride in rainbow trout when chloride was added to the water or the fish were tempered to chloride prior to the fluoride treatment. They suggest that chloride stimulated cells in the gills excreted not only chloride but also fluoride thereby reducing toxicity.

When fluoride enters the stomach of terrestrial animals, it reacts with the HCl in the stomach and HF is created and easily absorbed through the stomach wall (Whitford and Pashley, 1984). After ingestion, depending on the source of fluoride, about 30 to 50% of the fluoride is absorbed as HF in the stomach (Messer and Ophaug, 1993; Whitford, 1996). Peak plasma concentration is reached at about 30-60 min (Fig. 2). The uptake mechanism of fluoride in the intestinal tract has been reviewed by Whitford (1996) and both diffusion and active transport have been suggested. In humans, roughly 90% of the excreted fluoride is excreted through the kidneys (Maheshwari et al., 1981). This is linearly related to the glomerular filtration rate (Schiffl and Binswanger, 1982). Whitford (1994) also showed that fluoride from plasma can migrate into the intestinal lumen, be trapped, and account for a significant portion of fluoride exposure when rats were given fluoride through a miniosmotic pump.

Bone is a major sight for fluoride accumulation in fish, as it is for terrestrial animals. This has been shown for rainbow trout and carp exposed to water-borne fluoride (Neuhold and Sigler, 1960), and for rainbow trout exposed to dietary fluoride (Tiews et al., 1982; Landy, 1988). Whether fluoride is excreted through the kidneys and urine of fish is questionable. The Indian carp (*Labeo rohita*), showed toxic effects in the kidneys, similar to that observed in mammals, after being exposed to water-borne fluoride (Bhatnagar et al., 2007). This suggests that fish kidneys also excrete fluoride.

Fluoride concentrations are approximately the same in plasma and in the intercellular space and the fluoride level in a given organ is therefore dependent on the tissue-water-to-plasma-water (T/P) concentration ratio (Whitford et al., 1979; Armstrong and Singer, 1980). The

kidney is the only soft tissue that might contain a marked increase in fluoride level, about four times higher than other soft tissues (Whitford, 1996). HF is a weak acid with a pK_s=3.1. At a pH of 7, a small amount of HF is present. Studies show that the cell membrane is more or less impermeable to fluoride and that HF has a 10^6 times greater permeability coefficient than fluoride (Whitford and Pashley, 1984). This shows that the amount of HF is pH dependent, and a pH gradient between cells and fluids will increase the permeability of fluoride. This produces an increased reabsorption of fluoride with decreased pH of the tubular fluid in the kidney nephrones (Whitford et al., 1976).

2.2.2 Fluoride in krill

Several crustaceans from both the southern oceans and the Atlantic have been shown to accumulate levels of fluoride up to 2,400 mg kg^{-1} (Soevik and Braekkan, 1979; Sands et al., 1998). The fluoride concentrates in the exoskeleton with the highest levels found in the mouthparts with levels up to 13,000 mg kg^{-1} (Sands et al., 1998). The reason for the high fluoride levels in krill is unknown and the specific chemical form of the fluoride present has not been reported. Murano et al. (1979) suggested that the accumulation of fluoride in the exoskeleton was a detoxification of the body when the fluoride was lost with the shell during moulting. Zhang et al. (1993a; 1993b) suggested that the fluoride in krill acted as a hardener in the shell fraction as fluorapatite and that the inner layer of the exoskeleton contained more fluoride than the outer layer. They also believed that the fluoride was not linked to the chitin fraction. Sands et al. (1998), however, disagreed, suggesting that the fluoride probably was removed during analyses of the chitin conducted by Zhang et at. (1993a). They also showed that the fluoride in krill was not immobilized by formalin storage and therefore leached out of all sections during storage. Similar results have been reported previously for krill kept in frozen storage (Christians and Leinemann, 1983). This experiment together with the findings of Tenuta (1993) shows that the fluoride in krill is water soluble.

The high fluoride levels in krill were one reason for the decline in further development of the krill fisheries in the late 1980's. High dietary levels of fluoride are toxic for humans and terrestrial animals, and fluoride from krill has shown to be highly available for humans (Trautner and Siebert, 1986), rats (Alvarenga and Tenuta, 1993) and chickens (Soevik and Braekkan,

1981). The bioavailability of fluoride to fish from krill is dependent on several factors. One main factor is the hardness of the water and experiments have been shown that fish reared in brackish or freshwater (Grave, 1981; Yoshitomi et al., 2006) accumulate more fluoride than fish reared in salt water (Julshamn et al., 2004; Moren et al., 2007). Yoshitomi et al. (2006) fed rainbow trout in freshwater with Anatarctic krill and showed a reduction in growth when 30% of the fish meal was replaced with krill meal. This diet had 444 mg F^- kg^{-1} diet, and the authers suggested that the fluoride accumulation in the bones led to this growth depression. Limeted fish experiments have shown neagative effects of dietary fluoride attributed to krill with exept of Weirich et al. (2005), who reported decreased survival of channel catfish (*Ictalurus punctatus*) fry fed diets supplemented with high fluoride krill meal. The fish had increased fluoride levels in whole body and the authors suggesting that the reduced survival was due to fluorosis. Vertebral bones of Antarctic marine fish caugth at the South Shetland islands showed relatively high fluoride content between 616 and 1,207 mg kg^{-1} (Oehlenschläger and Manthey, 1982). This marine fish is feeding mainly on krill, indicating that there may be variability among marine fish species in their ability to accumulate fluoride.

2.2.3 Fluoride toxicity in fish

Water-borne fluoride toxicity has been widely examined in fish (reviewed by Camargo, 2003). Generally, the toxicity of water-borne fluoride is dependent on exposure time, water hardness, fish size (Angelovic et al., 1961; Pimentel and Bulkley, 1983) and water chloride concentration (Neuhold and Sigler, 1962). In soft water (less than 1.3 mg l^{-1} of calcium and magnesium), 50% of the rainbow trout died within 72 hours when exposed to 4 mg F^- l^{-1} (Neuhold and Sigler, 1962). While in hard water (45 mg calcium l^{-1}) no mortalities were shown for rainbow trout exposed to 75 mg F^- l^{-1} (Herbert and Shurden, 1964). Acute water-borne fluoride toxicity is displayed by rainbow trout as initial apathy and a non-feeding behavior followed by sporadic movements, loss of equilibrium and finally death (Neuhold and Sigler, 1960). This behavior is similar to terrestrial animals exposed to acute fluoride poisoning and is partly a result of reduced calcium ion concentration in the blood when fluoride is forming stable complexes with calcium. It has, however, been shown that fish reared in both salt and freshwater can tolerate high levels

of dietary fluoride. As mentioned previously, fish reared in salt water did not accumulate dietary fluoride indicating a low bioavailability of fluoride (Julshamn et al., 2004; Moren et al., 2007). It does, however, accumulate in bones in fish reared in freshwater (Landy, 1988; Yoshitomi et al., 2006). In addition, fish reared in freshwater appear to have an extreme ability to tolerate high dietary fluoride (Landy, 1988; Shi et al., 2009) compared to terrestrial animals (Whitford, 1996). Landy (1988) concluded that the differences between terrestrial animals and fish are due to differences in physiology, biochemistry and ionic regulatory mechanisms.

Landy (1988) also showed that both calcium and aluminum reduced the bioavailability of fluoride in rainbow trout as previously shown in rats (Havivi, 1972; Cerklewski and Ridlington, 1987) and rabbits (Reddy and Rao, 1973). In addition, magnesium has also been shown to reduce the bioavailability of fluoride in rats (Cerklewski, 1987). This shows that a possible toxic effect of dietary fluoride can be reduced by the addition of calcium, aluminum or magnesium to the diets.

In recent years, a large effort in fluoride research has shown that fluoride can induce oxidative stress and lipid peroxidation, alter gene expression and cause apoptosis in terrestrial animals (reviewed by Barbier et al., 2010). Fluoride given to mice in their drinking water contained higher levels of both reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) in their brains (Flora et al., 2009). In addition, the mice given fluoride developed DNA damage in the brain cells and oxidative stress is considered to be one of the mechanisms that induce these alterations in the brain and neurotransmitters (Flora et al., 2009). In fish, the Indian carp developed histological changes in gill, kidney and intestine after being exposed to 6.8 mg F^{-} l^{-1} (Bhatnagar et al., 2007). This experiment was, however, performed with no replications of the experimental treatments, which makes the results less trustful. Further, Tripathi et al. (2009) showed that water-borne fluoride exposure induced chromosomal aberration in the kidney of Asian catfish (*Clarias batrachus*). This experiment also lacked replication and the poor quality of the pictures made it impossible to verifying the results. To summarize, the latter experiments indicate that fluoride can induce changes as seen in terrestrial animals (Barbier et al., 2010).

3. Objectives of the research

The main objective of this research was to increase the knowledge regarding Antarctic krill as a feed ingredient for Atlantic salmon with emphasis on the krill shell fraction and fluoride metabolism in fish reared in fresh and salt water. The work was divided into the following sub-objectives:

- To investigate the effect of growth, nutrient digestibility and health in Atlantic salmon fed diets were fish meal was completely replaced with a whole krill meal or a partly deshelled krill meal (**Paper I**).
- To investigate the effects of growth, nutrient digestibility, mineral excretion and kidney histology in Atlantic salmon fed increased inclusion of a partially deshelled krill meal and a pea protein concentrate (**Paper II**).
- To determine the accumulation of fluoride from krill in various tissues of Atlantic salmon reared in both salt (**Paper I** and **II**) and freshwater (**Paper III**).
- To determine if dietary fluoride level of 150 and 350 mg kg^{-1} from krill or high doses of NaF caused histological changes in the kidney of Atlantic salmon reared in freshwater (**Paper III**).
- To evaluate the bioavailability of fluoride from either krill shell or NaF and the influence of dietary calcium in rainbow trout reared in freshwater (**Paper IV**).

4. Main results and discussion

4.1 Feed processing

The diets in **Papers I** and **II** were produced with a five-section Bühler twin-screw extruder. All parameters during extrusion were continuously recorded in the extruder system. This gave us an opportunity to adjust the extruder settings during extrusion to obtain similar conditions for each diet. High lipid content in the mash during extrusion leads to lubrication in the extruder barrel which further leads to less shear force and pressure (Lin et al., 1997). During extrusion, an increased krill meal inclusion led to increased lubrication due to the high lipid content of the PDKM, 187 mg lipids kg^{-1} compared with 88 mg kg^{-1} in the fish meal. To compensate for the lubrication from the krill lipids, addition of steam to the extruder was reduced. This resulted in relatively similar production parameters of the diets in **Papers I** and **II.** A simpler technology was employed when producing the diets in **Papers III** and **IV.** These diets were gelatin-based moist feeds and they were cold extruded using an Italgi pasta extruder (**Paper III**), and a Braun meat grinder (**Paper IV**).

Physical pellet qualities like hardness and durability were improved with a complete replacement of fish meal with WKM and PDKM (**Paper I**) and with increased PDKM/PPC inclusion (**Paper II**). Pellet water stability, however, decreased with increased dietary PDKM/PPC inclusion (**Paper II**). This was probably a result of decreased starch gelatinization and increased amounts of soluble proteins from the krill meal (Kolakowski, 1989; Aksnes et al., 1997). The decrease in water stability was unexpected and led to progressively inaccurate recording of un-eaten feed during the feeding trials with increasing inclusion of krill. Therefore, the adjusted feed intake values were biased and were not presented in **Papers I** and **II**.

4.2 Growth performance

Growth rates (SGR) for salmon fed WKM was lower than fish fed fish meal and PDKM as sole protein source during the first experimental period (**Paper I**). Final weight was lower for fish fed the WKM diet compared to the PDKM fed fish, but not significantly different from the fish meal control (**Paper I**). These results show that the krill shell fraction reduces growth when krill meal with shell is used at high inclusion level. Similar results have been reported when WKM partly (Yoshitomi et al., 2006) or completely (Beck et al., 1977; Koops et al., 1979) replaced fish meal in diets for rainbow trout raised in freshwater. Increasing replacement of fish meal with a PDKM/PPC mixture showed no decrease in growth compared to a fish meal control. The fish had, however, an optimum SGR with a PDKM/PPC inclusion of 400 g kg^{-1} (**Paper II**). Body weights at 56 days of feeding were higher for all PDKM/PPC fed fish compared to the control group, but no differences were seen at 100 days (**Paper II**). Both **Papers I** and **II** indicate that differences in growth were greater between fish fed fish meal and krill during the first part of the experiment. This is in keeping with Olsen et al. (2006), Rungruangsak-Torrisen (2007), and Suontama et al. (2007a) who reported increased SGR and/or weight gain in Atlantic salmon when 20 and/or 40% of the fish meal was replaced with WKM in the first part of the experimental period. As discussed in **Paper II**, this is maybe a palatability effect where krill has enhanced the feed uptake at the start of the experimental period, and after a given period the palatability effect may gradually disappear.

Most of the fish related research with high dietary fluoride levels is linked to high dietary inclusion of either Northern or Atlantic krill as summarized in the introduction. There are, however, some other studies where fluoride has been added as NaF. No effects on growth have been observed in Atlantic cod, Atlantic halibut, rainbow trout or Atlantic salmon reared in salt water when they were fed diets with 150 mg F^{-} kg^{-1} with NaF as the fluoride source (Moren et al., 2007). Rainbow trout, reared in fresh water, given 4,450 mg F^{-} kg^{-1} diet showed poor feeding behavior the first weeks of feeding but after six weeks the feed intake was similar to the control group (Landy, 1988). The poor growth in this group of fish is most likely a result of low feed intake. Fish fed dietary fluoride levels of 450 and 2,250 mg kg^{-1}, however, grew at a similar rate as trout fed the control diet without fluoride added (Landy, 1988). In **Paper III**, Atlantic salmon fed a diet with 3,500 mg F^{-} kg^{-1} and reared in fresh water had lower final weight than the control

fed fish, whereas no significant differences between the treatments were seen for feed intake, FCR or SGR. These results are, however, from a 30 day feeding trial and thus, there are reasons to believe that the changes seen between treatments could have developed into differences over time. Shi et al. (2009) reported decreased final weight, SGR and feed intake in freshwater reared Siberian sturgeon (*Acipenser baerii*) fed 710 and 1,478 mg F^{-} kg^{-1} diet for 84 days. It is, however, questionable whether sturgeon is more sensitive to NaF than salmon (**Paper III**), or if the differences are due to the longer feeding period.

4.3 Nutrient digestibility and mineral faecal excretion

Apparent digestibility of crude protein (nitrogen) was not affected by dietary treatment (**Papers I and II**). In **Paper I,** crude protein digestibility ranged from 83.5 to 85.6%, while in **Paper II**, the values ranged from 84.2 to 84.8% for salmon fed increased dietary PDKM/PPC. In comparison with Olsen et al. (2006), where salmon fed diets with increasing levels of WKM the digestibility of crude protein appeared higher with values ranging from 86.5 to 88.2. Whereas salmon fed diets with replacement of up to 60% of the fish meal with three types of macrozooplankton, including Antarctic krill, gave similar digestibility of crude protein as the present results ranging from 84.2 to 85.7% (Suontama et al., 2007a).

Digestibility of several amino acids was affected by dietary treatment (**Papers I and II**). Fish fed the WKM had lower digestibility of threonine, serine, glutamine, histidine, and lysine compared to the fish meal control (**Paper I**). In addition to these five amino acids, valine, isoleucine and leucine showed a linear decrease in digestibility with increased dietary inclusion of PDKM/PPC (**Paper II**). The reduction in digestibility of some amino acids in the WKM fed fish may be linked to the low trypsin activity in both the pyloric and mid intestine. The decrease in digestibility of several amino acids was not reflected by the crude protein or the total amino acid digestibility (**Paper II)**. Similar amino acid digestibility in salmon fed PPC and fish meal indicates that the PPC should not affect the amino acid digestibility negatively (Øverland et al., 2009). The reductions in amino acid digestibility may be linked to the reduced water content during extrusion because low moisture content in the extruder can increase the amount of covalent cross-linking in proteins (Li and Lee, 1998). The overall higher digestibility of total

amino acids compared to crude protein indicates a lower digestibility of the non-protein nitrogen fraction (**Paper II**). The nitrogen containing chitin fraction in krill has shown fairly low digestibility in Atlantic salmon (Olsen et al., 2006), thus, the undigested chitin fraction will contribute to increased difference between digestibilities of crude protein and total amino acids.

Digestibility of crude lipids in salmon fed the WKM tended to be lower than those fed the PDKM (**Paper I**) which is in agreement with Olsen et al. (2006). They described a diarrhea like effect in salmon fed a complete replacement of fish meal with WKM. This diarrhea effect was not observed in either of the first two experiments (**Papers I and II**). Krill shell contains chitin that has been shown to, even at low levels, to reduce lipid digestibility in tilapia (Shiau and Yu, 1999). This together with the decreased bile acid level in the pyloric intestine of fish fed WKM might explain the tendency toward a reduced lipid digestibility in **Paper I**. The linear increase in lipid digestibility reported in **Paper II** with increased dietary PDKM/PPC inclusion is supported by the tendency towards increased lipid digestibility of the PDKM fed fish compared to the fish meal control in **Paper I**. The reason for this increased lipid digestibility is unknown; however, the increased level of dietary phospholipids with increased krill inclusion may have enhanced the emulsifying of lipids in the intestine of the fish. This in turn may have increased the digestibility of lipid. The contradictory results of phospholipids on lipid digestibility from **Paper II** and from the literature (Tocher et al., 2008), makes it difficult to draw firm conclusions.

The differences in digestibility of starch in **Papers I** and **II** are probably due to differences in extruder parameters during feed processing. Increasing the level of lipid in the mash decreased the degree of starch gelatinization (Schweizer et al., 1986) as shown in both **Papers I** and **II**. This leads to decreased starch digestibility as shown in rainbow trout (Bergot and Breque, 1983) and European sea bass (Peres and Oliva-Teles, 2002). There are, however, examples both that starch from legumes, such as peas, have been shown reduced digestibility in Atlantic salmon (Aslaksen et al., 2007), and that starch from PPC was digested at same rate as starch from wheat in Atlantic salmon (Øverland et al., 2009).

The level of copper in krill meal is high and is reported to be 46 mg kg^{-1} which exceeds the EU's upper limit for fish diets by approximately 100% (Moren et al., 2006). The copper levels in the WKM and the PDKM used in **Papers I** and **II** were 78 and 81 mg kg^{-1}, respectively. This means that the EU's upper limit would have restricted the dietary krill inclusion to approximately 30% with the copper levels determined in our krill meals. The level

of copper in krill meal could limit the use of krill in fish feeds. High levels of copper are well known to affect the absorption of zinc (Cousins, 1985; Gunshin et al., 1997). The high dietary levels of copper in the present krill meals did, however, not affect the faecal excretion of zinc, or the zinc content in liver or whole body (**Papers I** and **II**). Copper accumulates in the liver and the absorption of copper is usually low and dependent on its chemical form (McDowell, 1992). Atlantic salmon fed increasing dietary copper from krill did not have increased copper accumulation in whole body or liver (**Papers I** and **II**). The faecal excretion of copper increased with increasing dietary copper (**Paper II**), which is in agreement with the findings of Berntssen et al. (1999). This shows that copper from krill in high doses is prevented for being absorbed, which in turn, prevents possible accumulatinon in liver. This is also in agreement with Moren et al. (2006) who reported no increased accumulation of copper in filet of Atlantic cod and Atlantic salmon fed variuos types and dietary levels of krill.

4.4 Fish health

When evaluating a new feed ingredient it is important to do a thorough investigation of possible negative health effects caused by the ingredient. In **Papers I** and **II** Atlantic salmon fed krill showed no major health problems with the exception of mild to moderate histological changes in the kidney. In **Paper I** the control fed fish showed no signs of histological changes in the kidney, whereas the krill fed fish showed changes classified as degeneration, mild to moderate apoptosis and/or necrosis of renal tubule cells. In **Paper II**, the increased inclusion of PDKM/PPC supported the findings in **Paper I** with an increased incidence of histological changes in the salmon kidney. Histological changes in the fish kidney such as nephrosis are often related to nephrotoxic therapeutants like anitibiotics and anti-inflammatory agents, and heavy metals like mercury, cadmium, nickel and arsenic (Reimschuessel and Ferguson, 2006). Heavy metal concentration in the WKM and PDKM diets (**Paper I**) did not exceed the current EU allowable limits in fish feed (Moren et al., 2006), with the exception of copper. However, as discussed earlier, no increased accumulation of copper in either whole fish or liver were found in krill fed fish, indicating that dietary copper is not causing the histological changes in kidney. Fluoride is, however, known to cause histological kidney changes in rats (Ogilvie, 1953). After dietary

fluoride exposure, renal tissue apoptosis and necrosis have been reported in rabbits (Shashi et al., 2002) and pigs (Zhan et al., 2006). In addition, the Indian carp exhibited renal changes after exposed to water-borne fluoride at a level of 6.8 mg l^{-1} (Bhatnagar et al., 2007). These studies reported, however, both changes in renal glomeruli and tubules, while results from **Papers I** and **II** only showed tubular changes. Despite the low bioavailability of fluoride given to salt water reared fish, these experiments indicate that the high dietary fluoride levels could explain the histological changes found in Atlantic salmon in **Papers I** and **II**. Because dietary fluoride is more bioavailable for fish reared in freshwater compared to salt water, we wanted to investigate if dietary fluoride caused histological changes in the kidney of freshwater reared Atlantic salmon (**Paper III**). The histological results from this experiment showed no changes in gill, liver or kidney in salmon fed fluoride levels of 150 and 350 mg kg^{-1} with krill shell as the fluoride source. From 18 salmon sampled that were fed a diet with 3,500 mg F^{-} kg^{-1} as NaF, 6 fish had crystalline material within distal tubules and/or collecting ducts in the kidney. This crystalline material is most likely urolithiasis, or kidney stones. This is in accordance with Singh et al. (2001) who showed increased kidney stone formation in humans with increased intake of fluoridated water. However, despite the high dietary fluoride level in **Paper III**, the result does not support our findings regarding histological changes in kidney in **Papers I** and **II**. The high glomerular filtration rate in the kidney of freshwater fish may prevent fluoride for being concentrated within the tubules, thus explaining the difference between kidney histology of fish fed fluoride in salt water (**Papers I and II**) and freshwater (**Paper III**). Another possibility is that fluoride did not cause the histology changes in Atlantic salmon in **Paper I** and **II**, and this is contrary to our hypothesis. Further investigations are needed to clarify the actual cause.

4.5 Fluoride uptake and accumulation in fish

In terrestrial animals, fluoride in the form of a soluble salt such as NaF, is rapidly absorbed in the stomach. The fluoride is reacting with the acid in the stomach and fluoride is absorbed as the highly reactive gas HF through the stomach wall and a peak in plasma fluoride is reached after 30-60 min (Whitford and Pashley, 1984). In **Paper IV**, the NaF fed rainbow trout reached a clear peak in plasma concentration at three hours, indicating that the rainbow trout also absorbs

fluoride in the stomach. The delay in peak concentration, three hour versus 30-60 min, may be due to the relatively high dietary fluoride level given to the rainbow trout. The rainbow trout fed krill shell as the fluoride source showed no clear peak in plasma concentration and the area under the curve (AUC) was only half of that seen in trout fed NaF (**Paper IV**). This is in agreement with Alvarenga and Tenuta (1993) who reported lower bioavailability of fluoride from krill paste compared to NaF, and with Whitford (1996) who described reduced bioavailability in organically bond fluoride as hydroxyfluorapatite in bone meal compared to NaF. Krill shell contains chitin, and Yin et al. (2010) reported reduced fluoride uptake in rats fed chitin in addition to sodium fluoride (NaF) which probably is a result of the ability of chitins to bind fluoride. The results from **Paper IV** indicate that fluoride from the krill shell is less available than NaF and the flattening of the uptake curve for krill shell fed fish may describe a longer duration of absorption and that the absorption occurs further down in the digestive tract than absorption of fluoride through the stomach wall.

