Biochemical composition of live feed used in cod larvae production – As good as it gets, or room for improvement?

by

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PREFACE

Ay, think of it – wish it done – will it to boot, - but do it…..
(Henrik Ibsen: Peer Gynt, 3rd act, 1st scene, translation by William and Charles Archer)

Well, now I have done it…. that is written a master thesis. Three years of hard work, disappointments and joy, exhaustion and excitement. Well, it was worth it!

The analytical work for this master thesis was performed at the Norwegian College of Fishery Science in the period 2008-2009 and I wish to thank both staff and students at the institute for supporting me during this period. Special thanks to Bjørn Tore, Rune and Ida, for valuable tips and hints during the writing process. Thanks to Marie Cooper for proof reading and necessary lessons in English grammar.

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Thanks to Thor Arne Hangstad and Kristin Skar at Troms Marin Yngel AS for providing the rotifers and for good answers to all of my questions regarding the production.
Thanks to Kurt Tande at the Department of Arctic and Marine Biosciences for providing krill and to Calanus AS for providing calanus.
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And last, but not least. I wish to express my gratitude towards my supervisor Edel O. Elvevoll, first for convincing me that a master degree was within reach and secondly for all help and encouragement, both personally and professionally, during the process. Her never-ending enthusiasm is impressive!

Hanne K. Mæhre
Tromsø, January 2010

ABSTRACT

Low survival rate, slow growth and developmental abnormalities during the larval stage are challenges related to aquaculture of marine species. Several studies have shown that feeding the larvae their natural feed source, zooplankton, improves these factors significantly compared to feeding them intensive feeds like rotifers. The nutritional quality of the feed is likely to play a crucial role. Components suggested to have an impact are, for instance, the typical marine fatty acids eicosapentaenoic (EPA) and docosehexaenoic acid (DHA). A high content of free amino acids (FAA) and peptides