Mineral metabolism is different for fish reared in fresh or salt water. In salt water, the fish continuously drinks water to compensate for the loss of water due to the difference in ion concentration between the fish's body and the salt environment. The opposite occurs in freshwater fish, where high amounts of diluted urine are excreted to compensate for the high influx of water, mainly through the gills (Evans, 1998). Fish reared in fresh and salt water have differences in their ability to accumulate dietary fluoride as shown in **Paper I**, **II** and **III**. This is in keeping with previous experiments were fish reared in brackish or freshwater (Grave, 1981; Yoshitomi et al., 2006) accumulate more fluoride than fish reared in salt water (Julshamn et al., 2004; Moren et al., 2007). We believe that this difference is caused by the calcium and magnesium content in salt water. Fish reared in salt water drink continuously and fluoride has a great ability to bind to calcium and magnesium in the digestive tract (Harrison et al., 1984; Cerklewski and Ridlington, 1987) which further reduces the bioavailability of fluoride. In agreement with this, supplementation of dietary calcium reduced the bioavailability of fluoride in rats (Tenuta and Alvarenga, 1999; Ekambaram and Paul, 2001) and rabbits (Reddy and Rao, 1973). Results from **Paper IV** support this, levels of plasma fluoride in freshwater reared rainbow trout decreased with supplementation of dietary calcium.

The results from **Paper IV** show that fluoride from both NaF and krill shell was taken up by rainbow trout reared in freshwater. To our knowledge, faecal excretion or digestibility of

fluoride in fish reared in freshwater has not been quantified previously. Thus, we wanted to investigate the level of dietary fluoride that was excreted through faeces and determine the accumulation of fluoride in various tissues of Atlantic salmon reared in freshwater in **Paper III**. The faecal excretions of fluoride were similar among fish fed 150 and 350 mg F^{-} kg^{-1} with krill shell as fluoride source and in the low-fluoride control. Whereas the fish fed the NaF diets obtained a lower faecal excretion of fluoride compared to fish fed the krill shell diets and the control. This demonstrates a higher uptake of fluoride when the dietary fluoride level was high. This could be due to the difference in availability of fluoride from NaF versus krill shell as shown in **Paper IV** and by Alvarenga and Tenuta (1993). As previously mentioned, calcium and magnesium have the ability to form low solubility molecules with fluoride in the digestive tract (Harrison et al., 1984; Cerklewski, 1987). When the dietary fluoride level is high, it is possible that the level of available calcium and magnesium ions might be limited in the digestive tract and thus, a higher portion of the ingested fluoride may remind available for absorption and cause an increased absorption of fluoride.

Salmon fed the two krill shell diets in **Paper III** showed no difference in accumulation of fluoride in any of the tissues measured compared to the low-fluoride control fed fish. Together with the histological results discussed previously Atlantic salmon reared in freshwater may be fed fluoride levels of 150 and 350 mg kg^{-1} with krill as fluoride source without any adverse health effects.

The salmon fed the NaF diets in **Paper III** showed a significant increase in fluoride accumulation in whole body, bone, liver, kidney and plasma compared to the control and the krill shell fed fish. Especially a marked increased in the fluoride levels in kidney were observed in fish fed the two NaF diets with 60 and 297 mg kg^{-1}, respectively. This suggests that fish fed the diet with 3,500 mg F^{-} kg^{-1} failed to excrete fluoride in the urine. This indicates that the kidney in freshwater fish is important for fluoride excretion. This increased level of fluoride in kidney could also be due to the presence of kidney stones and the possible high fluoride content in the kidney stones as shown by Sathish et al. (2008).

The bones in salmon fed the NaF diets in **Paper III** showed a high accumulation of fluoride, in keeping with previous experiments (Tiews et al., 1982; Landy, 1988). To our knowledge, there are no fish experiments describing negative effects of increased fluoride accumulation in fish bones. Opstad et al. (2006) suggested, however, that dietary fluoride from

amphipods was a possible reason for deformities in juvenile Atlantic cod. Further, Neuhold and Sigler (1960) reported that rainbow trout embryos exposed to highly fluoridated water produced a high frequency of spinal deformities, however, no numbers or statistics were presented for this conclusion. Landy (1988) reported fluoride levels of up to 24,377 mg kg^{-1} in bones of rainbow trout fed 4,450 mg F^{-} kg^{-1} diet for 30 weeks. The author described the color of these bones as brown compared to white for the control fed fish, however, no further health examination of the fish was done. Natural fluoride levels in Norwegian surface water ranged between 0.02 and 0.20 mg l^{-1} (Skjelkvale, 1994). Thus, the wild juvenile Atlantic salmon in the rivers are probably exposed to a low degree of both water-borne and dietary fluoride (Camargo, 2003). During intense fish farming, the salmon will be feed commercial diets containing a high proportion of marine ingredients during the freshwater stage. The fluoride content in marine fish meal may have up to 300 mg kg^{-1} (Julshamn et al., 2004), and commercial salmon pellets had a fluoride level at 167 mg kg^{-1} (**Paper III**). Vertebral deformities are a recurring problem for the Atlantic salmon farming industry (Witten et al., 2006). Thus, dietary fluoride should be investigated as a possible factor that may affect bone structure and development in the early stage of the life of salmonids.

5. Conclusions and further perspectives

- Complete replacement of fish meal with whole krill meal, including the shell fraction, in diets for Atlantic salmon reduced the growth rate and tended to reduce lipid digestibility compared to fish fed a partly deshelled krill meal or a fish meal as sole protein source. The chitin fraction in the whole krill meal is thought to be a major reason for the negative effects on growth rate and lipid digestibility. A follow-up experiment investigating both pure chitin and a krill shell fraction could provide further knowledge regarding the adverse effects of whole krill meal.

- Lipid digestibility increased linearly in Atlantic salmon fed increasing level of a 3.5:1 ratio mixture of partly deshelled krill meal: pea protein concentrate. This suggests that the use of partly or fully deshelled krill in fish feeds may enhance lipid uptake and save lipid in the feed formulation. A follow-up study should be designed to investigate if this was due to the high proportion of phospholipids with unique composition in krill meal.

- Atlantic salmon fed increasing replacement of fish meal with a partly deshelled krill meal and a pea protein concentrate had an optimum growth rate at a PDKM/PPC level of 400 g kg^{-1}. The differences in growth rate and weight gain were higher between the krill fed fish and those fed the fishmeal in the first part of the feeding experiment compared to the whole period. This suggests that krill can be used as a feed intake enhancer for fish during short periods known to be critical in commercial fish farming, such as during the coldest winter months or directly after sea transfer. The time-dynamics of feed intake stimulation by using krill in fish feeds should be followed up in further studies.

- Increasing the level of krill meal during extrusion reduced pellet expansion, pellet water stability, and starch gelatinization. This was most likely due to the high lipid content of the krill meals used, but the effects of krill products on physical feed quality should be further investigated.

- Dietary krill induced mild to moderate histological changes in the kidney of Atlantic salmon reared in saltwater at an inclusion level as low as 123 g kg^{-1} diet. The changes

were thought to be caused by the dietary fluoride, however, this was not verified in Atlantic salmon reared in freshwater, despite a higher bioavailability of dietary fluoride in these fish. Further research is needed to verify the histological kidney changes found when feeding krill to salmon reared in salt water.

- Dietary fluoride had low bioavailability in Atlantic salmon reared in salt water. Fluoride was accumulated especially in the bones of salmon reared in freshwater. Fluoride from krill shell was absorbed to a lower degree than fluoride from NaF in rainbow trout reared in freshwater. High levels of dietary fluoride increased the faecal excretion of both magnesium and calcium in Atlantic salmon reared in freshwater. Further, faecal excretion of fluoride was higher for salmon fed krill shell than NaF as fluoride source. Despite the short duration of the experiment, it indicated that a dietary fluoride level of 150 and 350 mg kg^{-1} from krill shell did not affect growth rate or health in freshwater reared Atlantic salmon. The use of fluoride containing marine ingredients in diets for Atlantic salmon and other salmonids in the freshwater period should, however, be further investigated with respect to a possible adverse effect on bone development.

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Paper I

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High inclusion of partially deshelled and whole krill meals in diets for Atlantic salmon (*Salmo salar*)

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Fluoride

ABSTRACT

The aim of this study was to investigate how diets containing partially deshelled or whole krill meals affected growth, digestibility of main nutrients, faecal excretion of minerals, fluoride accumulation, and organ indices and health parameters in Atlantic salmon (*Salmo salar*). Three extruded diets were fed for 100 days to salmon with an average weight of 550 g, distributed into 9 tanks equipped with flow through sea water. The dietary treatments comprised a control diet based on high-quality fish meal (FM) and two experimental diets where the FM was substituted with either partially deshelled krill meal (PDKM) or whole krill meal (WKM). Shell removal reduced the chitin content from 28 to 8 g kg^{-1} dry matter (DM), while fluoride was only reduced from 940 to 631 mg kg^{-1} DM.

Growth rate for fish fed WKM was significantly lower than for salmon fed control diet whereas the PDKM diet did not appear to alter growth during the first feeding period. Digestibility of lipid tended to be higher for PDKM and lower for WKM compared to the FM control. No significant difference was seen for digestibility of nitrogen, but fish fed the FM diet had higher digestibility of threonine, serine, glutamine, histidine and lysine compared to fish fed the WKM diet. No major differences in plasma were seen for triacylglycerols, free fatty acids, glucose, total protein, albumin, globulin, urea, and total bilirubin. Trypsin activities in the pyloric and mid intestine were lower in fish fed the WKM diet compared to FM. Bile acid concentration in the pyloric intestine were significantly lower in fish fed the WKM diet compared to FM and PDKM. Fish fed both diets containing krill meal had signs of mild to moderate nephrosis. To conclude, PDKM could successfully replace FM as a sole protein source for Atlantic salmon, whereas the WKM slightly reduced growth rate compared to the FM and the PDKM diet.

1. Introduction

Farming of marine carnivorous fishes is increasing; implying a comparable increase in the demand for high-quality protein feed ingredients. Antarctic krill (*Euphausia superba*) is receiving increased attention as a marine protein source. Knox (2007) describes the difficulty of obtaining a reliable estimate of the Antarctic krill biomass but suggests that this biomass is between 10^8 and 10^9 tons. Krill was examined as a source for fish feeds in the late 70's and early 80's (Storebakken, 1988), but it's commercial use did not increase due to the economics of the krill fisheries, problems with krill processing and increased supplies of fish meal (FM) (Ichii, 2000).

A limiting factor for use of krill in European fish feeds has been EU's restriction on fluoride level in feed of 150 mg kg^{-1} (Commission dir. 2002/32/EC). A recent EU directive increased the allowable fluoride level in complete feed for fish to 350 mg kg^{-1} (Commission dir. 2008/76/EC). Fluoride is mainly located in the exoskeleton of the krill and the fluoride level in whole Antarctic krill has been shown to be between 1000 mg kg^{-1} (Boone and Manthey, 1983; Zhang et al., 1993) and 2400 mg kg^{-1} (dry wt.) (Soevik and Braekkan, 1979). A reduction of fluoride concentration in krill meal can be obtained by separating the exoskeleton from the muscle fraction prior to meal production. The fluoride, however, is able to leach from the exoskeleton to the muscle fraction during cold storage in a temperature dependent manner (Christians and Leinemann, 1983; Adelung et al., 1987). A partial deshelling of the krill meal will also reduce the level of chitin, a long-chain polymer of N-acetyl-glucosamine that is known to reduce growth rate similarly to that of dietary fiber (Tharanathan and Kittur, 2003). Another limiting factor for use of krill is the natural high level of copper in krill; EU's allows a maximum of 25 mg kg^{-1} in complete feedstuff (Commission dir. 2003/100/EC).

Feeding rainbow trout (*Salmo gairdneri*) with krill resulted in reduced growth rate and feed efficiency when average quality FM was

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completely replaced with krill meal (Beck et al., 1977; Koops et al., 1979). Feeding Atlantic salmon (*Salmo salar*) with Antarctic krill, a complete replacement of high-quality FM also gave significantly lower final weights compared to fish fed a diet with 200 g kg^{-1} krill meal replacement (Rungruangsak-Torrissen, 2007). These experiments suggest that krill meal might contain components that reduce growth rate at high dietary inclusions. In contrast, Yoshitomi et al. (2007) reported no negative effect on growth in small rainbow trout reared in fresh water and fed graded levels, up to complete replacement of a brown FM with a low fluoride krill meal, produced by deshelling the meal after it was dried.

The aims of this study were to examine nutritional, physiological and histo-pathological responses to diets where krill meals, with and partly without shell, fully replaced FM as the source of protein.

2. Materials and methods

2.1. Krill meal and diet production

The experimental krill meals were produced at the Norwegian University of Life Sciences (UMB) (Ås, Norway) by a technology proprietary to Krillsea Group AS, using frozen Antarctic krill, *Euphausia superba* (United Ocean Co., Ltd, South Korea). Two types of krill meal were produced. One was a partially deshelled krill meal (PDKM), where parts of the shells had been mechanically removed prior to heating. The other was a WKM, with shells (Table 1). Both meals contained krill water solubles ("stickwater"), which is shown to be responsible for some of the palatability properties in krill meal (Tibbetts et al., 2010). The shell removal process reduced the chitin level from 28 to 8 g kg^{-1} dry matter (DM), while the fluoride level was only reduced from 940 to 631 mg kg^{-1} DM. The experimental krill meals were lower in ammonium and volatile nitrogen compared to the FM (Table 1). The levels of biogenic amines in all three meals were within the quality specifications for Norse Eco LT FM from Norsildmel A/L, Fyllingsdalen, Norway. The WKM contained higher level of the biogenic amine tyramine compared to the FM, but putrescine, cadaverine, and histamine levels were higher in the FM compared to the krill meals.

Three extruded diets were produced at the Centre for Feed Technology at UMB (Table 2). Yttrium oxide (Y_2O_3) was used as an inert marker for determination of digestibility (Austreng et al., 2000). The diets were mixed, ground, conditioned, extruded, dried and vacuum coated by the same equipment and procedures as used by Aslaksen et al. (2007).

After cooling, the coated pellets were packed in plastic bags and stored at 5 °C. The diets were formulated to be isonitrogenous and isolipidic (Table 3). Because FM and krill meals differed in lipid content, different levels of oil were added during vacuum coating to obtain similar dietary lipid levels.

2.2. Biological experiment and facilities

A total of 225 Atlantic salmon, weighing 550 g on average, were randomly distributed to nine fibreglass tanks of 1.5 m^3 each and kept in 34 g l^{-1} sea water supplied at 22 l min^{-1}. The mean water temperature was 8.1 °C for days 1–56 and 7.0 °C for days 57–100. The tanks were under constant light 24 h d^{-1}. Each diet was fed to triplicate groups of fish. Feed was supplied in excess of appetite in order to ensure maximum voluntary feed intake.

2.3. Sampling procedure

All fish were weighed at the start of the experiment and at days 56 and 100. At the end of the experiment, the fish were anaesthetized with metacaine (MS-222™; 50 mg l^{-1} water). Weight was recorded for all fish. Randomly selected fish from each tank were sampled for analyses. All fish in each tank, except those sampled for histology and gut enzymes, were stripped for faeces. Three fish per tank were homogenized and pooled prior to analyzing for whole body composition. Another five fish per tank were sampled for weight of the gastrointestinal tract (GIT), liver and carcass. Blood was drawn from six fish by caudal venipuncture using vacutainers containing lithium heparin. The gastrointestinal tracts were removed and carefully cleaned of adherent adipose. The intestine was then divided into four regions: stomach (ST), pyloric caeca (pyloric intestine, PI), mid intestine (MI) and distal intestine (DI). Digesta was collected from the PI (distal half), MI and DI (proximal half) for analysis of bile acid concentration and lipase and trypsin activities. The tissue of each region was individually weighed and samples taken for histology (fixed in neutral buffered formalin) and the remainder frozen in liquid N_2 for brush border enzyme activity analyses. Additional samples for histology were taken from the liver (LI) and trunk (filtrative) kidney (KI). The Norwegian quality cut, NQC (NS, 1994) was sampled from three fish per tank. A standard piece, approximately 10 g of the white dorsal muscle from the NQC was sampled with a cork punch. The remainder was microwaved in sealed plastic bags until the bone easily separated from the muscle. The bone and muscle were freeze dried and pooled within tank, ground with a pestle and mortal prior to fluoride analysis. Three whole fish from each tank were frozen and later thawed for radiography.

Table 1
Chemical composition of fish meal (FM), partly deshelled krill meal (PDKM), and whole krill meal (WKM) used in the experimental diets.

	FM	PDKM	WKM
Composition, g kg^{-1}			
Dry matter	969	964	925
Crude protein[b]	708	610	594
Crude lipid	88	187	151
Ash	139	118	134
Chitin	0.2	8	28
Ammonium nitrogen	26	8	9
Total volatile nitrogen	0.1	<.1	<.1
Volatile fatty acids, % of total 18:1	nd. [a]	29	31
Peroxide value	nd.	0.7	4.1
Amino acids[c], g kg^{-1}			
Total AA[d]	519.6	390.6	381.5
Cysteine	5.7	5.2	4.8
Methionine	16.1	11.7	11.0
Aspargine	54.7	43.8	43.4
Threonine	25.4	19.7	19.2
Serine	25.3	17.8	17.1
Glutamine	83.9	58.1	57.9
Proline	23.1	22.1	23.0
Glycine	30.8	20.2	21.1
Alanine	32.6	22.0	22.3
Valine	26.4	20.5	20.2
Isoleucine	23.7	20.6	19.7
Leucine	42.0	31.8	30.7
Tyrosine	15.2	14.0	13.1
Phenylalanine	22.0	18.4	17.9
Histidine	11.8	8.3	8.6
Lysine	45.5	31.4	30.0
Arginine	35.3	24.9	21.5
Minerals, mg kg^{-1}			
Fluoride	176	631	940
Copper	5.2	78	81
Zinc	63	58	55
Biogenic amines, mg kg^{-1}			
Tyramine	215	92.6	529
Putrescine	122	12.4	21.1
Cadaverine	496	<1	<1
Histamine	7.0	<1	<1

[a] nd. = not determined.
[b] Adjusted for chitin nitrogen.
[c] Presented in dehydrated form.
[d] Total sum of amino acids without tryptophan.

Table 2
Diet formulation.

Diet	FM [a]	PDKM [b]	WKM [c]
Formulation, g kg^{-1}			
Partly deshelled krill meal	0	752	0
Whole krill meal	0	0	689
Fish meal[a]	641	0	0
Wheat flour[d]	108	105	107
Vitamin and mineral premix[e]	5.0	5.0	5.0
Vitamin C[f]	0.60	0.60	0.60
MCP[g]	0	18.0	18.3
Yttrium oxide[h]	0.1	0.1	0.1
Fish oil[i]	245	119	180

[a] Norse Eco LT, Norsildmel, Egersund, Norway.
[b] PDKM; partly deshelled krill meal.
[c] WKM; whole krill meal.
[d] Felleskjøpet, Kambo, Norway.
[e] Farmix, Trouw Nutrition, LA Putten, The Netherlands. Per kg feed. Retinol 2500.0 IU, Cholecalciferol 32400.0 IU, α-tocopherol SD 0.2 IU, Menadione 40.000 mg, Thiamin 15.0 mg, Riboflavin 25.0 mg, d-Ca-Pantothenate 40.003 mg, Niacin 150.003 mg, Biotin 3000.0 mg, Cyanocobalamin 20.0 mg, Folic acid 5.0 mg, Pyridoxine 15.0 mg, Ascorbate polyphosphate 0.098 g, Cu: CuSulfate 5H_2O 11.998 mg, Zn: ZnSulfate 89.992 mg, Mn: Mn(II)Sulfate 34.993 mg, I: K-Iodide 1.999 mg, Se: Na-Selenite 0.200 mg, Cd Max. 0.003 mg, Pb Max. 0.028 mg, Ca 0.915 g, K 1.380 g, Na 0.001 g, Cl 1.252 g.
[f] Stay-C® 35. DSM Nutritional Products, Basel, Switzerland.
[g] Bolifor® MCP-F. KPP Oy, Animal Nutrition, Helsingborg, Sweden.
[h] Y_2O_3. Metal Rare Earth Limited, Shenzhen, China.
[i] NorSalmOil, Norsildmel, Egersund, Norway.

2.4. Chemical and physical analyses

Diets and freeze-dried faeces were ground with a pestle and mortar prior to analysis. Diets and faeces were analyzed for dry matter by drying to constant weight at 104 °C (Commission dir. 71/393/EEC), protein using Kjeldahl nitrogen (Commission dir. 93/28/EEC) × 6.25, lipid by HCl hydrolysis followed by diethyl ether extraction (Commission dir. 98/64/EC), starch (AOAC enzymatic method 996.11), ash (Commission dir. 71/250/EEC), minerals (ICP-AES/ICP-MS) (Nordic Committee on Food Analysis (NMKL) method 161), and yttrium oxide (ICP-AES) (NS-EN ISO 11885). Starch gelatinization was analyzed by the BioLab Analyse (Nofima Ingredients, Bergen) using a modification of Chiang and Johnson (1977) glucoamylase methodology. Biogenic amines were analyzed by using HPLC according to Smělá et al. (2003). Amino acids were analyzed according to Commission dir. 98/64/EC on a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Chitin in diets was determined as glucosamine-residues after post-column derivatization with ninhydrin on the same Biochrom 30 analyzer as used for the amino acid analyses. In brief, the 100 mg chitin samples were hydrolyzed at 110 °C for 4 h in Duran-Schott GL-18 screw capped tubes in a Labtherm® heating block (Liebisch Labortechnik GmbH, Bielefeld, Germany) using 10 ml of 6 M HCl. After cooling to room temperature, 8 ml of 7.5 M NaOH was gently added and the hydrolysates were diluted to 100 ml using 0.2 M sodium citrate loading buffer, pH 2.2 (Biochrom Ltd., Cambridge, UK). An aliquot (1 ml) of the hydrolysates was micro-filtrated (0.45 µm Spartan membrane filter, Schleicher & Schuell, Dassel, Germany) prior to injection (40 µl). All data were analyzed against external standards of glucosamine-HCl (Sigma Chemical, St. Louis, Mo., U.S.A.) using the Chromeleon® Chromatography Management Software (Dionex Corporation, Sunnyvale, CA). Fluoride concentration was determined using a fluoride ion selective combination electrode (VWR symphony, model 14002-788) using the procedure given by Malde et al. (2001).

Physical pellet quality was tested for durability, hardness and expansion 14 days after production. Pellet durability was tested with a DORIS pellet tester (AKVAsmart, Bryne, Norway) using 100 g of presieved pellets. After the pellets passed through the machine the pellet collector was emptied on a 4 mm screen with a collector and sieved for 60 s at 0.5 amplitude with a Retsch AS 200 Control (Haan, Germany). The material remaining on the 4.0-mm screen after sieving was weighed. The durability was defined as the percentage of pellets remaining on the screen. The testing was conducted in triplicate for each diet. Hardness was determined with a Texture Analyzer TA-XT2 (SMS Ltd, Surrey, UK), equipped with a 5 kg load cell as described by Øverland et al. (2009). The length of each pellet tested was recorded with an electrical caliper and the pellet width was recorded by the texture analyzer. Expansion was calculated as: ((pellet width − die diameter) × die diameter^{-1}) × 100.

2.5. Plasma clinical chemistry

The Central Laboratory at The Norwegian School of Veterinary Science performed plasma analyses according to standard methods. These methods are based on Mulder et al. (1983) for free fatty acids and Trinder (1969) and Allain et al. (1974) for cholesterol. The analyses for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), total protein, albumin, globulin, urea, creatinine, glucose, triglycerides, free fatty acids, bile acids, total bilirubin, inorganic phosphorus, Ca, Na, K, and Cl, are based on Tietz (1995). Bile acid in plasma was determined using the Enzabile® test kit (Cat. No. 550101, Bio-Stat Diagnostic Systems, Cheshire, UK). The concentrations of 3α-hydroxy bile acids were calculated from a standard curve generated using bovine serum based standards containing glycohenodeoxycholic acid.

Table 3
Chemical composition of the diets.

	FM	PDKM[a]	WKM[b]
Dry matter (DM), g kg^{-1}	959.8	955.9	951.1
In DM, kg^{-1}			
Crude protein, g	484.4	494.0	464.9
Total protein[c], g	484.4	490.4	455.9
Lipid, g	323.0	284.6	310.2
Starch, g	72.9	67.0	66.2
Ash, g	103.7	108.4	112.1
Chitin, g	0.1	8.5	20.9
Gross energy, MJ	23.6	22.6	22.9
Amino acids[d], g kg^{-1}			
Total AA[e]	336.0	315.8	296.9
Cysteine	3.7	4.3	3.8
Methionine	10.1	9.2	8.4
Aspargine	35.2	35.3	33.4
Threonine	16.2	15.9	14.9
Serine	16.6	14.8	13.6
Glutamine	56.5	48.4	46.6
Proline	16.1	18.5	18.1
Glycine	20.2	16.0	16.3
Alanine	20.8	17.6	17.3
Valine	16.7	16.1	15.5
Isoleucine	14.8	16.0	14.8
Leucine	26.8	25.5	23.6
Tyrosine	9.8	11.4	10.1
Phenylalanine	14.4	14.9	14.0
Histidine	7.5	6.7	6.3
Lysine	27.9	24.8	23.1
Arginine	22.7	20.4	16.9
Minerals, kg^{-1}			
Phosphorus, g	14.5	14.5	12.3
Copper, mg	10.0	72.0	64.0
Iron, mg	13.0	22.0	14.0
Zinc, g	0.19	0.20	0.19
Fluoride, mg	110	470	640
Yttrium, mg	82.0	87.0	84.0

[a] PDKM; partly deshelled krill meal.
[b] WKM; whole krill meal.
[c] Adjusted for chitin nitrogen.
[d] Presented in dehydrated form.
[e] Total sum of amino acids without tryptophan.

2.6. Histological evaluation

Samples were fixed in neutral buffered formalin (40 g formaldehyde l^{-1}; pH 7.4) for 24 h and subsequently transferred to 70% ethanol until processing. Initially three fish sampled from each tank were processed for tissue histology (n = nine per treatment). Tissues were processed at the Pathology Laboratory, NVH using standard histological methods. Sections for routine histological analysis were stained with haematoxylin and eosin (H&E). When initial screening indicated differences among groups (i.e. in kidney samples) additional samples were examined (n = 18 total per treatment).

2.7. Brush border enzyme activities

Brush border membrane bound alkaline phosphatase (ALP), leucine aminopeptidase (LAP) and maltase activities were determined in homogenates of intestinal tissue. The tissues were thawed, weighed and homogenized (1:20) in ice-cold 2 mM Tris/50 mM mannitol, pH 7.1, containing the serine protease inhibitor phenyl-methyl-sulphonyl fluoride (Sigma no. P-7626; Sigma Chemical Co., St. Louis, MO, USA). Aliquots of homogenates were frozen in liquid nitrogen and stored at −80 °C until analysis. Enzyme activities were determined colorimetrically as previously described by Krogdahl et al. (2003). Incubations were performed at 37 °C. Enzyme activities are expressed as mol substrate hydrolyzed min^{-1} and related to kg fish (specific activity).

2.8. Trypsin and lipase activities and bile acid content of digesta

Trypsin and lipase activities and bile acid concentration were determined on pooled, freeze dried digesta from the distal PI, MI and proximal DI. Trypsin activity was determined colorimetrically (Kakade et al., 1973) using the substrate benzoyl-arginine-*p*-nitroanilide (BAPNA) (Sigma no. B-4875) and a curve derived from a standardized bovine trypsin solution.

Lipase activity was analyzed in freeze dried digesta (1.25 mg ml^{-1} sonicated suspension in 25 mM Tris-buffer, pH 8.0) spectrophotometrically by hydrolysis of 4-nitrophenol-myristate (4-NPM) as described by Gjellesvik et al. (1992). The reaction rate was measured at 37 °C and pH 8.0. Bile acid concentration was determined using the same method described for plasma chemistry.

2.9. Calculation and statistical analysis

Specific mechanical energy (SME) during extrusion was calculated as: $(2\times\pi\times60^{-1})\times(S_{rpm}\times T_{kNm}\times T_{t/h}^{-1})$, where S_{rpm} is screw speed, T_{kNm} is torque and $T_{t/h}$ is throughput. Specific growth rate (SGR) was calculated as: SGR = 100 × (ln(end wt) − ln(start wt)/Δt), where end wt = end weight of fish, start wt = start weight of fish, Δt = number of experimental days. Apparent digestibility coefficients (%) of individual nutrients were calculated as follows: ADC = $100\times(1-(D_i\times F_i^{-1}\times F_n\times D_n^{-1}))$, where; D_i and F_i represent the concentrations of inert marker in the diet and faeces, and D_n and F_n represent the concentrations of nutrients in the diet and faeces, respectively. Faecal excretion for minerals was calculated as follows: $(-100\times(1-(D_i\times F_i^{-1})\times(F_n\times D_n^{-1})))+100$.

The results were statistically analyzed by one-way analysis of variance to differentiate between the diets (SAS, 1990). Results are presented as means and pooled standard errors of means (s.e.m.). Significant ($P<0.05$) differences among means were ranked by Tukey's multiple range test and are indicated in the tables by different superscripts [a,b,c]. Tendency is indicated for $0.10>P\geq0.05$.

Table 4
Processing parameters and physical pellet quality and starch gelatinization in diets based on fish meal (FM), partially deshelled krill meal (PDKM) and whole krill meal (WKM).

	FM	PDKM	WKM	s.e.m[1]	*P*-value
Feed mash					
Lipid content [2], g kg^{-1}	77.9	162	130		
Moisture content [3], g kg^{-1}	268	162	185		
Extrusion					
Section 3, °C	118	129	124		
SME [4], Wh kg^{-1}	37.0	45.0	35.0		
Physical pellet quality					
Hardness, N	16.7[b]	29.9[a]	31.4[a]	0.23	0.0002
Durability, %	64.4[c]	83.8[b]	92.2[a]	0.77	<.0001
Expansion, %	23.6[a]	15.0[b]	8.6[c]	1.12	<.0001
Starch gelatinization [5]	91.3	73.4	84.1		

[1] Pooled standard error of mean. Different letters denote significant ($P<0.05$) difference among diets. n = 3 replicates per treatments. Tukey multiple range test is used. [2] Lipid content in feed mash prior to processing. No lipid was added before vacuum coating. [3] Total moisture content, calculated based on analyzed moisture in raw materials and added amounts of steam and water in the conditioner. [4] Specific mechanical energy, W h kg^{-1}. [5] Percent of total starch content in finished feed.

Table 5
Growth for Atlantic salmon fed diets with fish meal (FM), partially deshelled krill meal (PDKM) or whole krill meal (WKM).

	FM	PDKM	WKM	s.e.m.[1]	*P*-value
Start weight, g	544	554	553	3.38	0.17
Mid weight 56 d, g	758[ab]	809[a]	711[b]	12.2	0.004
End weight, 100 d, g	1060[ab]	1100[a]	956[b]	32.0	0.045
SGR, 1–56 d	0.76[a]	0.86[a]	0.57[b]	0.035	0.003
SGR 1–100 d	0.66	0.69	0.55	0.033	0.055

[1] Pooled standard error of mean. Different letters denote significant ($P<0.05$) difference among diets. n = 3 replicates per treatments.

3. Results

3.1. Feed production and pellet quality

Different amounts of water and steam were added into the extruder to obtain equal conditions between the dietary productions, because the amount of lipid in mash prior to extrusion differed among the diets (Table 4). The SME varied from 35 to 45 for the experimental diets (Table 4). The krill meal based diets had increased pellet hardness and durability compared to the FM diet, while expansion was lower in the krill diets. The degree of starch gelatinizing was 91.3, 73.4, and 84.1% of total starch for the FM, PDKM, WKM diets, respectively (Table 4).

3.2. Growth performance

No fish died during the experimental period and there were no differences among treatments for initial weights. The fish grew from an average of 550 g to between 956 and 1100 g during 100 days of feeding (Table 5). During the first 56 days of feeding, growth rates were significantly higher for the fish fed PDKM and FM than fish fed WKM. For the entire 100 days of feeding, there was a strong tendency for reduced growth rate for the WKM diet compared to the PDKM and FM diets.

3.3. Nutrient digestibility and mineral faecal excretion

There was a clear tendency for reduced lipid digestibility (Table 6), whereas the fish fed the WKM were ranked below the PDKM and FM treatments. Replacement of FM with WKM did not appear to affect starch digestibility, whereas PDKM reduced starch digestibility. Nitrogen digestibility did not significantly differ from the two dietary krill diets compared to the FM control. However, digestibility of several amino acids was affected. Cysteine digestibility ranged from 80.2 to 83% and tended to be higher for the PDKM fed fish compared to the FM fed fish. Fish fed the WKM diet had significantly lower

Table 6
Apparent digestibility (%) of main nutrients, amino acids and faecal excretion of minerals in Atlantic salmon fed diets with fish meal (FM), partially deshelled krill meal (PDKM) or whole krill meal (WKM).

	FM	PDKM	WKM	s.e.m.[1]	*P*-value
Crude lipid	93.3	95.2	89.9	1.26	0.062
Nitrogen	84.2	85.6	83.5	0.81	0.23
Starch	74.3^{b}	59.4^{a}	66.2^{ab}	2.16	0.008
Amino acids					
Total	90.5	90.4	88.8	0.44	0.065
Cysteine	80.2	83.0	81.5	0.73	0.089
Methionine	90.3	91.2	89.2	0.51	0.079
Aspargine	85.1	84.4	82.2	0.72	0.068
Threonine	89.8^{a}	88.6^{ab}	86.8^{b}	0.50	0.015
Serine	89.9^{a}	89.3^{a}	86.9^{b}	0.48	0.001
Glutamine	93.0^{a}	91.6^{ab}	90.2^{b}	0.35	0.004
Proline	90.1^{b}	93.3^{a}	92.4^{a}	0.31	0.0009
Glycine	88.3	90.2	89.4	0.44	0.062
Alanine	92.0	92.5	91.3	0.34	0.12
Valine	90.5	90.5	89.2	0.45	0.15
Isoleucine	90.2	90.3	88.7	0.50	0.10
Leucine	92.0	91.8	90.2	0.44	0.057
Tyrosine	89.7	91.0	89.1	0.44	0.062
Phenylalanine	89.4	90.2	88.8	0.52	0.25
Histidine	89.2 ^{a}	86.7 ^{b}	84.6 ^{b}	0.56	0.003
Lysine	93.3 ^{a}	92.5 ^{ab}	91.4 ^{b}	0.35	0.026
Arginine	92.5 ^{ab}	93.9 ^{a}	91.9 ^{b}	0.33	0.013
Faecal excretion of minerals, % of intake					
Phosphorus	68.3	57.5	54.9	3.27	0.059
Copper	77.4	93.2	81.2	5.13	0.15

[1] Pooled standard error of mean. Different letters denote significant ($P<0.05$) difference among diets. $n=3$ replicates per treatments.

digestibilities for threonine, serine, glutamine, histidine and lysine compared to fish fed the FM control.

Faecal excretion of phosphorus tended to be higher in the two diets with krill meal than the FM diet (Table 6). Faecal excretion of copper was not significantly affected by dietary treatment.

3.4. Whole body and liver mineral, tissue fluoride concentrations, and radiography

There were no clear trends among fish fed the dietary treatments for whole body and liver minerals (Table 7). The muscle tissue fluoride level ranged from 5.7 to 11.5 mg kg^{-1} on a dry weight basis with no effect of dietary treatment (Table 7**).** There appeared to be more fluoride accumulation in bone compared to muscle, varying from 7.3 to 18.4 mg kg^{-1}. The salmon fed the WKM diet contained significantly more bone fluoride than the other dietary treatments. Radiography showed no differences in bone density or incidence of skeletal deformities that could be specifically ascribed to dietary treatment.

3.5. Blood plasma parameters, tissue somatic indices, intestinal enzyme activities and bile acid levels

Salmon fed the diets with krill meal had significantly lower plasma cholesterol and creatinine levels than fish fed the FM diet (Table 8). No significant differences were seen for TG, FFA, glucose, bile acids, total protein, albumin, globulin, or urea. The same applied to the enzymes AST, ALT, and AP. Inorganic phosphorous was significantly higher in fish fed the two diets with krill meal compared to the FM control, while calcium, sodium, potassium and chloride plasma levels were similar among treatments.

The tissue somatic indices of stomach, PI and DI were higher for fish fed the WKM compared to fish fed the FM control (Table 9). There was no significant difference among treatments for liver and MI somatic index.

Table 7
Fluoride content in muscle tissue, bone tissue and faeces (mg kg^{-1} dry wt.), minerals in whole body and liver of Atlantic salmon fed diets with fish meal (FM), partially deshelled krill meal (PDKM) or whole krill meal (WKM).

	Start	FM	PDKM	WKM	s.e.m.[1]	*P*-value
Fluoride, mg kg^{-1}						
Muscle		10.3	5.65	11.5	2.43	0.27
Bone		8.64^{b}	7.27^{b}	18.4^{a}	1.98	0.014
Faeces		519^{b}	2308^{a}	2417^{a}	156	0.0002
Whole body minerals, kg^{-1}						
Calcium, g	4.49	4.49	4.71	5.00	0.31	0.56
Phosphorus, g	5.2	5.46	5.45	5.53	0.13	0.89
Magnesium, mg	558	440	450	450	4.01	0.53
Zinc, mg	65.9	61.8	57.7	59.3	3.32	0.69
Copper, mg	3.64	2.53	2.31	2.27	0.09	0.19
Liver minerals, mg kg^{-1}						
Zinc		2.51	2.67	2.64	0.05	0.13
Copper		11.1	11.0	11.0	0.60	0.99

[1] Pooled standard error of mean. Different letters denote significant ($P<0.05$) difference among diets. $n=3$ replicates per treatments. Tukey multiple range test is used.

Brush border enzyme (ALP, LAP and maltase) activities are shown in Table 9. Fish fed the krill containing diets had higher ALP and LAP activities in the DI compared to fish fed FM. Similar findings were observed in the PI, but the differences only tended to be significant for the ALP activity. In contrast, there was lower maltase activity in fish fed the WKM diet compared to FM, though this effect was only significant in the PI.

Trypsin activity in digesta was lower in fish fed the WKM diet compared to fish fed the FM diet, particularly in the PI and MI (Table 10). Lipase activity was lower in fish fed the PDKM diet compared to the WKM and the FM diet in the MI. Bile acid levels in the PI were lower in fish fed the WKM diet compared to FM control, and both dietary treatments with krill meal resulted in reduced bile acid levels in the DI when compared to FM.

3.6. Histology of the GIT, liver and kidneys

No histological differences were found in the intestines and livers between fish from the different dietary groups. In the trunk kidney,

Table 8
Blood chemistry in Atlantic salmon fed diets with fish meal (FM), partially deshelled krill meal (PDKM) or whole krill meal (WKM).

	FM	PDKM	WKM	s.e.m.[1]	*P*-value
Metabolites [2]					
Cholesterol, mM	15.7^{a}	11.1^{b}	10.5^{b}	0.30	<.0001
TG, mM	2.58	2.68	2.69	0.05	0.31
FFA, mM	0.53	0.47	0.48	0.04	0.57
Glucose, mM	4.60	5.03	4.84	0.11	0.082
Bile acids, μM	13.9	6.2	6.0	2.64	0.13
Total protein, g l^{-1}	48.2	50.2	48.8	1.22	0.53
Albumin g l^{-1}	24.5	25.8	25.1	0.65	0.41
Globulin g l^{-1}	23.8	24.4	23.6	0.69	0.70
Urea, mM	1.07	1.05	1.05	0.05	0.96
Creatinine, μM	55.1^{a}	19.2^{b}	19.0^{b}	3.23	0.0003
Enzymes [2], U l^{-1}					
AST	362	458	385	42.4	0.32
ALT	23.1	25.0	28.1	3.89	0.68
AP	207	224	202	7.45	0.18
Electrolytes [2], mM					
Inorganic P	3.1^{a}	4.2^{b}	4.1^{b}	0.05	<.0001
Calcium	3.0	3.1	3.0	0.03	0.47
Sodium	162	164	163	0.89	0.35
Potassium	2.8	2.9	2.8	0.16	0.75
Chloride	138	138	138	1.02	0.79

[1] Pooled standard error of mean. Different letters denote significant ($P<0.05$) difference among diets. $n=3$ replicates per treatments. [2]Abbreviations: TG: triglycerides, FFA: free fatty acids, AST: aspartate aminotransferase, ALT: alanine aminotransferase, AP: alkaline phosphatase, Inorganic P: inorganic phosphorous.

Table 9
Tissue somatic indices, activities of alkaline phosphatase (ALP), leucine aminopeptidase (LAP) and maltase for Atlantic salmon fed diets with fish meal (FM), partially deshelled krill meal (PDKM) or whole krill meal (WKM).

	FM	PDKM	WKM	s.e.m.[1]	*P*-value
Tissue somatic indices					
Stomach	0.45[b]	0.50[ab]	0.50[a]	0.01	0.023
Pyloric intestine	1.61[b]	1.84[a]	2.06[a]	0.05	0.003
Mid intestine	0.25	0.21	0.25	0.01	0.11
Distal intestine	0.38[b]	0.46[ab]	0.50[a]	0.02	0.028
Liver	1.17	1.21	1.19	0.03	0.70
ALP activity, μmol min^{-1} kg^{-1} fish					
Pyloric intestine	418	610	622	50.9	0.052
Mid intestine	80.0	94.3	107	11.3	0.31
Distal intestine	147[b]	242[a]	241[a]	16.4	0.010
LAP activity, μmol min^{-1} kg^{-1} fish					
Pyloric intestine	166[b]	230[ab]	246[b]	15.8	0.026
Mid intestine	21.0	24.7	29.6	2.65	0.15
Distal intestine	57.8[b]	87.2[a]	88.5[a]	5.28	0.010
Maltase activity, μmol min^{-1} kg^{-1} fish					
Pyloric intestine	48.3[a]	44.4[ab]	37.6[b]	1.94	0.022
Mid intestine	32.2	36.0	32.6	2.05	0.42
Distal intestine	33.9	33.3	28.1	1.47	0.060

[1] Pooled standard error of mean. Different letters denote significant ($P<0.05$) difference among diets. $n=3$ replicates per treatments.

degeneration and mild to moderate apoptosis and necrosis of renal tubule cells with concomitant exfoliation into the tubular lumen (Fig. 1) were observed in fish fed the krill containing diets. The renal glomeruli appeared normal.

During sampling grossly observable lesions were noted in the stomachs of between three and eight (of 18) fish per treatment (Fig. 2A). There was no apparent correlation to dietary treatment. The lesions appeared as focal to multifocal (2–3) round to oblong, pale, raised, slightly soft masses. Some of the lesions had a central depression, but no discoloration was noted. Histologically the lesions exhibited altered tissue architecture (Fig. 2B). The mucosa appeared as long folds extending to the basal lamina propria. The epithelia consisted of a single layer of low columnar cells. In more advanced lesions the normal glandular tissue was completely absent and epithelial sloughing was observed.

4. Discussion

The observed trend for increased pellet hardness and pellet durability with decreased pellet expansion with krill meal inclusion in the present experiment is in keeping with previous findings (Lue et al., 1990; Hansen and Storebakken, 2007). Increased lipid in the mash prior to extrusion has been shown to increase the lubrication of the extruder barrel resulting in reduced dough temperature (Lin et al., 1997) and to function as an insulating agent preventing water from being absorbed by the starch granules resulting in reduced starch gelatinization (Schweizer et al., 1986). This is in line with the present results, where increased lipid and reduced water in the mash reduced starch gelatinization, resulting in lower starch digestibility. This is in line with earlier results for rainbow trout (Bergot and Breque, 1983) and European sea bass (*Dicentrarchus labrax*) (Peres and Oliva-Teles, 2002).

Reduced growth in salmon fed the WKM diet agrees with data of Beck et al. (1977) and Koops et al. (1979), who completely replaced FM with WKM in diets for fresh water raised rainbow trout and observed reduced growth and feed efficiency. Olsen et al. (2006) reported the same effect, while Yoshitomi et al. (2006) reported decreased growth of rainbow trout fed 30% replacement of FM with WKM. Also in line with our results, Yoshitomi et al. (2007) found no effect on growth in rainbow trout when replacing all of the FM with a deshelled krill meal. These data indicate that the krill shell fraction can partially explain the reduced growth when a high fraction of FM is replaced by WKM.

The chitin content of whole Antarctic krill is approximately 30 g kg^{-1} dry weight (Nicol and Hosie, 1993). In the present experiment the PDKM and the WKM diets had chitin levels of 8.5 and 20.9 g kg^{-1} diet, respectively. Lindsay et al. (1984) reported reduced growth in juvenile rainbow trout fed 40, 100 and 250 g kg^{-1} dietary chitin compared to a control diet without chitin. Shiau and Yu (1999) reported that feeding

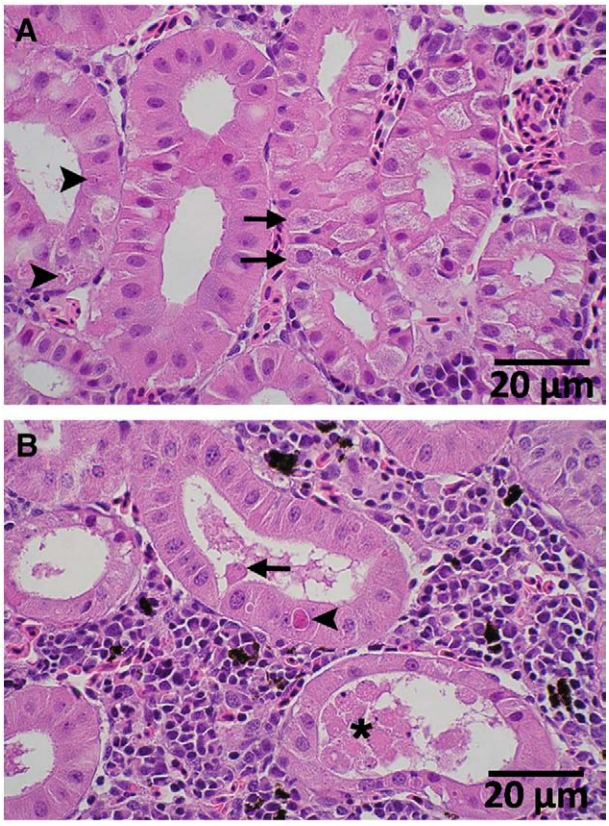

Fig. 1. Mild to moderate nephrotic changes of fish fed WKM (A) and PDKM (B) diets. Panel A shows degenerative renal tubule epithelial cells (arrows) and apoptotic bodies (arrowheads). Panel B shows extrusion of a renal tubule epithelial cell (arrow), apoptotic bodies (arrowhead) and cellular debris within the tubule lumen (asterisk).

Table 10
Dry matter, trypsin and lipase activity, bile acids concentration in the digesta of Atlantic salmon fed diets with fish meal (FM), partially deshelled krill meal (PDKM) or whole krill meal (WKM).

	FM	PDKM	WKM	s.e.m.[1]	*P*-value
Dry matter (g 100 g^{-1})					
Pyloric intestine	11.8[b]	11.7[b]	14.1[a]	0.36	0.005
Mid intestine	14.9	14.3	14.5	0.53	0.76
Distal intestine	16.7[a]	15.2[b]	15.1[b]	0.33	0.026
Trypsin (U mg^{-1} dry matter)					
Pyloric intestine	216.1[a]	221.1[a]	129.3[b]	6.42	<.0001
Mid intestine	174.5[a]	151.0[ab]	120.7[b]	7.51	0.007
Distal intestine	80.8	62.0	60.1	7.49	0.18
Lipase (U mg^{-1} dry matter)					
Pyloric intestine	0.044	0.041	0.044	0.003	0.56
Mid intestine	0.038[a]	0.028[b]	0.037[a]	0.002	0.018
Distal intestine	0.035	0.024	0.032	0.004	0.19
Bile acid (mg g^{-1} dry matter)					
Pyloric intestine	127.0[a]	107.5[ab]	82.0[b]	5.95	0.005
Mid intestine	115.6	111.0	67.2	15.6	0.12
Distal intestine	53.2[a]	38.8[b]	34.9[b]	2.39	0.004

[1] Pooled standard error of mean. Different letters denote significant ($P<0.05$) difference among diets. $n=3$ replicates per treatments.

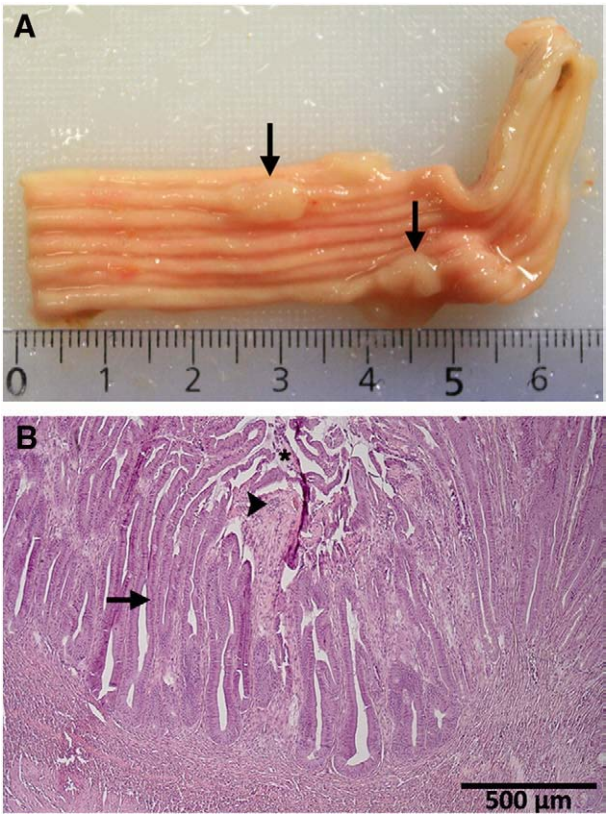

Fig. 2. Gross (A) and microscopic (B) appearances of stomach lesions. Histologically the lesions exhibited altered tissue architecture. The mucosa appeared as long folds (arrow) extending to the basal lamina propria. The epithelia consisted of a single layer of columnar cells. There is an area of widened lamina propria and leukocyte infiltration (arrowhead) accompanied by epithelial necrosis and sloughing (asterisk). The normal glandular tissue is completely absent. This condition appeared both for the FM and krill meal dietary treatments.

juvenile tilapia (*Oreochromis niloticus*×*O. aureus*) diets with 0, 20, 50 and 100 g kg^{-1} chitin resulted in a linear decrease in weight gain. Moreover, adding 10 g kg^{-1} chitin to diets depressed growth in juvenile carp compared to a control diet (Gopalakannan and Arul, 2006). Thus, it appears that even small amounts of dietary chitin can depress growth in fish.

The tendency of reduced lipid digestibility in fish fed the WKM diet in the present experiment indicates a negative effect of the krill shell fraction on lipid digestibility. Similarly, Olsen et al. (2006) found a tendency toward reduced lipid digestibility when all of the FM was replaced by WKM and suggested that the chitin produced a diarrhoea-like effect in the gastrointestinal tract. However, no such diarrhoea-like effect was observed in the present study, although a small difference in faecal dry matter content was noted among fish fed with FM and those fed diets with krill meal. Furthermore, Shiau and Yu (1999) reported decreased lipid digestibility (from 96.1 to 89.5%) in tilapia fed diets with increasing levels of dietary chitin (varying from 0 to 100 g kg^{-1}). Chitin is a natural biopolymer found in exoskeletons of crustaceans and has characteristics in common with insoluble plant fibre. However, the chemical properties of chitin differ mainly in its ability to form strong hydrogen bonds between the amino acetyl and hydroxyl groups (Minke and Blackwell, 1978). These chemical properties make chitin able to reduce activity of digestive enzymes as demonstrated for porcine pancreatic lipase (Kilinç et al., 2006). Furthermore, Zacour et al. (1992) reported that rats fed 5% chitin showed higher levels of triglycerides in faeces, suggesting that chitin may interfere with lipid absorption in the intestine. Our results did not reveal reduced lipase activity at high dietary chitin level, and thus, we cannot explain the reduction in lipid digestibility for fish fed the WKM diet by chitin's lipase inhibitory effects. The explanation for the lack of effect of WKM on lipase activity may be the fact that fish lipase are of the bile salt dependent type and as such different from the co-lipase dependent porcine pancreatic lipase (Gjellesvik et al., 1992). Rather, the decreased bile acid level in the pyloric intestine for fish fed the WKM diet might contribute to the reduced lipid digestibility because the pyloric region is the main area for lipid digestion and bile acids are essential for lipase activation as well as for efficient fatty acid absorption.

The reduction in digestibility of threonine, serine, glutamine, histidine, and lysine in fish fed the WKM compared to the FM fed fish may be related to the low trypsin activity seen in the fish fed the WKM diets compared to the control fed fish. A study by Rungruangsak-Torrissen (2007) supports our results by showing a significant reduction of *in vitro* amino acid digestibility with increased dietary WKM. One possible reason for this could be that chitin from a partially digested krill shell fraction is able to immobilize proteolytic enzymes as shown in previous experiments (Muzzarelli, 1980; Spagna et al., 1998).

In contrast to fish, that use hemoglobin for oxygen transport, crustacean blood utilizes respiratory proteins, with copper as the oxygen binding atom. This, results in high copper content in krill meals. Information on copper concentration in whole Antarctic krill, from several publications has been summarized by Locarnini and Presley (1995). The review shows variation from 12 to 82 mg copper kg^{-1} krill. The results of the present study are in line with previous studies showing 81 mg copper kg^{-1} of WKM. The high copper level was, however, not considered to be a factor in the observed growth reduction as Berntssen et al. (1999) found no effects on growth in juvenile Atlantic salmon fed up to 500 mg copper kg^{-1} diet.

In the present study, dietary krill inclusion did not affect muscle fluoride content, which is in line with previous experiments with Atlantic salmon, rainbow trout, Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) (Julshamn et al., 2004; Moren et al., 2007). In contrast, there was an increase in bone fluoride level in the fish fed the WKM diet with a dietary fluoride level of 640 mg kg^{-1} in the present study. This increase in bone fluoride was not seen in rainbow trout, Atlantic halibut, Atlantic cod or Atlantic salmon reared in sea water and fed krill diets containing up to 1080 mg fluoride kg^{-1} (Moren et al., 2007). The reason for increased bone fluoride in fish fed the WKM diet in the present study remains unclear. However, the amount of fluoride accumulated in the salmon bones in the present study was small (18.4 mg kg^{-1} dry bone) compared to fluoride accumulation found in rainbow trout fed krill in fresh water (2400 mg kg^{-1} dry bone) (Yoshitomi et al., 2006). The possible negative effect of a high content of fluoride in diets for fish has not been well examined. Yoshitomi, et al. (2006) found a significant reduction in growth for fresh water raised rainbow trout fed diets with 30% replacement of FM with WKM containing 444 mg fluoride kg^{-1} diet compared to a FM control group. In contrast, up to a complete replacement of FM with a deshelled krill meal (222 mg fluoride kg^{-1} diet) did not depress growth in rainbow trout reared in fresh water (Yoshitomi et al., 2007). Yoshitomi et al. (2007) suggested increased fluoride deposition in bone could depress fish growth. However, Landy (1988) showed reduction in feed intake and growth in fresh water raised rainbow trout fed 4450 mg fluoride kg^{-1} diet during the first four weeks of feeding, but obtained similar feed intake and growth as the control group after six weeks of the total 30 weeks feeding period. Growth was similar in fish fed <30, 450 and 2250 mg fluoride kg^{-1} diet during the total feeding period.

The blood parameters for Atlantic salmon fed the three experimental diets are within normal levels (Sandnes et al., 1988; Stoskopf, 1993). The reason for the decrease in blood cholesterol seen in krill fed fish is uncertain. It has been shown that chitin can prevent uptake of TG and reduce liver cholesterol for rats (Zacour et al., 1992). However, this seems not to be a pure effect of chitin, because the

PDKM and WKM diet contained 8.45 and 20.9 g chitin kg^{-1} diet and gave the same plasma cholesterol level. In contrast, Olsen et al. (2006) did not see any differences in plasma cholesterol level of Atlantic salmon when WKM totally replaced FM. However, blood lipid parameters are highly dependent on sampling and feeding time, thus, it is difficult to draw any firm conclusions. The plasma enzymes AST, ALT, AP can serve as indicators of liver and kidney functions in fish (Sandnes et al., 1988). The present results for these enzymes indicate that replacing all the FM with a PDKM or WKM for Atlantic salmon did not affect liver or kidney functions.

Increased concentrations of creatinine and urea in plasma may reflect renal structural damage or kidney dysfunction (Bernet et al., 2001). The reason for the elevated creatinine plasma levels of the FM compared to the krill fed fish is uncertain, however, all values were close to normal levels (Sandnes et al., 1988). The levels of inorganic phosphorous in plasma of fish fed the krill diets reflect the reduced faecal excretion values of phosphorous in these diets. The decreased faecal excretion of phosphorous in the krill diets, which were supplemented with mono calcium phosphate, indicates that phosphorous from FM is less available than phosphorous from this phosphorus source. This is in line with previous results (Nordrum et al., 1997).

Intestinal brush border enzyme activities can be sensitive indicators of enterocyte alterations. Marked reductions in intestinal brush border enzyme activities are observed in the DI of salmon fed soy-based diets (Krogdahl et al., 2003; Kraugerud et al., 2007; Øverland et al., 2009) indicating enterocyte dysfunction or lack of maturation. Differences in brush border enzyme activities of alkaline phosphatase (ALP) and leucine aminopeptidase (LAP) were found between dietary treatments in the current work in the PI and DI. However, activities were higher in krill fed fish compared to FM fed fish. While the exact cause of the increased activities is not clear, it may be related to increased tissue mass as indicated by higher organosomatic indices for PI and DI in krill fed fish compared to FM fed fish.

The lesions observed in the stomach are consistent with intestinal metaplasia, based on hematoxylin and eosin staining. In humans, intestinal metaplasia of the stomach is most often associated with chronic gastritis, and is generally considered to be a pre-neoplastic change. No signs of chronic gastritis, however, were observed in the present experiment. Whether or not these lesions are pre-neoplastic in Atlantic salmon is unknown. To our knowledge there are no reports describing this type of lesion in salmon. Spontaneously occurring as well as chemically induced gastro-intestinal neoplasms have been reported in fish (Bunton, 1996; Spitsbergen and Kent, 2003; Dale et al., 2009) though very little is known regarding their behavior. The lesions observed in the present experiment did not appear to be correlated with inclusion of krill meal in the diets. If any correlation with diet exists, then it is more likely to be associated with a common component(s) or characteristic all diets.

The histological findings in the kidney are consistent with mild to moderate nephrosis. Nephrosis has been associated with nephrotoxic therapeutants (antibiotics, anti-inflammatory agents), heavy metals (mercury, uranium, arsenic, nickel and cadmium) and xenobiotic pollutants (Reimschuessel and Ferguson, 2006). The fish in the current study have no history of treatment with potential nephrotoxic therapeutants. No analysis for xenobiotic pollutants was performed. Based on the reported levels of heavy metals in krill (Gasparics et al., 2000; Moren et al., 2006), levels in the diets were expected to be less than current EU limits (Commission dir. 2002/32/EC, 1334/2003, 2005/87/EC), except for copper. Copper levels exceeded EU limits in both the krill diets. However, neither whole body nor liver copper tissue levels were higher in fish fed diets containing krill meal compared to fish fed the FM control, nor were any significant histological changes observed in the liver. Excessive dietary fluoride has been shown to produce morphological changes in the rat kidney (Ogilvie, 1953), and renal tissue apoptosis and necrosis in both rabbits (Shashi et al., 2002) and pigs (Zhan et al., 2006). The fresh water teleost *Labeo rohita* exhibited renal damage after 30, 60, 90, and 120 days of exposure to water-borne fluoride at a level of 6.8 mg l^{-1} (Bhatnagar et al., 2007). However, in all of these studies the authors note changes in both renal glomeruli and tubules, whereas in the current study only tubular changes were observed. Even if there was no, or a low level, of fluoride accumulation in bone in fish fed krill, the dietary fluoride may explain the changes in kidney histology of fish fed the two krill diets. Additional studies are necessary to clarify the cause(s) of the observed kidney changes.

To conclude, replacing fish meal with a whole krill meal to Atlantic salmon reared in sea water gave reduced growth rate and reduced digestibility of several amino acids compared to a fish meal control. Replacing fish meal with a partially deshelled krill meal, on the other hand, generally resulted in similar or better growth performance and similar nutrient digestibilities than fish fed the fish meal control. Both krill containing diets gave signs of mild to moderate nephrosis.

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Paper II

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Replacement of LT fish meal with a mixture of partially deshelled krill meal and pea protein concentrates in diets for Atlantic salmon (*Salmo salar*)

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ABSTRACT

The aim of the study was to investigate how the replacement of LT-fish meal by a 3.5:1 mixture of an experimentally produced partially deshelled krill meal (PDKM) and a pea protein concentrate (PPC) affected growth rate, digestibility of main nutrients and minerals, fluoride accumulation and histology in Atlantic salmon (*Salmo salar*). Five extruded diets were fed to salmon with an average weight of 546 g, distributed into 15 tanks equipped with flow through sea water. During extrusion, increased krill meal resulted in increased lipid content in the feed mash and subsequently decreased pellet expansion and decreased pellet water stability. The salmon had an optimum growth rate with a PDKM/PPC inclusion of 400 g kg^{-1}. Starch digestibility decreased and lipid digestibility increased with increasing PDKM/PPC inclusion. Fluoride in faeces increased linearly with increasing dietary fluoride levels. Plasma cholesterol decreased with increased PDKM/PPC inclusion, whereas no major differences were seen for triglycerides, free fatty acids, glucose, or bile acids. Fish fed increased dietary PDKM/PPC showed an increased prevalence of mild to moderate nephrosis.

1. Introduction

Pea protein concentrate (PPC) produced by air classification can partially replace high quality LT fish meal (FM) without compromising growth rate or feed conversion in Atlantic salmon (*Salmo salar*) (Øverland et al., 2009). Recently, we have also found that an experimentally produced partly deshelled krill meal (PDKM) from Antarctic krill (*Euphausia superba*) can fully replace FM without compromising growth rate, and that Atlantic salmon fed PDKM tended to grow faster than fish fed the FM control (Hansen et al., 2010). Salmon fed a diet with a high level of krill meal with shells, on the other hand, tended to show reduced growth rate, probably associated with the chitin fraction (Shiau and Yu, 1999). The first aim of the present experiment was therefore to evaluate the effect of replacing FM with graded levels of a PDKM and PPC mixture (3.5:1) in diets for Atlantic salmon on growth performance and nutrient digestibility.

Replacing FM with PPC and PDKM in extruded diets may affect the physical quality of the feed. The PPC results in harder and more durable extruded pellets than FM (Øverland et al., 2009), while PDKM results in less expanded pellets due to lubrication of the extruder by the high lipid content (Hansen et al., 2010). Accordingly, the second aim was to investigate the affect of graded levels of PDKM/PPC in extruded diets with respect to pellet physical quality.

Fluoride leaches from the shell into the flesh of krill during frozen storage at temperatures higher than −30 °C (Christians and Leinemann, 1983; Adelung et al., 1987). The krill used for production of the PDKM had been stored at −15 to −20 °C prior to partial deshelling and further processing. In our previous study, we found no differences in fluoride contents in flesh of salmon fed diets with FM, PDKM or whole krill meal with shells, in keeping with the observations of Moren et al. (2007). We found, however, that the whole krill meal resulted in increased concentration of fluoride in the bones of the salmon, in contrast to the findings of Julshamn et al. (2004) and Moren et al. (2007). The experimentally produced PDKM allowed us to study the responses of fluoride from krill, relatively independently of the contents of chitin in the feed. Thus, a third aim was to investigate how salmon reared in sea water respond to increasing dietary fluoride concentrations, how efficiently it is excreted in faeces and how much is stored in the muscle and bone.

In contrast to vertebrates, that use haemoglobin for oxygen and CO_2 transport, crustaceans such as krill use hemocyanin with copper as the prosthetic group, resulting in high copper content in krill meals (Locarnini and Presley, 1995). The fourth aim was, therefore, to investigate the effect of feeding excess copper from increasing levels of krill meal in diets for salmon on mineral excretion and how the excess copper affected the utilisation of iron and zinc, two typical nutritional copper agonists.

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The previous study (Hansen et al., 2010) revealed a mild to moderate nephrosis in Atlantic salmon fed diets in which krill meal fully replaced FM. Therefore, the fifth aim was to determine if morphological changes in the kidney occurred in salmon fed diets containing increasing levels of a PPC/PDKM mixture, partially replacing FM.

2. Materials and methods

2.1. Diets

The experiment was designed as a regression study employing five extruded diets where FM was increasingly replaced by a mixture of a PDKM and PPC in a ratio of 3.5:1 (Table 1). The PDKM contained 610 g kg^{-1} crude protein and 187 g kg^{-1} lipids. To obtain a similar protein contribution from krill as for fishmeal, we needed to combine the krill meal with a protein ingredient with a low fat content. A ratio of 3.5:1 with PDKM and PPC was chosen to solve this problem since this ratio provided the same protein content as FM. The chemical composition of the PDKM, FM and the PPC is shown in Table 2 and the dietary chemical composition is shown in Table 3. The fish meal used was a Low Temperature (LT) dried FM with quality specifications, which ensure a low level of biogenic amines, given by Norsildmel A/L, Fyllingsdalen, Norway. The experimental krill meal and the extrusion technology used for producing the diets were as described in detail by Hansen et al. (2010) and Aslaksen et al. (2007).

2.2. Fish and facilities

A total of 375 Atlantic salmon of 546 g initial individual weight were used in a growth experiment at Nofima Marine AS, Sunndalsøra, Norway. The fish were randomly distributed to 15 fibreglass tanks of 1.5 m^3 each and kept in 34 g l^{-1} sea water supplied at 22 l min^{-1}. The average water temperature was 8.1 °C for the first period (days 1–56) and 7.0 °C for the second period (days 57–100). The tanks were equipped with individual light 24 h d^{-1}. Each diet was fed to triplicate groups of fish and the feed was supplied in excess of appetite, about 120% of measured feed intake, in order to facilitate maximum voluntary feed intake.

2.3. Sampling

All fish were individually weighed at the start of the experiment, and after 56 and at the end at 100 days. At the end of the experiment, prior to weighing and sampling, the fish were anaesthetized with metacaine (MS-222™; 50 mg l^{-1} water). All fish in each tank, except those sampled for histology, were stripped for faeces. Three fish per tank were sampled for whole body composition analysis. Blood was sampled from six fish by caudal venipuncture using vacutainers containing lithium heparin. The gastrointestinal tracts were removed and carefully cleaned. Histology samples were taken from four regions of the intestine: stomach, pyloric caeca, mid intestine and distal intestine. The liver and trunk (filtrative) kidney were also taken for histology analysis. The Norwegian quality cut, NQC (NS, 1994) was sampled from three fish per tank. A standard piece, approximately 10 g of the white dorsal muscle from the NQC was sampled with a cork punch. The remainder was microwaved in sealed plastic bags

Table 1
Diet formulation.

	Dietary PDKM/PPC[a] inclusion (g kg^{-1})				
	0	158	327	487	750
Formulation, g kg^{-1}					
Fish meal	641	504	357	217	0
Partly deshelled krill meal	0	123	257	383	598
Pea protein concentrate	0	35	70	104	152
Wheat flour[b]	108	107	107	96	84
Vitamin, mineral and inert marker[b]	5.7	5.7	5.7	5.7	5.7
Mono calcium phosphate[b]	0	0	0	9.2	10.0
Fish oil[b]	245	224	203	185	149

[a] PDKM and PPC mixture (3.5:1), PDKM; partly deshelled krill meal. PPC; pea protein concentrate.
[b] Specified by Hansen et al. (2010).

Table 2
Chemical composition[a] of partly deshelled krill meal (PDKM), fish meal (FM), and pea protein concentrate (PPC) used in the diets.

	PDKM	FM[b]	PPC[c]
Composition, g kg^{-1}			
Dry matter	964	969	904
Crude protein	610	708	496
Crude lipid	187	88	38
Starch	–	–	79
Ash	118	139	52
Chitin	8	0.2	–
Non-starch polysaccharides	nd[d]	nd	121
Minerals, mg kg^{-1}			
Fluoride	631	176	nd
Copper	78	5.2	nd

[a] Detailed composition of PDKM and FM is presented by Hansen et al. (2010).
[b] LT Eco, Norsildmel, Egersund, Norway.
[c] AgriMarine, Stavanger, Norway.
[d] Not determined.

Table 3
Chemical composition of the diets (g kg^{-1}).

	Dietary PDKM/PPC[a] inclusion (g kg^{-1})				
	0	158	327	487	750
Dry matter (DM)	960	953	964	968	958
In DM					
Crude protein	484	493	484	476	474
Total protein[b]	484	492	482	475	472
Total lipid	323	300	297	292	291
Starch	72.9	76.6	77.8	73.3	71.0
Ash	104	102	96.0	98.1	94.7
Chitin	0.06	2.08	3.37	3.86	5.06
Gross energy, MJ/kg	23.6	23.4	23.4	23.3	22.8
Amino acids[c], g kg^{-1}					
Total AA[d]	336.0	320.6	327.1	324.9	308.0
Cysteine	3.7	3.4	3.9	3.8	4.1
Methionine	10.1	9.5	9.3	8.9	7.9
Aspargine	35.2	34.3	35.5	36.0	35.0
Threonine	16.2	15.7	16.0	15.8	15.0
Serine	16.6	15.9	16.2	15.9	14.9
Glutamine	56.5	52.5	53.4	52.4	49.1
Proline	16.1	15.4	16.4	16.9	17.2
Glycine	20.2	19.1	18.4	17.5	15.1
Alanine	20.8	19.8	19.3	18.5	16.5
Valine	16.7	15.9	16.2	16.1	15.4
Isoleucine	14.8	14.4	14.9	15.3	15.1
Leucine	26.8	25.6	26.1	26.0	24.7
Tyrosine	9.8	9.6	10.3	10.4	10.5
Phenylalanine	14.4	14.0	14.5	14.9	14.7
Histidine	7.5	7.0	7.1	7.2	6.9
Lysine	27.9	26.7	26.9	26.3	24.2
Arginine	22.7	21.8	22.6	22.9	21.7
Minerals, kg^{-1}					
Phosphorus, g	14.5	13.4	12.4	13.6	11.8
Copper, mg	10	19	31	41	59
Iron, mg	130	130	140	170	180
Yttrium, mg	82	81	83	88	86
Zinc, mg	190	180	190	190	200
Fluoride, mg	110	140	240	290	430

[a] PDKM and PPC mixture (3.5:1), PDKM; partly deshelled krill meal. PPC; pea protein concentrate.
[b] Adjusted for chitin nitrogen.
[c] Presented in dehydrated form.
[d] Total sum of amino acids without tryptophan.

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until the bone easily separated from the muscle. The bone and muscle were freeze-dried and pooled by tank, and ground with a pestle and mortar prior to fluoride analysis.

2.4. Chemical and physical analyses

Fish scale contamination was removed from the faeces prior to grinding. The extruded diets and freeze-dried faeces were ground with a mortar and pestle prior to analysis. Diets and faeces were analysed for dry matter by drying to constant weight at 104 °C (Commission dir. 71/393/EEC), protein using Kjeldahl nitrogen (Commission dir. 93/28/EEC) × 6.25, lipid by HCl hydrolysis followed by diethyl ether extraction (Commission dir. 98/64/EC), starch (AOAC enzymatic method 996.11), ash (Commission dir. 71/250/EEC), minerals (ICP-AES/ICP-MS) (Nordic Committee on Food Analysis (NMKL) method 161), and yttrium oxide (ICP-AES) (NS-EN ISO 11885). Starch gelatinisation was analysed by the BioLab Analyse (Nofima Ingredients, Bergen) using a modification of Chiang and Johnson (1977) glucoamylase methodology. Fluoride concentration was determined using a fluoride ion selective combination electrode (VWR symphony, model 14002-788) using the procedure given by Malde et al. (2001). Amino acids were analysed according to the Commission dir. 98/64/EC on a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Chitin in diets was determined with a Biochrom 30 analyzer as described in Hansen et al. (2010).

Physical pellet quality was tested for durability, hardness, expansion, and water stability 14 days post-production. Pellet durability was tested with a DORIS pellet tester (AKVAsmart, Bryne, Norway) as described by Hansen et al. (2010). Pellet hardness was determined with a Texture Analyzer TA-XT2 (SMS Ltd., Surrey, UK), equipped with a 5 kg load cell as described by Øverland et al. (2009). The texture analyzer measured the pellet width and the length was recorded electronically. Pellet water stability was determined following the procedure of Baeverfjord et al. (2006).

2.5. Plasma clinical chemistry and histology evaluation

Blood samples were kept on ice until centrifugation (1500 × *g* for 10 min). Plasma samples were divided into three Eppendorf tubes, frozen on dry ice and stored at −80 °C until analysis. Plasma was analysed for glucose, cholesterol, triglycerides (TG), free fatty acids (FFA), and bile acids. The plasma analyses were performed according to the standard methods at the Central Laboratory at The Norwegian School of Veterinary Science. The method for FFA was based on Mulder et al. (1983) and the method for cholesterol was based on Trinder (1969) and Allain et al. (1974). The glucose and TG analyses were based on Tietz (1995). Bile acid in plasma and the histology evaluation was determined as described in Hansen et al. (2010).

2.6. Calculation, hypotheses and statistical analysis

Specific mechanical energy (SME) during extrusion was calculated as: $(2\times\pi\times60^{-1})\times(S_{rpm}\times Tk_{Nm}\times T_t/h^{-1})$, where S_{rpm} is screw speed, Tk_{Nm} is torque and T_t/h^{-1} is throughput. Pellet expansion was calculated as: ((pellet width − die diameter) × die diameter^{-1}) × 100. Specific growth rate (SGR) was calculated as: SGR = 100 × (ln(end wt) − ln(start wt)/Δt), where; end wt = end weight of fish, start wt = start weight of fish, Δ*t* = number of experimental days. Apparent digestibility coefficients were calculated as (%) ADC = $100\times((a-b)\times a^{-1})$, where

a = nutrient in feed × yttrium in feed^{-1}.

b = nutrient in faeces × yttrium in faeces^{-1}.

Faecal excretion of minerals was calculated as follows: $(-100\times((a-b)\times a^{-1}))+100$.

The experiment was designed to test the following main hypotheses:

Ho(1). Partial replacement of FM by a PDKM/PPC mixture improves physical quality of extruded salmon feed.

Ho(2). A PDKM/PPC mixture gives similar growth performance and utilisation of nutrients as high-quality FM as when used as a source of dietary protein for Atlantic salmon.

Ho(3). Increasing concentration of dietary krill meal results in increased uptake of fluoride and copper and causes pathological changes in salmon.

This experiment employed a completely randomised design. Regression analysis was used for evaluation of the relationship between different variables and the inclusion level of the PDKM/PPC mixture. Linear, polynomial and exponential models were tested for all regressions, and the best model was selected based on R^2, residual plots and significance level. One-way analysis of variance was used to differentiate among the diets, and significant ($P<0.05$) differences among diet means were ranked by Tukey's multiple range test and are indicated by different superscript letters in the tables. Trends are indicated for $0.10\leq P<0.05$. All statistical analyses were conducted with the software package SAS System Release 8.01 (SAS, 1990).

Table 4
Processing parameters, starch gelatinisation and physical pellet quality in diets based on fish meal (FM), and increasing levels of a partly deshelled krill meal/pea protein concentrate mixture (PDKM/PPC, ratio 3.5:1).

	Dietary PDKM/PPC inclusion (g kg^{-1})					S.E.M.[1]	Regression[2]	
	0	158	327	487	750		Equation	R^2/significance
Feed mash								
Lipid content[3], g kg^{-1}	77.9	90.0	103	114	134			
Moisture content[4], g kg^{-1}	268	257	228	201	215			
Extrusion								
Temperature in Section 3, °C	118	123	125	127	120			
SME[5], Wh kg^{-1}	37	37	39	43	39			
Starch gelatinisation[6]	93.1	92.2	92.3	92.5	84.5			
Physical pellet quality								
Hardness, N	16.7^{D}	20.1^{D}	27.6^{C}	35.7^{AB}	37.9^{A}	1.25	9.4 + 6.5x	0.93***
Durability, %	64.4^{D}	76.2^{C}	86.6^{B}	94.1^{A}	93.1^{A}	0.54	46.0 + 19.8x − 2.0x^2	0.99***
Expansion, %	23.6^{A}	16.5^{B}	14.6^{BC}	13.7^{C}	9.7^{D}	0.55	28.4 − 6.14x	0.90***

[1]Pooled standard error of mean. [2]x, dietary PDKM/PPC inclusion level. Significance for each polynomial factor is given by * = P<.05, ** = P<.01, *** = P<.001, and ns = nonsignificant (P>.05). [3]Lipid content in feed mash prior to processing. No lipid was added before vacuum coating. [4]Total moisture content, calculated based on analysed moisture content in raw materials and added amounts of steam and water in the conditioner. [5]Specific mechanical energy given in W h kg^{-1}. [6]Percent of total starch content in finished feed. [A,B,C,D]Different letters denote significant (P<0.05) difference among diets. n = 3 replicates per treatments.

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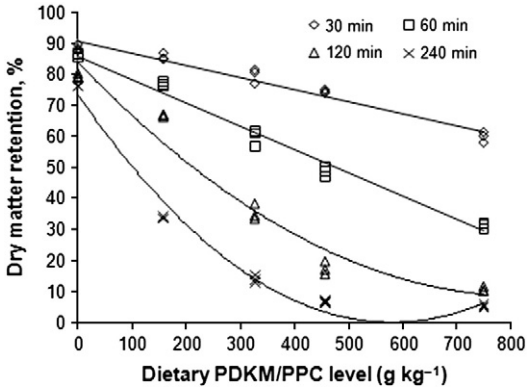

Fig. 1. Pellet water stability given in percent dry matter retained after given minutes in water shaking bath. Dry matter retention after 30 min ($P_{model}<0.001$, $R^2=0.90$, Equation = 98 − 6.9x), after 60 min ($P_{model}<0.001$, $R^2=0.99$, Equation = 101.7 − 13.7x), after 120 min ($P_{model}<.001$, $R^2=0.97$, Equation = 110.7 − 29.5x(***) + 1.8x^2(*)), and after 240 min ($P_{model}<.001$, $R^2=0.99$, Equation = 124.9 − 57.2x(***) + 6.7x^2(***)) as a function of dietary PDKM/PPC levels given with linear or second degree polynomial regression. Significance for each polynomial factor is given by * = P<.05, ** = P<.01, *** = P<.001.

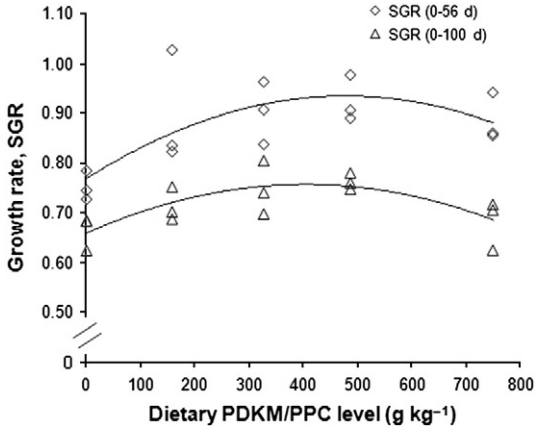

Fig. 2. Growth rates for SGR 0–56 d ($P_{model}=0.014$, $R^2=0.51$, Equation = 0.61 + 0.18x (*) − 0.02x^2(*)), and SGR 0–100 d ($P_{model}=0.012$, $R^2=0.52$, Equation = 0.54 + 0.13x (ns) − 0.02x^2(**)) as a function of dietary PDKM/PPC levels given with second degree polynomial regression. Significance for each polynomial factor is given by * = P<.05, ** = P<.01, *** = P<.001, and ns = nonsignificant (P>.05).

3. Results

3.1. Feed production and pellet quality

Increased lipid content in the feed mash with increasing PDKM/PPC inclusion resulted in decreased water added as steam during conditioning to obtain approximately the same extruder parameters (Table 4). The SME varied from 37 to 43 Wh kg^{-1} in the diets, while extrusion temperature remained fairly constant. Starch gelatinisation was reduced at the highest PDKM/PPC inclusion level. Pellet durability followed a second order polynomial model and appeared to reach a maximum at dietary PDKM/PPC level between 450 and 500 g kg^{-1} (Table 4). Pellet hardness increased linearly with increasing PDKM/PPC inclusion, whereas the expansion rate decreased with increasing PDKM/PPC. Increasing levels of PDKM/PPC gave decreasing pellet water stability (Fig. 1). After 30 min in the water bath, the average dry matter retention was reduced by 30% for the highest PDKM/PPC inclusion level compared to the fish meal control.

3.2. Growth performance

No fish died during the experimental period and there were no differences among treatments for initial weights. The fish grew from an average of 546 g to between 1060 and 1167 g after 100 days of feeding (Table 5). After 56 days, all krill and pea fed fish had gained significantly more weight than the FM fed fish. Tukey's range test showed no differences in SGR among the different dietary groups (Fig. 2). Using regression analysis, the SGR during the first feeding period (0–56 d) displayed a second degree polynomial fit, and appeared to reach an optimum inclusion level at a dietary PDKM/PPC level of approximately 500 g kg^{-1} (Fig. 2). The same applied for SGR (0–100 d), though, the optimum level had changed to approximately 400 g kg^{-1}.

3.3. Nutrient and mineral digestibility

Lipid digestibility of fish fed the FM control averaged 93.2% and increased linearly with increasing PDKM/PPC levels up to 97.1% (Fig. 3). Fish fed the highest PDKM/PPC level had significantly higher lipid digestibility compared to fish fed the FM control and the two lowest PDKM/PPC diets. Starch digestibility decreased linearly with increasing dietary PDKM/PPC levels, ranging from 74.3 to 60.5% (Fig. 3), whereas protein digestibility was not affected. Digestibility of threonine, serine, glutamine, valine, leucine, histidine, and lysine decreased linearly with increasing PDKM/PPC inclusion (Table 6). Digestibilities of these amino acids were significantly lower in fish fed the highest PDKM/PPC inclusion compared to the FM fed fish. In contrast, proline digestibility followed a second order polynomial regression, with a minimum digestibility at dietary PDKM/PPC level of 150 g kg^{-1}. Faecal excretion of copper increased with increasing dietary PDKM/PPC level, while iron excretion followed a second degree polynomial regression, and appeared to have a minimum at dietary PDKM/PPC level between 300 and 400 g kg^{-1}(Table 6).

3.4. Whole body and liver mineral, tissue fluoride concentrations, and radiography

The whole body mineral content did not differ among the dietary groups. Zinc content in liver increased linearly with increasing dietary PDKM/PPC levels, whereas the liver copper content fitted a second polynomial model, and appeared to have a minimum concentration at a dietary PDKM/PPC level between 300 and 400 g kg^{-1}.

The muscle tissue fluoride level ranged from 8.1 to 10.6 mg kg^{-1} and bone fluoride level ranged from 6.7 to 11.3 mg kg^{-1} on a dry weight basis with no effect of dietary treatment (Table 7). Fluoride content in faeces increased with increasing dietary PDKM/PPC level and followed a linear regression (y = 133 + 2.97x, $R^2=0.85$, P<.0001) (Fig. 4). The results of radiography showed no changes in

Table 5
Growth for Atlantic salmon fed increasing levels of a partly deshelled krill meal/pea protein concentrate mixture (PDKM/PPC, ratio 3.5:1).

	Dietary PDKM/PPC inclusion (g kg^{-1})					S.E.M.[1]	Regression[2]	
	0	158	327	487	750		Equation	R^2/significance
Start weight, 0 d, g	544	551	541	544	549	3.50		ns
Mid weight, 56 d, g	758^{B}	818^{A}	805.6^{A}	817^{A}	812^{A}	9.90	718 + 56x(*) − 7.5x^2(*)	0.55**
End weight, 100 d, g	1060	1123	1148	1167	1084	25.4	940 + 137x(ns) − 21x^2(**)	0.50*

[1]Pooled standard error of mean. [2]x, dietary PDKM/PPC inclusion level. Significance for each polynomial factor is given by * = P<.05, ** = P<.01, *** = P<.001, and ns = nonsignificant (P>.05). [A,B]Different letters denote significant (P<0.05) difference among diets. n = 3 replicates per treatments.

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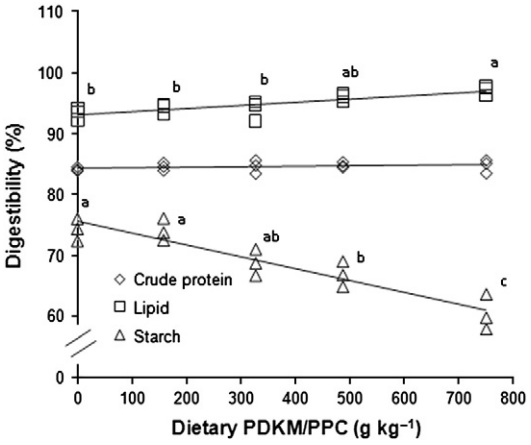

Fig. 3. Digestibilities of lipids (P_{model} = 0.003, R^2 = 0.65, Equation = 92.1 + 0.94x), starch (P_{model} = <.0001, R^2 = 0.82, Equation = 79.5 − 3.5x), and crude protein (ns) as a function of dietary PDKM/PPC levels given with linear regression. Different superscript letters denote significant (p>0.05) difference. ns = nonsignificant (P>.05).

bone density with increased fluoride accumulation. The incidence of abnormalities such as fused vertebrae or, shortened vertebrae approx 2/3 distance between cranium and tail was seen in 6 of total 36 fish examined independent of dietary treatment. All of the abnormalities were considered mild.

3.5. Blood plasma parameters, histology of the GIT, liver and kidneys

Plasma cholesterol decreased with increasing dietary PDKM/PPC levels (Table 8). Fish fed the FM control had significantly higher plasma cholesterol than fish fed the three diets with the highest PDKM/PPC inclusion. No major differences were seen for TG, FFA, glucose, and bile acids in blood plasma. No histological differences were found in the intestines and livers among fish from the five different dietary groups. In the trunk kidney, there was a significant correlation between PDKM/PPC in the diet and nephrotic changes, characterised by degeneration and/or apoptosis/necrosis of tubular epithelium and exfoliation of cells into the tubular lumen. The changes, when present, varied from mild to moderate. The numbers of fish with mild and moderate changes in the different dietary groups were; 0 and 0 for the control group; 2 and 0; 9 and 3; 8 and 7; and 10 and 3 for fish fed the highest PDKM/PPC inclusion, respectively. Renal glomeruli appeared normal.

4. Discussion

The reduced pellet expansion with increasing dietary PDKM/PPC inclusion was probably due to the increased lipid level in the mash, leading to increased lubrication in the extruder barrel, reduced dough temperature and decreased starch gelatinisation. This is in line with results of the work of Lin et al. (1997) and Schweizer et al. (1986). The observed negative relationship between hardness and expansion is in line with previous findings (Hsieh et al., 1989; Lue et al., 1990; Hansen and Storebakken, 2007). Pellet durability is recognised as a better measurement of physical quality than pellet hardness, as it correlates better with physical stress on pellets during transportation. Thus, the optimal physical quality of the pellets was obtained with a PDKM, FM and, PPC inclusion of approximately 380, 200 and 100 g kg^{-1} diet, respectively. In keeping with this, the PPC has been shown to improve pellet quality in extruded salmon feed (Øverland et al., 2009), and this could be associated with the higher amylase to amylopectin ratio in the pea starch compared to wheat starch (Vose, 1977; Zhou et al., 2004). In addition, krill meal can contribute to increased pellet durability as reported by Hansen et al. (2010). Water stability of the pellet can be influenced by processing parameters during extrusion (Baeverfjord et al., 2006) as well as by the ingredient composition (Lim, 1994). The reduction in water stability with increasing dietary krill meal in this experiment may be associated with the high amount of soluble proteins in krill meal compared to FM (Kolakowski, 1989; Olsen et al., 2006). In keeping with this, Aksnes et al. (1997) found

Table 6
Apparent digestibility (%) of amino acids and minerals faecal excretion in Atlantic salmon fed increasing levels of a partly deshelled krill meal/pea protein concentrate mixture (PDKM/PPC, ratio 3.5:1).

	Dietary PDKM/PPC inclusion (g kg^{-1})					S.E.M.[1]	Regression[2]	
	0	158	327	487	750		Equation	R^2/significance
Amino acids								
Total	90.5	89.1	89.6	89.4	88.8	0.41		ns
Cysteine	80.2^{A}	76.0^{B}	79.2^{AB}	77.5^{AB}	78.2^{AB}	0.82		ns
Methionine	90.3	88.9	89.3	89.6	89.0	0.47		ns
Aspargine	85.1	83.4	84.5	84.2	83.4	0.64		ns
Threonine	89.8^{A}	88.3^{AB}	88.5^{AB}	87.9^{AB}	86.7^{B}	0.49	90.2 − 0.67x	0.60***
Serine	89.9^{A}	88.5^{AB}	89.0^{AB}	88.6^{AB}	87.8^{B}	0.44	90.0 − 0.41x	0.40*
Glutamine	93.0^{A}	91.7^{AB}	91.9^{AB}	91.5^{AB}	90.6^{B}	0.32	93.2 − 0.48x	0.62***
Proline	90.1^{AB}	89.0^{B}	90.4^{AB}	90.8^{A}	91.5^{A}	0.36	90.3 − 0.66x(**) + 0.18x^2(ns)	0.54**
Glycine	88.3	87.2	88.1	88.4	88.6	0.46		ns
Alanine	92.0	91.0	91.2	91.2	90.8	0.34		ns
Valine	90.5^{A}	89.0^{AB}	89.1^{AB}	89.0^{AB}	88.2^{B}	0.47	90.5 − 0.44x	0.40*
Isoleucine	90.2	88.7	88.9	88.8	87.9	0.50	90.2 − 0.44x	0.38*
Leucine	92.0^{A}	90.8^{AB}	90.9^{AB}	90.6^{AB}	89.9^{B}	0.39	92.2 − 0.44x	0.51**
Tyrosine	89.7	88.0	89.0	89.3	89.1	0.46		ns
Phenylalanine	89.4	88.0	88.5	88.6	88.1	0.48		
Histidine	89.2^{A}	87.4^{AB}	87.6^{AB}	87.2^{AB}	86.1^{B}	0.50	89.4 − 0.63x	0.54**
Lysine	93.3^{A}	92.3^{AB}	92.4^{AB}	92.1^{AB}	91.4^{B}	0.30	93.5 − 0.40x	0.57**
Arginine	92.5	91.8	92.5	92.6	92.8	0.26		ns
Faecal excretion of minerals, %								
Phosphorus	68.3	72.3	66.7	64.0	64.0	2.25		ns
Copper	77.4^{A}	78.1^{A}	93.7^{BC}	91.8^{B}	105.0^{C}	2.71	68.6 + 6.8x	0.76***
Iron	108.0^{BC}	82.4^{AB}	96.7^{ABC}	76.8^{A}	112.4^{C}	6.08	138.3 − 37.3x(ns) + 6.3x^2(*)	0.42*
Zinc	67.2	65.4	66.7	66.7	72.4	1.97		ns

[1]Pooled standard error of mean. [2]x, dietary PDKM/PPC inclusion level. Significance for each polynomial factor is given by * = P<.05, ** = P<.01, *** = P<.001, and ns = nonsignificant (P>.05). ^{A,B,C}Different letters denote significant (P<0.05) difference among diets. n = 3 replicates per treatments.

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Table 7
Fluoride content in muscle tissue, bone tissue and faeces, minerals in whole body and liver of Atlantic salmon fed increasing levels of a partly deshelled krill meal/pea protein concentrate mixture (PDKM/PPC, ratio 3.5:1).

	Dietary PDKM/PPC inclusion (g kg^{-1})						S.E.M.[1]	Regression[2]	
	Start	0	158	327	487	750		Equation	R^2/significance
Fluoride, mg kg^{-1}									
Muscle		10.3	9.23	9.66	10.6	8.10	0.74		ns
Bone		8.64	6.73	9.25	11.3	9.40	1.0		ns
Faeces		519^{C}	627^{C}	772^{BC}	989^{B}	1533^{A}	67.8	y = 133 + 2.97x(***)	0.85***
Whole body minerals, mg kg^{-1}									
Calcium	4490	4490	3940	3830	4450	4480	351		ns
Phosphorus	5200	5460	5140	5170	5420	5420	157		ns
Zinc	65.9	61.8	65.7	63.4	61.7	57	4.36		ns
Copper,	3.64	2.53	2.11	2.41	2.16	2.59	0.15		ns
Liver minerals, mg kg^{-1}									
Zinc		2.51^{B}	2.46^{B}	2.56^{AB}	2.61^{AB}	2.72^{A}	0.04	2.40 + 0.06x(**)	0.58**
Copper		11.1^{A}	9.10^{AB}	8.17^{B}	8.97^{AB}	11.1^{A}	0.58	14.6 − 4.32x(ns) + 0.70x^2(***)	0.67**

[1]Pooled standard error of mean. [2]x, dietary PDKM/PPC inclusion level. Significance for each polynomial factor is given by * = P<.05, ** = P<.01, *** = P<.001, and ns = nonsignificant (P>.05). [A,B,C]Different letters denote significant (P<0.05) difference among diets. n = 3 replicates per treatments.

reduced water stability of pellets with increased levels of soluble proteins in extruded fish feed.

The replacement of FM with a PDKM/PPC mixture had no adverse effect on the growth rate of Atlantic salmon reared in sea water. The significant growth enhancement of fish fed krill during the first period is in line with previous results from Olsen et al. (2006) who described increased initial growth rate in Atlantic salmon reared in sea water fed moderate levels of krill while there were no differences in growth rate during the second period. The reason for this could be an initial increase in feed intake due to increased feed palatability, while after a given period, the palatability effect may fade. In agreement with this, Toften and Jobling (1997a) reported a short-term increase in feed intake with the addition of squid extract to a medicated feed for Atlantic salmon. The uses of feed attractants have, in addition, shown to improve feed acceptability and growth performance of Atlantic salmon (Toften et al., 1995, 2003; Toften and Jobling, 1997b).

The amount of phospholipids in Antarctic krill has been reported to between 41 and 54% of the total lipid content (Clarke, 1980; Fricke et al., 1984), but this varies with sex and life stage (Pond et al., 1995). The level of phospholipids in refined fish oils, on the other hand is low. The triglycerides/phospholipid ratio in the residual lipid content in fish meal is about 2:1 of total lipids (Tocher et al., 2008). These values indicate that the FM control and the highest PDKM/PPC diet contained approximately 5 and 15% phospholipids of total lipids, respectively. Phospholipids acts as surfactants in the intestine during lipid digestion and may thus improve lipid emulsification and increase lipid digestibility in fish (Hung et al., 1997; Tocher et al., 2008), which might explain the increased lipid digestibility with the increasing PDKM/PPC inclusion in the present experiment.

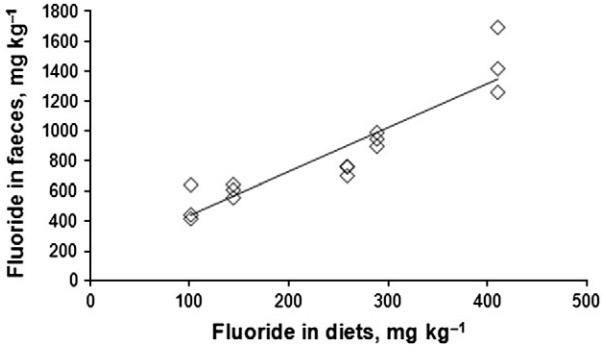

Fig. 4. Fluoride content in faeces for Atlantic salmon as a function of dietary fluoride given with linear regression (P_{model}<.0001, R^2 = 0.85, Equation = 133 + 2.97x).

The reason for the reduced starch digestibility observed with increasing inclusion of PDKM/PPC was most likely due to a higher resistance of legume starch to amylase catalysed hydrolysis, than observed for cereal starch (Bednar et al., 2001). Low digestibility of starch from legumes has previously been described for Atlantic salmon (Aslaksen et al., 2007). In contrast, Øverland et al. (2009) found no negative effect on starch digestibility when adding 70% of the total dietary starch (8.8%) from pea starch to diets for Atlantic salmon reared in sea water. However, the increased starch gelatinisation in the FM based diet compared to the diets with the highest PDKM/PPC inclusion level would be expected to be reflected in the starch digestibility. This is in keeping with previous findings for rainbow trout (*Oncorhynchus mykiss*) (Bergot and Breque, 1983) and European sea bass (*Dicentrarchus labrax*) (Peres and Oliva-Teles, 2002).

Digestibility of crude protein did not differ among dietary groups in the present experiment, but a negative relationship was found between dietary PDKM/PPC and digestibility of threonine, serine, glutamine, valine, isoleucine, leucine, histidine, and lysine. The higher digestibility of total amino acids than of the crude protein indicates lower digestibility of the non-protein nitrogen compounds in the krill meal, which increases with dietary inclusion of PDKM/PPC. The reason for the decrease in digestibility of several amino acids with increasing dietary PDKM/PPC inclusion is uncertain. Amino acids from the PPC has shown similar digestibilities in Atlantic salmon compared to a FM control (Øverland et al., 2009), and should thus not affect the amino acid digestibility negatively. Hansen et al. (2010), however, reported similar amino acids digestibility in salmon fed a diet based on PDKM compared with diets based on FM indicating that the PPC rather than the krill reduced the amino acid digestibility in the present experiment. Interestingly, proline digestibility was lowest at a dietary PDKM/PPC level of 160 g kg^{-1}, indicating a different digestibility pattern for this amino acid. This could be related to the increased dietary copper level, considering that proline forms various complexes with copper ions (Twiss, 1996). Increased dietary copper from bacterial protein meal fed to Atlantic salmon and rainbow trout, however, did not result in the same pattern for proline digestibility as in our study (Aas et al., 2006a,b).

The copper content of the PDKM in this experiment is similar to levels reported previously (Locarnini and Presley, 1995), showing 78 mg copper kg^{-1} krill meal. The reduced apparent copper uptake with increasing PDKM/PPC inclusion, indicated by increased faecal excretion, is in line with previous findings for Atlantic salmon parr showing significantly decreased copper digestibility with diets containing 35 mg copper compared to diets with 5 mg copper kg^{-1}

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Table 8
Blood chemistry in all groups of Atlantic salmon fed increasing levels of a partly deshelled krill meal/pea protein concentrate mixture (PDKM/PPC, ratio 3.5:1).

	Dietary PDKM/PPC inclusion (g kg^{-1})					S.E.M.[1]	Regression[2]	
	0	158	327	487	750		Equation	R^2/significance
Metabolites								
Cholesterol, mM	15.7^{A}	16.4^{A}	13.7^{B}	12.2^{BC}	11.7^{C}	0.38	17.6 − 1.23x	0.79***
TG[3], mM	2.6	2.7	2.8	2.6	2.8	0.08		ns
FFA[4], mM	0.53	0.73	0.69	0.52	0.68	0.09		ns
Glucose, mM	4.6	4.5	4.9	5.1	5.0	0.17		ns
Bile acids. μM	13.9	8.9	7.6	6.9	8.3	2.65		ns

[1]Pooled standard error of mean. [2]x, dietary PDKM/PPC inclusion level. Significance for each polynomial factor is given by * = P<.05, ** = P<.01, *** = P<.001, and ns = nonsignificant (P>.05). [3]Triglycerides. [4]Free fatty acids. [A,B,C]Different letters denote significant (P<0.05) difference among diets. n = 3 replicates per treatment.

(Berntssen et al., 1999). Dietary copper, iron, and zinc compete for binding sites on the transporters involved in intestinal mineral transport (Gunshin et al., 1997). No increase in zinc faecal excretion or whole body zinc content was, however, seen in this study. This indicates no antagonistic effect of zinc uptake with increasing copper levels from krill meal, which is in line with previous results (Berntssen et al., 1999).

The liver is the main organ for accumulation of absorbed copper (Bury et al., 2003). Fish fed increasing levels of copper from increasing levels of krill, however, showed no increase in copper accumulation in whole body or liver in the present experiment. This, together with the increased copper excretion, indicates a regulatory function in dietary copper uptake in Atlantic salmon as also shown by Berntssen et al. (1999). The reason for the difference among the dietary treatments in liver copper and zinc content in this experiment is uncertain.

The fluoride levels in muscle and bone of Atlantic salmon fed increased dietary PDKM/PPC levels in sea water are in agreement with previous experiments with Atlantic salmon, rainbow trout, Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) (Julshamn et al., 2004; Moren et al., 2007). There was a strong linear relationship between dietary fluoride and fluoride in faeces in the present study. The slope given by the regression showed that the faecal fluoride concentration was three times higher than in the diets, and that fish are effectively eliminating the dietary fluoride through the faeces.

The observed blood parameters in the present experiment showed values within normal ranges for Atlantic salmon (Sandnes et al., 1988; Stoskopf, 1993). There was a linear decrease in blood cholesterol with increased dietary PDKM/PPC level. Also in our previous study investigating effects of complete replacement of FM with PDKM or a whole krill meal for Atlantic salmon decreases in plasma cholesterol were observed (Hansen et al., 2010). In contrast to this, Olsen et al. (2006) did not see any differences in plasma cholesterol in Atlantic salmon fed up to a complete replacement of FM with a whole krill meal. The decreased plasma cholesterol observed in the present work could be due to the increased dietary inclusion of PPC since the cholesterol level in plant meals and oils is low compared to levels found in marine meals and oils (Tocher et al., 2008). In addition, soy fed to salmonids can have hypocholesterolemic effect (Kaushik et al., 1995; Kraugerud et al., 2007).

The increasing numbers and severity of kidney changes with increasing dietary PDKM/PPC level suggest that the nephrosis is dose dependent. The current results support our previous findings (Hansen et al. 2010) where nephrotic changes were seen in fish fed krill based diets (whole krill meal or PDKM as the sole protein source). These changes may be caused by one or several factors in the krill meal. As discussed in the previous study, fluoride is capable of causing these types of changes, as seen in rabbits (Shashi et al., 2002) and pigs (Zhan et al., 2006). Further investigations are needed to clarify the possible role of fluoride and what, if any, consequence these changes may have.

In conclusion, increased levels of PDKM/PPC in extruded salmon diets resulted in increased pellet durability and decreased water stability. The salmon had a maximum growth rate with a PDKM/PPC inclusion of 400 g kg^{-1}. Starch digestibility decreased with increasing PDKM/PPC inclusion, possibly as a result of the reduced gelatinisation during extrusion. The lipid digestibility increased while the digestibility for several amino acids decreased with increasing PDKM/PPC inclusion level. There was a correlation between fluoride in diets and fluoride in faeces, indicating a steady elimination of fluoride through the faeces. The same trend was shown for copper. Fish fed increased dietary PDKM/PPC had an increased level of mild to moderate nephrosis.

Acknowledgements

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Paper III

Photo previous page: Knut Werner Alsén

Tissue fluoride accumulation and kidney lesions in freshwater reared Atlantic salmon (*Salmo salar*) fed high dietary fluoride concentrations

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Abstract

Antarctic krill (*Euphausia superba*) and other marine zooplankton may contain high levels of fluoride. The aim of the present experiment was to determine if dietary fluoride from Antarctic krill at levels similar to the old and the new EU allowable limits in fish feeds (150 and 350 mg kg^{-1}) would induce kidney lesions in freshwater reared Atlantic salmon (*Salmo salar*). In addition to the diets containing krill, two high sodium fluoride (NaF) diets (1,500 and 3,500 mg kg^{-1}) were used to investigate the effect on growth, feed intake, faecal excretion of minerals, and accumulation of fluoride in various tissues. No major effects of growth or feed intake were seen among dietary treatments. A higher proportion of the ingested fluoride was absorbed in salmon fed the NaF diets compared to fish fed

krill shell. Fluoride accumulated in liver, kidney and especially bone. Faecal excretion of calcium and magnesium were higher for the NaF fed fish compared to fish fed the control and krill shell diets, whereas the levels of these minerals in plasma were unaffected. Dietary fluoride from krill shells did not induce kidney lesions. One-third of the salmon fed the highest NaF diet showed signs of crystal formation within the distal tubules and/or collecting ducts in the kidney.

Key-words: Fluoride excretion; Antarctic krill, *Euphausia superba*; Atlantic salmon, kidney histology.

1. Introduction

Fluoride is one of the most electronegative elements and is toxic at high doses. In humans, chronic fluoride toxicity from intake of a moderate or high level of fluoridated water is known to produce fluorosis related diseases such as osteosclerosis and symptoms such as pain and stiffness in joints (Whitford 1996). For freshwater fish, water-borne fluoride is toxic at low levels, but this is highly dependent on exposure time and water hardness (Camargo 2003). Dietary fluoride toxicity has only marginally been examined in fish. Experiments with rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmon salar*), Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) fed various krill meals containing fluoride levels up to 1,000 mg kg^{-1} diet and reared in salt water showed no fluoride accumulation in bone or muscle, reduction in growth or effects on health (Olsen *et al.* 2006, Moren *et al.* 2007, Julshamn *et al.* 2004). In contrast, Hansen *et al.* (2010) found a small but significant fluoride accumulation in bone of Atlantic salmon

reared in salt water when fed a diet containing whole krill meal compared to a partially deshelled krill meal. Fish reared in fresh or brackish water, however, have shown increased fluoride accumulation in bone with increasing levels of dietary fluoride (Grave 1981, Tiews *et al.* 1982, Yoshitomi *et al.* 2006).

In mammals, fluoride is excreted through the kidneys, and the kidney is the organ that accumulates the greatest amount of fluoride among the soft tissues (Whitford 1996). To our knowledge, no studies examining increased dietary fluoride levels and soft tissue accumulation of fluoride have been reported for fish reared in freshwater. Thus, our first aim was to investigate the possibility of fluoride accumulation not only in muscle and bone, but also in kidney and liver from Atlantic salmon fed fluoride from Antarctic krill (*Ephausia superb*a). Two levels of dietary fluoride were investigated; the previous EU dietary limit of 150 mg kg^{-1} (Commission dir. 2002/32/EC) and the new EU limit levels of 350 mg kg^{-1} (Commission dir. 2008/76/EC). In addition, salmon were fed two diets containing 1,500 and 3,500 mg fluoride kg^{-1} as sodium fluoride (NaF). NaF was chosen as the fluoride source for these extreme diets because it was impossible to obtain a fluoride level of 3,500 mg kg^{-1} using krill shell as the fluoride source and chitin from the krill would have been a confounding factor for the second aim of this experiment which was designed to examine growth rate and feed conversion ratio (FCR) in salmon fed extreme levels of fluoride. Further, the third aim of the present experiment was to investigate the faecal excretion of fluoride and other minerals from fish fed the five experimental diets.

Histological changes in gill, kidney and intestine have been reported for the Indian Major carp rohu (*Labeo rohita*) exposed to 6.8 mg fluoride l^{-1} water for 120 days, in freshwater (Bhatnagar *et al.* 2007). Differences in gill histology have also been reported for rainbow trout exposed to water-borne fluoride (Neuhold and Sigler 1962). Fluoride has

been shown to produce histological changes in the gastrointestinal tract of rats fed 30 mg fluoride day^{-1} (Das *et al.* 1994). Ogilvie (1953) reported histological changes in rat kidneys after both acute and chronic dietary fluoride exposure. Newer findings report kidney changes such as degeneration of tubular epithelia, tissue necrosis, extensive vacuolization in renal tubules, hypertrophy and atrophy of glomeruli in rabbits injected daily with 5, 10, 20, and 50 mg NaF kg^{-1} body weight (Shashi *et al.* 2002). In previous experiments, we found signs of mild to moderate nephrosis in Atlantic salmon reared in salt water and fed krill meal (Hansen *et al.* 2010, 2011). Thus, the fourth aim of this experiment was to determine if fluoride, even at low levels, could induce histological kidney changes in Atlantic salmon reared in freshwater.

2. Materials and methods

2.1. Diets

Five experimental moist diets were produced using gelatin as a binder (Table 1). One low-fluoride control diet and two diets with fluoride levels of 150 and 350 mg kg^{-1} with krill shell as fluoride source (KS150 and KS350). In addition, two diets containing 1,500 and 3,500 mg fluoride kg^{-1} with NaF as fluoride source were produced (NaF1500 and NaF3500). The krill shell meal was produced from frozen Antarctic krill (United Ocean Co., Ltd, South Korea) at the Norwegian University of Life Sciences (UMB) (Ås, Norway) by a technology proprietary to Krillsea Group AS. Yttrium oxide (Y_2O_3) was used as an inert marker for determination of apparent faecal excretion (Austreng *et al.* 2000). All dry ingredients except gelatine were mixed with a Moretti Foreni kneading machine (Spiry 25, Mondolfo, Italy). The gelatine was mixed in cold water, heated to 60

°C in a microwave oven and mixed with 5 g kg^{-1} attractant (7468 L-030, Protein extract, Seagarden ASA, Norway) and fish oil. The hot gelatine-oil mixture was added to the dry mix resulting in a firm dough that was cold pelleted through an Italgi pasta extruder (P35A, Carasco, Italy) equipped with a 2 mm die. After cooling to room temperature, 2 ml of water was mixed with 5 g kg^{-1} attractant and spray coated on the pellets. The pellets were stored at –20 °C prior to feeding.

2.2. Biological experiment and sampling procedure

A total of 600 Atlantic salmon were used in this experiment which was carried out at the fish laboratory at the Norwegian University of Life Sciences, Ås, Norway. The fish weighed 12.1 g on average and were randomly distributed to 15 fiberglass tanks (300 l). Recirculated freshwater (average water temperature of 14.2 °C) was supplied to each tank at 6-7 l min^{-1} during the 30 day experiment. The fish were kept under a constant light regime. Each diet was fed to triplicate tanks, in excess of appetite, to ensure maximum feed intake. Uneaten feed was sieved from the outlet water of each tank and feed intake was monitored according to Helland *et al.* (1996). The daily rations were approximately 10% in excess, based on the level of uneaten feed. Uneaten pellets were collected daily and stored in sealed boxes at –18 °C prior to dry matter analysis.

2.3. Sampling procedure

All fish were bulk weighed per tank at the start and at the end of the 30 days experiment. At the final sampling, prior to weighing and sampling, all fish were anaesthetized with metacaine (MS-222^{TM}; 50 mg l^{-1} water). Five fish per tank were sampled for whole body composition analysis. Blood was taken from six fish per tank by

caudal venipuncture using a heparinised 1 ml syringe. Blood from each fish was transferred to a two ml Eppendorf tube, centrifuged (1,500xg for 10 min), frozen in liquid nitrogen and stored at –80 prior to analysis. Prior to plasma mineral analysis, equal amounts of plasma from each fish were pooled due to small sample sizes. The liver, trunk (filtrative) kidney, and second left gill arch were taken for histological analysis from six fish per tank (18 fish per treatment). The same six fish were stored at –20 °C prior to further processing when a transverse section between the cranial part of the dorsal fin and the cranial part of the anal fin was taken. Each sample was microwaved in a sealed plastic bag until the bone easily separated from the muscle. Only the dorsal part of the muscle was used. The bone and muscle were freeze dried and pooled within tank, ground with a mortar and pestle prior to fluoride analysis. The last 24 fish in each tank were frozen and stored at –20 prior to further dissection when liver, trunk (filtrative) kidney and the gut content of the distal intestine where pooled within each tank and freeze-dried for fluoride analysis.

2.4. Chemical analyses

The diets and faeces were freeze-dried and ground with a mortar and pestle prior to analysis. Diets and faeces were analyzed for dry matter by drying to constant weight at 104°C (Commission dir. 71/393/EEC), ash (Commission dir. 71/250/EEC), protein using Kjeldahl nitrogen (Commission dir. 93/28/EEC) × 6.25, lipid by HCl hydrolysis followed by diethylether extraction (Commission dir. 98/64/EC), starch (AOAC enzymatic method 996.11), and minerals according to Denstadli *et al.* (2006) using a Perkin Elmer Optima 5300 DV ICP (Perkin Elmer, Inc 2004 Shelton, USA). Gross energy was determined with a PARR 1281 oxygen bomb calorimeter (Moline, IL, USA). Chitin in the krill shell was determined with a Biochrom 30 analyzer as described in Hansen *et al.* (2010), and the

dietary chitin levels were calculated based on the chitin levels in the krill shell. Fluoride in tissues was determined with a VWR® sympHony® fluoride ion selective combination electrode (Illinois, US) according to Malde *et al.* (2001) with minor changes. Instead of using a 3:1 ratio (w:w) of NaOH to sample we used a ratio of 9:1. Sample sizes down to 50 mg were used when the expected fluoride levels were high. Standards were prepared from a 1,000 mg l^{-1} fluoride stock solution (Hanna Instruments, Italy). Plasma fluoride was analyzed based on the known addition method described by Ekstrand (1977). Low level total ionic strength adjustment buffer (TISAB) was made by diluting 57 ml glacial acetic acid and 58 g NaCl, both analytical-reagent grade, in one liter deionized water and adjusted to pH 5.0–5.5 with 5 M NaOH. Sarstedt centrifuge tubes, 8 ml, (60.542) were used as a sample container. A 0.15 ml blood plasma and 0.15 ml of low level TISAB were pipetted into the tube and mixed and the potential (mV) was recorded. A buffered fluoride standard with approximately 100 times the ion concentration and 1 % volume was then added to the sample. A new potential recording was made and the fluoride concentration was calculated according to formulae described by Ekstrand (1977).

2.5. Histological evaluation

Samples were fixed in neutral buffered formalin (40 g formaldehyde l^{-1}; pH 7.4) for 24 h and subsequently transferred to 70% ethanol until processing. Initially, three fish sampled from each tank were processed for tissue histology (n =nine per treatment). Tissues were processed at the Pathology Laboratory, NVH using standard histological methods. Sections stained with haematoxylin and eosin (H&E). When initial screening indicated differences among groups (i.e. in kidney samples) all remaining samples were examined (n =18 total per treatment).

2.6. Calculation and statistical analysis

The following calculations were used:

- Feed conversion ratio (FCR) = $DM_{feed} \times (FW–IW)^{-1}$

  DM_{feed}=feed intake

  FW=final weight of fish

  IW=initial weight of fish

- Specific growth rate (SGR) = $100 \times (\ln(FW)–\ln(IW)/\Delta t)$;

  Δt=number of experimental days.

- Apparent faecal excretion : = $(–100 \times ((a–b) \times a^{-1}))+100$;

  a = nutrient in feed×(yttrium in feed)$^{-1}$.

  b = nutrient in faeces×(yttrium in faeces)$^{-1}$.

The results were analyzed by one-way analysis of variance (ANOVA), followed by Tukey`s multiple range test to differentiate between the diets (SAS 1990). Results are presented as means and pooled standard errors of means (s.e.m.). The level of significance was chosen at $P<0.05$ and tendency is indicated for $0.05<P\leq0.10$.

3. Results

During the experimental period, two fish died, which appeared to be independent of dietary treatment, and no fish showed signs of abnormal behavior. The salmon grew from an average weight of 12.0 g to 32.2 g during the 30 day experimental period (Table 3). Feed intake varied among dietary treatments, with significantly higher feed intake in fish

fed the KS350 diet compared to the NaF3500 diet. The final weights were similar among fish fed the experimental diets, except for a significantly lower weight for fish fed the NaF3500 diet. The SGR ranged from 3.02 to 3.38 among the dietary treatments. According to ANOVA there was a significant differences in SGR among the dietary treatments, but the Tukey`s multiple range test was too conservative to separate the means. However, fish fed the NaF3500 grew less than the other groups. The fish fed KS350 also had a higher FCR compared to the control.

Faecal excretion of minerals is shown in Table 4. Fish fed the krill shell had similar faecal excretion of fluoride compared to fish fed control diet whereas the NaF fed fish and lower excretion compared to fish fed the control. Faecal excretion of calcium was higher for fish fed the NaF3500 diet compared to fish fed the control and the KS150 diet. Potassium excretion was significantly affected by dietary treatment ($P = 0.024$), but the Tukey's multiple range test did not show any significant difference among the means. The faecal excretion of magnesium was highest for fish fed the NaF3500 diet while excretion of sodium and phosphorus were unaffected by dietary treatments.

The fluoride levels in whole body, muscle, liver, kidney and faeces were significantly affected by the dietary treatment (Table 5). Fluoride level in whole body of the control fish was higher at the start of the experiment compared to the end of the experiment. Fish fed the NaF3500 diet showed higher fluoride levels in whole body compared to both fish fed the control and the krill shell diets. The fluoride levels in muscle were similar among dietary treatments, except in fish fed the NaF3500 diet. These fish had18.3 mg fluoride kg^{-1} muscle. Both liver and kidney from fish fed the two NaF diets had significantly more fluoride accumulation compared to fish fed the control and krill shell diets. Fluoride levels in kidney for fish fed the NaF1500 and NaF3500 diets were

59.6 and 297.3 mg fluoride kg^{-1}, respectively. The fluoride level in bone and faeces showed patterns similar to liver and kidney but with a higher degree of accumulation. Levels were 2,302 and 5,300 mg fluoride kg^{-1} for bone and faeces in fish fed the NaF3500 diet. Dietary fluoride affected the plasma fluoride levels significantly with 50 times higher plasma fluoride in the NaF3500 fed fish compared to fish fed the KS350diet. Plasma levels of calcium and magnesium remained unchanged among dietary treatments.

Histological evaluation of liver and gills revealed no differences among dietary treatments. These tissues appeared normal in all groups. However, tissue changes were observed in the trunk (filtrative) kidney in fish fed the NaF3500 diet (Fig. 1). Crystalline material was observed within distal tubules and/or collecting ducts in 6 of 18 fish fed the NaF3500 diet. These observations were not seen in any other diet group. The presence of crystalline material was often accompanied by degenerative/necrotic cellular debris. No other significant differences in tissue appearance were noted between groups. In all groups, few samples had rare or occasional tubules that contained exfoliated epithelial cells.

4. Discussion

The overall growth rate of the dietary groups was higher than growth rates presented by Austreng *et al.* (1987) and similar than newer growth rate estimates (Skretting 2010). The reduced weight gain of fish fed the NaF3500 diet is in line with that reported by Shi *et al.* (2009b) who described reduced weight gain for white sturgeon fed 710 and 1,450 mg fluoride kg^{-1} diet. Feed intake of fish fed the NaF3500 diet in the present experiment did not statistically differ from the control group, however, the reduced weight gain is likely an effect of reduced feed intake. In contrast, Shi *et al.* (2009b) reported reduced feed

intake and reduced condition factor in sturgeon fed 710 and 1,450 mg fluoride kg^{-1}. This indicates that Atlantic salmon accept a higher dietary level of fluoride without markedly reducing their feed intake compared to the white sturgeon that differ from other teleosts with high percentage of the skeleton as cartilage (Bemis *et al.* 1997). In line with the present results, rainbow trout reared in freshwater and fed krill diets containing 2,500 mg fluoride kg^{-1} did not show any growth reduction compared to a control group (Tiews *et al.* 1982). The FCR was not affected by dietary NaF in either the present experiment or for the white sturgeon (Shi *et al.* 2009b). Fish fed the KS350 containing 12 g chitin kg^{-1} diet had, however, increased FCR of fish in the present experiment. This is in line with Bromley and Adkins (1984) describing how fish increase their feed intake to compensate for the reduced nutrient content.

To our knowledge, literature reporting values for faecal excretion or digestibility of fluoride in fish reared in freshwater is limited. Fish fed the control diet and the krill shell diets indicate similar uptake of fluoride over a dietary range of 30 to 350 mg kg^{-1} diet from either SKP fish powder or krill shell. When the dietary levels of fluoride increased, a higher portion of the ingested fluoride was taken up in the fish. The contrary was shown for rats, where the net absorption of fluoride decreased with increasing dietary fluoride (Whitford 1994). The reason for this could be that fluoride from SKP fish powder and krill shell is less bioavailable due to chemical bonding compared to NaF as shown for hydroxyfluorapatite in bone meal (Whitford 1996). Fluoride is known to create indigestible compounds with both calcium and magnesium in the intestinal tract (Harrison *et al.* 1984, Cerklewski 1987, Cerklewski and Ridlington 1987). Thus, another reason for the low faecal excretion of fluoride can be an overload of fluoride in the digestive tract and the

availability of free calcium and magnesium ions can be limited. A higher portion of the ingested fluoride can, therefore, be taken up.

The high faecal excretion of both calcium and magnesium in fish fed the NaF diets are reflected by the low solubility and bioavailability of CaF_2 and MgF_2 which are easily created in the intestinal tract. The high faecal excretion of calcium of fish fed the NaF diets may lead to calcium deficiency over time, when the fish are kept in freshwater. The level of calcium and magnesium in plasma in the present experiment were, however, not affected by dietary treatments. Acute toxicity of fluoride in fish, characterized by anorexia, loss of equilibrium and sporadic movements, is likely caused by reduced blood calcium ion concentration due to stable complex formation between fluoride and calcium (Neuhold and Sigler 1960, Landy 1988). In the present experiment, however, no such behavior was observed. There were no differences in faecal excretion of phosphorous and sodium and this is probably due to the lower ability to bind fluoride compared to calcium.

The higher fluoride levels in whole body for the initial fish compared to fish fed the control and the krill shell diets is likely a result of the fairly high fluoride level in the commercial feed used prior to the start of the experiment (167 mg fluoride kg^{-1}) in combination with a low fluoride level for the control feed and a dilution of the fluoride concentration caused by rapid growth. The level of fluoride in the whole body of fish fed the NaF diets reflects the high fluoride levels found in the other tissues, especially in bone. As described previously for fish reared in freshwater, the accumulation of fluoride was highest in bone in all dietary groups (Tiews *et al.* 1982, Yoshitomi *et al.* 2006). Rainbow trout fed 450, 2,250 and 4,450 mg fluoride kg^{-1} had, however, had clearly higher fluoride levels in vertebral bone after five weeks of feeding compared to observations in the present experiment (Landy 1988).

Limited information is available regarding fluoride metabolism in fish. Siberian sturgeon exposed to fluoridated water showed no increase in fluoride in muscle or liver, but showed a clear increase in gills, skin, cartilage and bone (Shi *et al.* 2009a). For terrestrial animals, the fluoride concentration in plasma and interstitial fluid are assumed to be the same and fluoride distribution among soft tissues is dependent on the tissue-water-to-plasma-water (T/P) concentration ratio (Whitford *et al.* 1979). High lipid tissues such as adipose tissue and brain, therefore, have low concentrations of fluoride. In agreement with this, the increased level of plasma fluoride in fish fed increased dietary fluoride in the present experiment reflects the fluoride levels in various soft tissues. The fluoride level in muscle was low compared to liver and kidney as has been shown in rats (Armstrong and Singer 1980). The increased fluoride levels in liver are, however, in contrast to results of Chouhan *et al.* (2010) who reported no increased liver fluoride level in rats fed increased dietary fluoride whereas the kidneys showed a significant increase. The kidney is the only soft tissue in terrestrial animals that clearly shows increased fluoride concentration because it is the major fluoride excreting organ (Chen *et al.* 1956). In humans, the kidney is responsible for approximately 90% of fluoride excretion (Maheshwari *et al.* 1981) which is linearly related to glomerular filtration rate (Schiffl and Binswanger 1982). In freshwater fish, the kidneys excrete large quantities of dilute urine because the influx of water, mainly through the gills, can average 50% of the body water per hour (Evans 1998). High urine flow may prevent concentration of fluoride within kidney tubules, thus explaining why nephrotic changes, as previously reported in Atlantic salmon fed diets containing high levels of krill meal and reared in sea water (Hansen *et al.* 2010), were not observed in the current experiment. The extent to which the gills participate in excretion of ingested (i.e. dietary) fluoride in freshwater fish is uncertain. Neuhold and Sigler (1962) showed reduced

toxicity of waterborne fluoride in rainbow trout when the fish were either pre-adapted or exposed to an increased water chloride level. They suggested that the increased chloride level increased the numbers of chloride cells in the gills thus enhancing the ability of fish to excrete fluoride. This suggests that the gills may also play a role in fluoride excretion though little direct evidence is available.

In the present experiment, fluoride concentrations in the kidney were fairly similar to that found in liver, except for fish fed the NaF3500 diet which the kidneys showed a markedly higher level compared to the liver. This suggests that these fish failed to excrete the high levels of fluoride in the urine and indicates that the kidney in freshwater fish is important for fluoride excretion. The higher levels of fluoride appear to be responsible for the urinary crystals observed histologically in fish fed the NaF3500 diet and are in agreement with (Singh *et al.* 2001) who reported increased kidney stone formation in humans with increased intake of fluoridated water. In contrast, low levels of dietary fluoride have been shown to reduce the incident of kidney stones in rat (Hering *et al.* 1985). A fluoride level of 3,500 mg kg^{-1} is unlikely to be used commercially, despite fluoride levels up to 4,000 mg kg^{-1} in meal from the amphipod *Themisto libelulla* (Moren *et al*. 2007). Under practical conditions, urolithiasis is, therefore, unlikely to occur in freshwater fish fed diets containing krill meal.

In conclusion, fluoride levels of 150 and 350 mg kg^{-1} from krill shell did not induce kidney lesions in freshwater reared Atlantic salmon, whereas some of the salmon fed 3,500 mg fluoride kg^{-1} showed signs of urolithiasis within distal tubules and/or collecting ducts. Salmon fed NaF absorbed more of the ingested fluoride compared to the krill shell fed fish and the fluoride accumulated in the liver, kidney and especially bone. High levels of

dietary NaF increased faecal excretion of calcium and magnesium whereas the levels of these minerals in plasma were unaffected.

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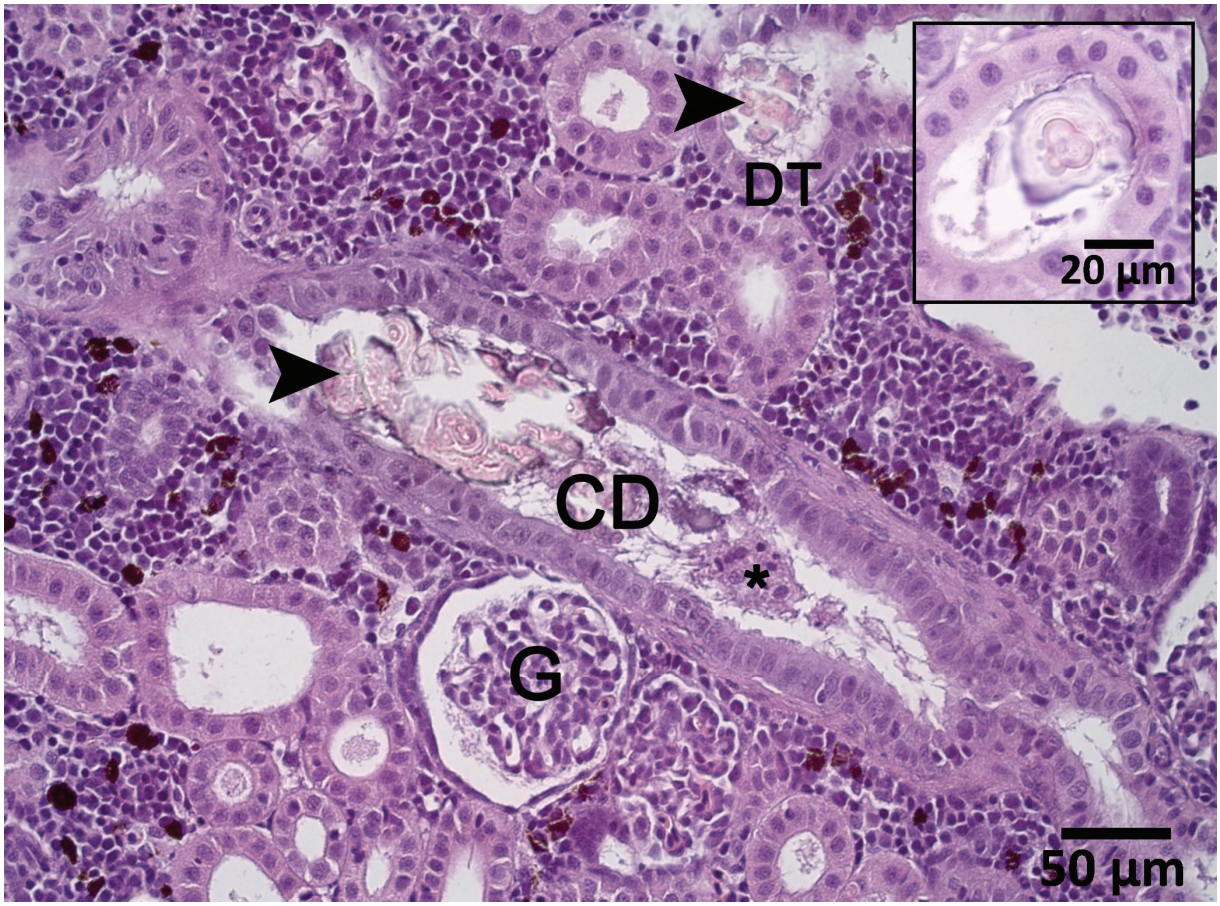

Figure 1. Photomicrograph of trunk kidney tissue of a fish fed the NaF3500 diet. Urinary calculi (arrowheads) are visible within the lumen of collecting ducts (CD) and distal tubules (DT). Necrotic cellular debris (asterisk) is also visible within the collecting duct lumen. The concentric crystalline structure of the calculi is clearly visible. The inset photomicrograph shows a higher magnification view, clearly showing the concentric crystalline structure.

Table 1. Diet formulation, g kg^{-1}

	Control	Krill shell		NaF	
Dietary fluoride, mg kg^{-1}	29.9	150	350	1,500	3,500
Fish powder[a]	567	542	487	564	560
Krill shell[b]	0	45	120	0	0
Gelatinized potato starch[c]	130	130	130	130	130
Gelatin[d]	80	80	80	80	80
Fish oil[e]	160	150	140	160	160
Sodium fluoride[f]	0	0	0	3.2	7.5
Cellulose[g]	20	10	0	20	20
Flavor concentrate[h]	10	10	10	10	10
Sodium phosphate[i]	15	15	15	15	15
Magnesium dichloride[j]	7	7	7	7	7
Choline[k]	5	5	5	5	5
Mineral and vitamin premix[l]	5.5	5.5	5.5	5.5	5.5
Yttrium oxide[m]	0.1	0.1	0.1	0.1	0.1

[a]SKP 0268, Rieber og Søn, Bergen, Norway. [b]Krill shell (crude protein 450 g kg^{-1}; lipid 130 g kg^{-1}; ash 150 g kg^{-1}; chitin 97 g kg^{-1}; fluoride 2,880 mg kg^{-1}. [c]Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden. [d]Rousselot® 250 PS, Rousselot SAS, Courbevoie, France. [e]NorSalmOil, Norsildmel, Egersund, Norway. [f]Sigma-Aldrich, S7920-500g, St. Louis, USA. [g] Alpha-Cel™ C100, International Fibre Europe NV, Belgium. [h]Protein extract,7468 L-030, Seagarden ASA, Norway. [i]Sodium phospate, 1.06580, Merck, Darmstadt, Germany. [j]Magnesium Cloride, 25107.361, VWR, Haasrode, Belgium. [k]Choline chloride, 70 % Vegetable, Indukern s.a., Spain. [l]Farmix, Trouw Nutrition, LA Putten, The Netherlands. Per kg feed. Retinol 2500.0 IU, Cholecalciferol 32400.0 IU, α-tocopherol SD 0.2 IU, Menadione 40.000 mg, Thiamin 15.0 mg, Riboflavin 25.0 mg, d-Ca-Pantothenate 40.003 mg, Niacin 150.003 mg, Biotin 3000.0 mg, Cyanocobalamin 20.0 mg, Folic acid 5.0 mg, Pyridoxine 15.0 mg, Ascorbate polyphosphate 0.098 g, Cu: CuSulfate $5H_2O$ 11.998 mg, Zn: ZnSulfate 89.992 mg, Mn: Mn(II)Sulfate 34.993 mg, I: K-Iodide 1.999 mg, Se: Na-Selenite 0.200 mg, Cd Max. 0.003 mg, Pb Max. 0.028 mg, Ca 0.915 g, K 1.380 g, Na 0.001 g, Cl 1.252 g. [m]Y_2O_3. Metal Rare Earth Limited, Shenzhen, China.

Table 2. Chemical composition of the experimental diets

	Control	Krill shell		NaF*	
Dietary fluoride, mg kg^{-1}	29.9	150	350	1,500	3,500
Dry matter (DM), g kg^{-1}	586	607	607	586	614
In DM, kg^{-1}					
Crude protein, g	618	611	605	601	594
Lipid, g	185	182	176	183	186
Starch, g	121	124	121	119	122
Ash, g	68.9	75.4	83.5	72.8	79.5
Chitin, g		5.0	12.0		
Gross energy, MJ	22.8	22.7	22.6	22.9	22.7
Minerals					
Fluoride, mg kg^{-1}	29.9	142	370	1,366	3,222
Calcium, g kg^{-1}	7.5	9.0	10.6	7.2	7.4
Potassium, g kg^{-1}	6.7	7.0	7.2	6.5	6.4
Magnesium, g kg^{-1}	2.1	2.4	2.8	2.0	2.0
Sodium, g kg^{-1}	8.9	9.6	10.3	10.4	12.2
Phosphorus, g kg^{-1}	9.6	10.5	11.3	9.6	9.4
Yttrium, g kg^{-1}	0.08	0.08	0.08	0.08	0.08

* Sodium fluorid

Table 3. Feed intake, feed conversion ratio (FCR), and specific growth rate (SGR) in Atlantic salmon fed dietary fluoride (F^-) level of 150 and 350 mg kg^{-1} with krill shell and 1,500 and 3,500 mg kg^{-1} with sodium fluoride (NaF) as fluoride source

	Control	Krill shell		NaF		S.E.M. *	P-value
Dietary F^-, mg kg^{-1}	29.9	150	350	1,500	3,500		
Initial weight (g $fish^{-1}$)	12.1	11.9	11.9	12.1	11.9	0.22	0.90
Final weight (g $fish^{-1}$)	33.4[a]	32.4[a]	33.1[a]	32.8[a]	29.5[b]	0.61	0.008
Feed intake (g $fish^{-1}$)	11.8[ab]	11.8[ab]	13.1[a]	12.3[a]	10.3[b]	0.35	0.003
FCR	0.56[a]	0.58[ab]	0.62[b]	0.59[ab]	0.58[ab]	0.009	0.008
SGR	3.37	3.31	3.38	3.33	3.02	0.078	0.046

*Pooled standard error of mean.

[ab]Different subscripts denote significant ($P<0.05$) difference among diets. n= 3 replicates per treatments.

Table 4. Faecal excretion in Atlantic salmon fed dietary fluoride (F^-) level of 150 and 350 mg kg^{-1} with krill shell as fluoride source or 1,500 and 3,500 mg kg^{-1} with sodium fluoride (NaF) as fluoride source

	Control	Krill shell		NaF		S.E.M. *	P-value
Dietary F^-, mg kg^{-1}	29.9	150	350	1,500	3,500		
Faecal excretion, % of dietary intake							
Fluoride	87.7 [a]	86.8 [a]	79.1 [a]	61.8 [b]	54.3 [b]	2.05	<.0001
Calcium	83.7 [b]	85.0 [b]	87.4 [ab]	95.6 [ab]	108.7 [a]	4.78	0.022
Potassium	67.1	82.3	63.4	64.1	81.7	4.47	0.024
Magnesium	39.6 [c]	45.8 [bc]	52.4 [b]	49.2 [bc]	74.5 [a]	2.41	<.0001
Sodium	28.3	31.0	29.7	24.2	29.1	1.68	0.12
Phosphorus	50.6	58.4	54.8	51.9	58.7	2.01	0.056

*Pooled standard error of mean.

[abc]Different subscripts denote significant ($P<0.05$) difference among diets. n= 3 replicates per treatments.

Table 5. Fluoride (F^-) content in whole body, muscle, bone, liver, kidney (mg kg^{-1} dry wt.) and plasma minerals of Atlantic salmon fed dietary fluoride level of 150 and 350 mg kg^{-1} with krill shell or 1,500 and 3,500 mg kg^{-1} with sodium fluoride (NaF) as fluoride source

	Start	Control	Krill shell		NaF		S.E.M. *	P-value
Dietary F^-, mg kg^{-1}		29.9	150	350	1,500	3,500		
Tissues, mg kg^{-1}								
Whole body	37.0 ^{c}	10.7 ^{d}	12.6 ^{cd}	29.5 ^{cd}	200.0 ^{b}	284.8 ^{a}	4.80	<.0001
Muscle		3.97 ^{b}	3.27 ^{b}	5.17 ^{b}	8.40 ^{b}	18.3 ^{a}	1.69	0.0005
Liver		3.80 ^{c}	4.63 ^{c}	13.8 ^{c}	50.0 ^{b}	81.6 ^{a}	6.11	<.0001
Kidney		8.5 ^{c}	9.33 ^{c}	15.9 ^{c}	59.6 ^{b}	297.3 ^{a}	7.45	<.0001
Bone		19.9 ^{c}	27.5 ^{c}	97.5 ^{c}	1,381 ^{b}	2,303 ^{a}	32.1	<.0001
Faeces		88.8 ^{d}	309.3 ^{cd}	729.6 ^{c}	2,279 ^{b}	5,300 ^{a}	110.6	<.0001
Plasma								
Fluoride, µmol l^{-1}		1.30 ^{c}	1.78 ^{c}	3.81 ^{c}	63.4 ^{b}	195.3 ^{a}	4.37	<.0001
Calcium, mmol l^{-1}		3.30	3.23	3.43	3.30	3.30	0.07	0.4
Magnesium, mmol l^{-1}		1.34	1.29	1.36	1.33	1.32	0.05	0.9

*Pooled standard error of mean.

^{abcd}Different subscripts denote significant ($P<0.05$) difference among diets. n= 3 replicates per treatments.

Paper IV

Photo previous page: Knut Werner Alsén

Dietary calcium supplementation reduces the bioavailability of fluoride from krill shell and NaF in rainbow trout (*Oncorhynchus mykiss*) reared in fresh water

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Abstract

The use of Antarctic krill (*Euphausia superba*) in fish feeds may be limited by a high fluoride level. The aim of this experiment was to investigate the bioavailability of dietary fluoride from either krill shell or sodium fluoride (NaF) to rainbow trout (*Oncorhynchus mykiss*). The second aim was to investigate if the fluoride bioavailability was affected by adding calcium to the feed. Five experimental diets were produced; one control diet and four test diets containing 700 mg fluoride kg^{-1} either from NaF or from krill shell, with or without added calcium chloride (15g Ca kg^{-1}). Triplicate groups of fish were given one meal in excess of appetite prior to sampling. Five fish from each replicate tank were terminally sampled for blood; 0, 1, 3, 6, 9, 12, 15, 18, 24, 36 h postprandial. Fluoride in blood plasma was used as an indicator of fluoride absorption. Fish fed NaF showed a clear peak in plasma fluoride of 1.17 mg l^{-1} 3 h postprandial, followed by a rapid decline. The fish fed krill shell did not obtain a

clear peak but had a maximum fluoride plasma concentration (C_{max}) of 0.32 mg l^{-1} 3 h postprandial. The addition of dietary calcium reduced the fluoride uptake in fish fed the NaF diet by nearly 100%, while adding calcium to the diets with krill shell reduced the uptake of fluoride to nearly the half. To conclude, fluoride from krill shell had a lower bioavailability than fluoride from NaF. Dietary calcium was an efficient way to reduce the fluoride uptake of rainbow trout reared in fresh water.

Key-words: Fluoride bioavailability; Calcium fluoride; Antarctic krill, *Euphausia superba*; Rainbow trout.

1. Introduction

Antarctic krill (*Euphausia superba*) contains a high amount of fluoride which is a limiting factor for the utilization of krill meal in both aquaculture feeds and human food (Boone and Manthey, 1983). The bioavailability of fluoride is dependent on chemical bonding of the fluoride and what type of feed-matrix it is incorporated into (Whitford, 1996). Fluoride found in the bones of mammals is in the form of fluorapatite which is highly insoluble and has a low bioavailability (Whitford, 1991). The fluoride in krill is mainly located in the exoskeleton (Sands et al., 1998), but the chemical form is uncertain. Zhang et al. (1993) suggeted that the fluoride is linked to the hardening of the krill exoskeleton by the formation of $Ca_5(PO_4)_3F$, which has a very low solubility (2×10^{-51}). Further, Sands et al. (1998) suggested that the high fluoride concentration in the mouth parts of the krill exoskeleton acts as a hardener, because the mouth needs to be hard to ingest food. The bioavailability of fluoride derived from krill has been shown to be high for rats (Tenuta and Alvarenga, 1999). Soevik and Braekkan (1981) have shown similar bioavailabilities of fluoride to young

growing chicks with fluoride derived from either northern krill (*Meganyctiphanes norvegica*) or from fish protein concentrate. The first aim of the present experiment was to compare the bioavailability of fluoride from Antarctic krill to that of sodium fluoride (NaF) in rainbow trout (*Oncorhynchus mykiss*).

Fluoride has a strong affinity for calcium and forms CaF_2 with a solubility product as low as 3.9×10^{-11}. Preventing fluoride absorption by adding dietary calcium was first tried by Lawrenz and Mitchell (1941) who showed reduced uptake of dietary fluoride from food and water in rats fed increased calcium. In rainbow trout reared in fresh water, Landy (1988) reported reduced clinical sign of fluoride poisoning and reduced fluoride accumulation in vertebrae when adding 2 or 4% calcium to the diets compared to a control with 0.2% calcium. The latter experiment, however, only used NaF as the fluoride source. Thus, the second aim of this experiment was to investigate whether dietary calcium supplementation reduced fluoride uptake in rainbow trout reared in fresh water fed Antarctic krill shells or NaF.

2. Materials and methods

2.1. Diets

Five moist experimental diets were produced with gelatin as a binder (Table 1). The diets containing krill shell and NaF were designed to obtain 700 mg kg^{-1} of fluoride (Table 2). Calcium ($CaCl_2$) was added to one krill shell diet (krill shell+Ca) and one NaF diet (NaF+Ca). The diets without calcium will henceforth be referred as the NaF diet and the krill shell diet. The krill shell meal was produced by deshelling frozen krill at the Norwegian University of Life Sciences (UMB, Ås, Norway) using a technology proprietary to Krillsea Group AS. All dry ingredients except gelatine and $CaCl_2$ were mixed in a Kenwood mixer (MX270, Hampshire, UK). Gelatine and $CaCl_2$ were mixed in cold water, heated to 60 °C in a

microwave oven and mixed with the fish oil. The dry mix was added to the hot gelatine-oil mixture resulting in a firm dough that was formed trough a Braun meat grinder (Power Plus 1300, Kronberg, Germany) equipped with a 3 mm die. After cooling to room temperature the pellets were cut with a knife to a pellet size of approximately 4 mm and stored at –20 °C prior to feeding.

2.2. Biological experiment and sampling procedure

A total for 750 rainbow trout, weighing of average 34.1 g, were randomly distributed to 15 fiberglass tanks of 300-l each and kept in recirculated fresh water under constant light. The water supply to each tank was 6–7 l min^{-1} and the water temperature was 16.4 °C. The fish were acclimated in the experimental tanks for three weeks and fed a low fluoride control diet similar to the control diet in the present experiment. Triplicate groups of fish were given one meal in excess of appetite prior to sampling. The fish were fed for 20 min. Five fish from each replicate tank were netted, given a lethal dose of MS-222 and sampled for blood at; 0,1, 3, 6, 9, 12, 15, 18, 24, 36 h postprandial. Blood was sampled by caudal venipuncture using 1-ml syringes treated with heparin. Blood from five fish was pooled in sterile 5-ml vacutainers and stored on ice prior to centrifuging. The blood samples were centrifuged at 1450×g in a CENCOM II (Athens, Greece), divided into three Eppendorf tubes and stored at –80 °C prior to fluoride analysis. After blood sampling, the fish were stored at –20 °C in plastic bags. After the experiment ended, the stomach from fish sampled one h postprandial was removed from half thawed fish and frozen digesta was collected. The digesta was pooled from the five fish, freeze-dried and weighed. Ten ml of water was sampled from the outlet of each tank after each sampling, stored capped at 5 °C prior to fluoride analysis.

2.3. Chemical and physical analyses

Freeze-dried diets and stomach content were ground with a pestle and mortar prior to analysis. Diets were analyzed for dry matter by drying to constant weight at 104 °C (Commission dir. 71/393/EEC), protein using Kjeldahl nitrogen (Commission dir. 93/28/EEC) × 6.25, lipid by HCl hydrolysis followed by diethylether extraction (Commission dir. 98/64/EC), starch (AOAC enzymatic method 996.11), ash (Commission dir. 71/250/EEC), and minerals were done according to Denstadli et al. (2006) using a Perkin Elmer Optima 5300 DV ICP (Perkin Elmer, Inc 2004 Shelton, USA). Chitin in the krill shell was determined with a Biochrom 30 analyzer as described in Hansen et al. (2010), and the dietary chitin levels were calculated based on the chitin levels in the krill shell.

Plasma fluoride was measured with a VWR® sympHony® fluoride ion selective combination electrode (Arlington Heights, IL, USA) based on the known addition method described by Ekstrand (1977). Standards were prepared from a 1000 mg l^{-1} fluoride stock solution (Hanna Instruments, Padova, Italy), deionized water, and a low level total ionic strength adjustment buffer (TISAB). The low level TISAB was made by diluting 57 ml glacial acetic acid and 58 g sodium chloride, both analytical-reagent grade, in one liter deionized water and adjusted pH to 5.0–5.5 with 5 M NaOH. Sarstedt centrifuge tubes, 8 ml, (60.542) were used as a sample container. A 0.4 ml blood plasma sample and 0.4 ml of low level TISAB were pipetted into the tube and mixed and the potential (mV) was recorded. A buffered fluoride standard with approximately 100 times the ion concentration and 1 % volume was then added to the sample. A new potential recording was made and the fluoride concentration was calculated according to formulae described by Ekstrand (1977). Water fluoride was measured directly (0.5 ml water + 0.5 ml low level TISAB) with a fluoride electrode.

2.4. Calculation and statistical analysis

The pharmacokinetic variables as maximum fluoride plasma concentration (C_{max}), time when C_{max} occur (T_{max}), and the area under the curve (AUC) values were calculated with NCSS software (Hintze, 2007). The data are presented as means±standard error (n=3). The results were statistically analyzed by using General Linear Model (GLM) (SAS, 1990). Significant ($P<0.05$) differences among means for the dietary treatments were ranked by Tukey's multiple range test. In addition, effect of fluoride source (krill shell or NaF) and calcium supplementation (without or with calcium) were tested on pharmacokinetic variables for fluoride in rainbow trout by using a 2×2 factorial model.

3. Results

No fish died during the experimental period. The feed intake, measured by stomach content 1 h postprandial, ranged from 0.70 to 0.81 g DM $fish^{-1}$ among the five dietary treatments and showed no significant difference (Table 3). The reduction in dry matter content in the stomach at a given h postprandial did not differ among the dietary treatments (Fig. 1). The gastric evacuation was close to finished 36 h postprandial. The water fluoride level in the tanks ranged from 0.10 to 0.15 mg l^{-1} (data not shown) and was not significantly affected by dietary treatment or sampling time.

Fish fed the control diet showed no increase in plasma fluoride during the sampling period (Fig. 2). There were significant interaction effects between fluoride source and calcium for C_{max}, T_{max}, and AUC. In the absence of supplemental calcium there were large differences in response to fluoride from either krill shell or NaF whereas these differences were reduced in the presence of supplemented calcium. Fish fed NaF showed a clear peak and a C_{max} in plasma fluoride of 1.17 mg l^{-1} and a T_{max} at 3 h, followed by a rapid decline that reduced the

plasma fluoride level by 50% after approximately 10 h (Fig. 2). After 36 h, the NaF fed fish had a fluoride plasma level close to the control group. The fish fed NaF had a significantly higher C_{max} and AUC compared to the other dietary groups. The fish fed krill shell showed a C_{max} of 0.32 mg l^{-1} and AUC of 8.59 mg h l^{-1}, both significantly lower than the NaF fed fish, but higher than the control and the fish fed diets supplemented with calcium. In contrast to fish fed NaF, the fish fed krill shell had a slower uptake of fluoride into the blood plasma and at 36 h the plasma fluoride level was still significantly higher than the control group with a level of 0.2 mg l^{-1}. Adding calcium to the NaF diet resulted in a reduction in AUC from 15.40 to 3.37 mg h l^{-1}, whereas, the reduction for the krill fed fish was from 8.59 to 4.29 mg h l^{-1}, respectively.

4. Discussion

Measuring the dry weight of the stomach contents postprandial is a commonly used method for estimating the rate of gastric evacuation (Bromley, 1994). The rates of gastric evacuation in the present experiment did not differ among dietary treatments indicating same passage speed trough the intestinal tract. The present evacuation was slower compared to those observed in some experiments with rainbow trout (Windell et al., 1969; Windell et al., 1972), even with a higher water temperature in the present experiment.

NaF is water soluble and may leak into the tanks and be taken up from the water by the fish. In the present experiment, however, no significant differences in water fluoride level were detected among tanks, probably due to the short time (approximately 2 min) it was possible for the uneaten pellet to stay within the tank before entering the outlet.

The T_{max}, the time it took for the plasma fluoride concentration to peaked (C_{max}) was the time when the amount of absorbed fluoride was equal to fluoride being distributed to the tissues or being eliminated (Whitford, 1996). The AUC could be described as the total amount of fluoride entering the blood circulation system from the GI–tact, and is a good estimate of bioavailability (Whitford et al., 2008). In the present experiment, fluoride in blood plasma was used as an indicator of fluoride absorption. The fish given the NaF diet in the present experiment showed a rapid uptake of fluoride, and close to a doubling of the area under the curve compared to fish fed krill shell. This is in line with Whitford (1996) who describing NaF as a highly available form of fluoride, but in contrast to Soevik and Braekkan (1981) who reported similar bioavailability of fluoride from northern krill and NaF in growing chicks. Further, in agreement with the present results, Hattab (1988) obtained a lower bioavailability of fluoride from a single meal of seafood compared to a single dose of NaF in an aqueous solution. In the present experiment, the higher calcium concentration in the krill shell compared to the fish meal resulted in approximately 6 g kg^{-1} diet more calcium in the krill shell diet. This may have contributed to the lower fluoride uptake in krill shell fed fish compared to the NaF fed fish. Dietary calcium levels were not balanced in this experiment, since the exact chemical form of the calcium in the krill shell and its association with fluoride was unknown.

Soluble fluoride is absorbed in the stomach as hydrogen fluoride and the level is dependent on the gastric pH and the amount of fluoride ingested (Whitford and Pashley, 1984). In the present experiment, fish fed the NaF diet reached a clear peak in plasma fluoride at 3 h, whereas the krill fed fish did not obtain a clear peak, but showed a higher fluoride level during the sampling period compared to the NaF fed fish. This may indicate a more rapid absorption of fluoride in the stomach of the NaF fed fish, whereas the absorption of fluoride occurred for a longer period and further down in the GI tract in the krill fed fish. This is in

line with Zhang et al. (1993), who suggested that fluoride is a hardener in the krill exoskeleton and occurs as fluorapatite, a molecule with low solubility and low digestibility. On the contrary, Sands et al. (1998) and Tenuta (1993) suggest that fluoride in krill is in a water soluble form. Furthermore, Yin et al. (2010) showed decreased fluoride absorption and decreased bone mineral density in rat fed fluoride (NaF) in combination with pure chitin compared to rats fed only fluoride, suggesting that chitin prevents fluoride absorption. This is in line with the present results, showing reduced fluoride uptake in fish fed krill shell, containing approximately 23.5 g chitin kg^{-1} diet. Moreover, Yin et al. (2010) also found that about 2/3 of of the total skeleton fluoride in penguins was organically bound, and not in form of calcium bound fluoride, but probably in form of fluorinated derivates from chitin. These penguins mainly eat Antarctic krill. This, together with the fluoride reducing effect by chitin in rat, indicates that fluoride from krill is linked to chitin in the exoskeleton.

Fluoride has a strong affinity to calcium and can easily form the insoluble CaF_2 within the intestinal lumen of mammals (Harrison et al., 1984; Cerklewski and Ridlington, 1987). Supplementation of dietary calcium has been shown to reduce the bioavailability of fluoride in rats (Havivi, 1972; Harrison et al., 1984; Cerklewski and Ridlington, 1987; Ekambaram and Paul, 2001) and rabbits (Reddy and Rao, 1973). In the present experiment, the addition of dietary calcium greatly reduced fluoride uptake in fish fed the NaF+Ca diet, showing fluoride values close to the control group. During feed production, the $CaCl_2$ was added to the water and heated prior to mixing which led to a totally dissociation of the $CaCl_2$. This dissociation facilitated the formation of CaF_2 during feed production. In contrast to the NaF+Ca group, fish fed krill shell+Ca approximately halved the fluoride uptake compared to fish fed krill shell. This is in line with Tenuta and Alvarenga (1999) who reported reduced fluoride absorption from Antarctic krill paste from 33.8 to 45.8% by adding 1.0 and 2.0% calcium ($CaCO_3$) above the minimum recommended dietary level for rats (0.5%). Further, Cerklewski

and Ridlington (1987) showed that the solubility of calcium was not important in influencing fluoride bioavailability, however, the difference in ability of calcium to prevent fluoride absorption from either NaF or krill shell may be related to the molecular structure and how available fluoride is for the calcium ion. This implies that a fluoride molecule with high solubility and high digestibility such as NaF probably also has a high ability to bind calcium.

Zinc is thought to create insoluble calcium phosphate complexes in the intestine that reduce the absorption of zinc. Rainbow trout fed diets containing high-ash white fish meal developed zinc deficiency symptoms and cataracts (Ketola, 1979). By adding dietary calcium they also showed an increased incidence and severity of the cataract. In the present experiment, 15 g kg^{-1} calcium was added to the diets, however, the addition of 20 g calcium and 15 g phosphorous per kg feed to rainbow trout did not reduce the zinc retention or whole body zinc concentration (Hardy and Shearer, 1985). This suggests that 15 g kg^{-1} additional added calcium may not contribute to a zinc deficiency.

Several studies, conducted in sea water, using krill meal with high levels of fluoride in diets for Atlantic salmon (*Salmon salar*), rainbow trout, Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) showed no fluoride accumulation in bones or muscles (Julshamn et al., 2004; Moren et al., 2007). On the basis of mainly these studies, the EU increased the legal fluoride level in animal feed, including fish, from 150 to 350 mg kg^{-1} (Commision dir. 2008/76/EC). Fish reared in fresh or brackish water fed krill meal obtained, however, increased fluoride accumulation in bones with increasing dietary fluoride (Grave, 1981; Tiews et al., 1982; Yoshitomi et al., 2006). Moren et al. (2007) suggested that the difference in fluoride accumulation in freshwater relative to saltwater is caused by the water salinity and Yoshitomi et al. (2006) suggested that fish reared in fresh water needs to accumulate minerals. Fish reared in sea water drink continuously, thus, we believe that dietary fluoride binds to water-borne calcium in the stomach and the GI tract and that calcium inhibits

fluoride uptake in the fish. There are limited reports of negative health effects of fish fed high fluoride diets reared in fresh water, but Weirich et al. (2005) reported decreased survival of channel catfish (*Ictalurus punctatus*) fry fed diets supplemented with high fluoride krill meal. The fish had increased fluoride levels in whole body and the authors suggesting that the reduced survival was due to fluorosis. In addition, extreme fluoride levels up to 4,500 mg kg^{-1} diet fed to rainbow trout resulted in spasmodic behaviour (Landy, 1988). Nevertheless, the difference in the ability to accumulate fluoride between fish reared in fresh or salt water should be taken into consideration when diets for fresh water fish are formulated.

In conclusion, fluoride from krill shell had lower bioavailability than sodium fluoride in diets fed to rainbow trout reared in fresh water. Calcium supplementation as $CaCl_2$ was an efficient way to reduce the fluoride uptake in rainbow trout reared in fresh water.

5. Acknowledgements

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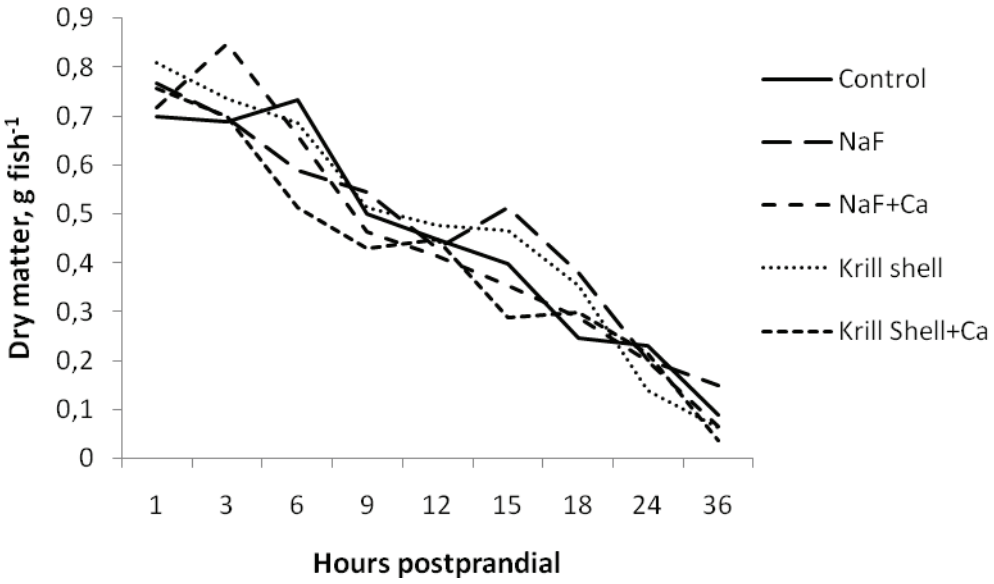

Figure 1. Postprandial dry matter content in stomach, g fish^{-1} in rainbow trout fed diets supplemented with krill shell or sodium fluoride (NaF), with or without calcium (Ca).

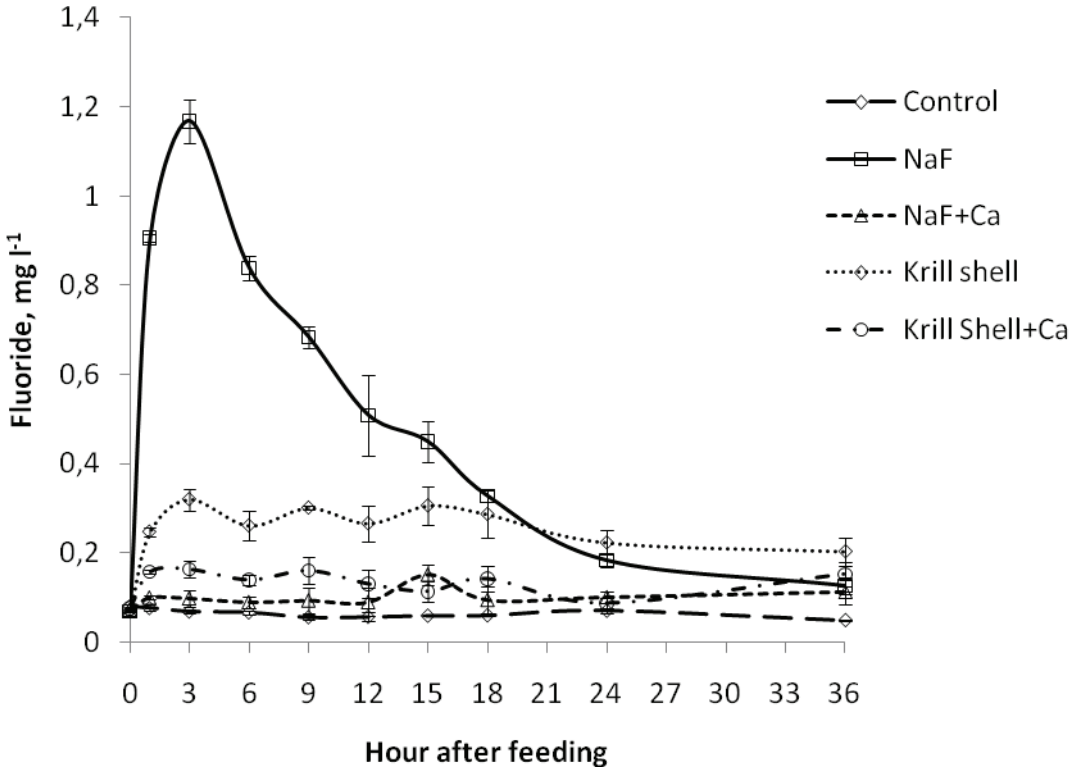

Figure 2. Plasma fluoride, mg l^{-1}, in rainbow trout at 0, 1, 3, 6, 9, 12, 15, 18, 24, 36 h postprandial after ingestion of diets supplemented with krill shell or sodium fluoride (NaF), with or without calcium (Ca), or a low fluoride control. The data are presented as means±standard error (n=3).

Table 1. Composition of the experimental diets

	Control	Krill shell		NaF	
Calcium supplementation[a]		–	+	–	+
Formulation, g kg^{-1}					
Fish powder[b]	519.9	375.9	365.9	520.4	520.4
Krill shell[c]	0	240	240	0	0
Gelatinized potato starch[d]	200	124	89	198	153
Gelatin[e]	120	120	120	120	120
NaF[f]	0	0	0	1.5	1.5
$CaCl_2$[g]	0	0	45	0	45
Fish oil[h]	160	140	140	160	160
Yttrium oxide[i]	0.1	0.1	0.1	0.1	0.1

[a]Diets without calcium are denoted "–" and diets with calcium "+". [b]SKP 0268, Rieber og Søn, Bergen, Norway. [c]Krill shell (crude protein 450 g kg^{-1}; lipid 130 g kg^{-1}; ash 150 g kg^{-1}; chitin 97 g kg^{-1}; fluoride 2,880 mg kg^{-1}. [d]Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden. [e]Rousselot® 250 PS, Rousselot SAS, Courbevoie, France. [f]Sigma-Aldrich, S7920-500g, St. Louis, USA. [g]Food grade Calcium Chloride 77, Tetra Chemicals, Kokkola, Finland. [h]NorSalmOil, Norsildmel, Egersund, Norway. [i]Y_2O_3. Metal Rare Earth Limited, Shenzhen, China.

Table 2. Chemical composition of the experimental diets

	Control	Krill shell		NaF[b]	
Calcium supplementation[a]		–	+	–	+
Dry matter (DM), g kg^{-1}	621	617	628	624	626
In DM, kg^{-1}					
Crude protein, g	602	596	593	592	588
Lipid, g	154	183	177	167	175
Starch, g	206	128	9.9	201	151
Ash, g	47.0	73.4	114	49.2	87.9
Chitin, g		23.5	23.5		
Minerals, kg^{-1}					
Fluoride, mg	31	637	638	667	688
Calcium, g	6.4	12.6	27.2	6.4	21.2
Magnesium, g	1.1	2.6	2.5	1.1	1.1
Phosphorus, g	7.3	10.4	10.0	7.2	7.4
Iron, mg	16	71	53	13	15

[a]Diets without calcium are denoted "–" and diets with calcium "+".

[b]Sodium fluoride.

Table 3. Stomach content 1 h postprandial, maximum concentration (C_{max}), time of maximum concentration (T_{max}) and area under the curve ($AUC_{(0–36)}$) for plasma fluoride (F^-) in rainbow trout fed diets with krill shell or sodium fluoride (NaF), supplemented with or without calcium (Ca)

	Control[1]	Krill shell		NaF		P-value[2]		
Calcium supplementation[3]		–	+	–	+	F^--source	Calcium	F^- × Ca
Stomach content, DM g $fish^{-1}$	0.70±0.01	0.81±0.06	0.76±0.14	0.77±0.17	0.72±0.16	0.78	0.72	0.99
C_{max}, mg l^{-1}	0.082± 0.00 ^{c}	0.32±0.02 ^{b}	0.16±0.02 ^{c}	1.17±0.05 ^{a}	0.13±0.02 ^{c}	<.0001	<.0001	<.0001
T_{max}, h	0.33±0.00	10.0±4.36	4.43±2.4	3.0±0.00	20.0±8.2	0.39	0.27	0.045
$AUC_{(0–36)}$, mg h l^{-1}	2.18±0.12 ^{c}	8.59±0.82 ^{b}	4.29±0.52 ^{c}	15.4±0.11 ^{a}	3.37±0.42 ^{c}	0.0005	<.0001	<.0001

[1]The control diet was not included in the statistics for the 2×2 factorial design. A one way ANOVA (n=3) including the control diet was performed to determined significant differences between treatmen means±st.error (P<0.05, Tukey's) indicated by different superscripts $^{a, b, c}$.
[2]P-values are given for the main effects; fluoride source and calcium, and the interaction between fluoride source and calcium.
[3]Diets without calcium are denoted "–" and diets with calcium "+".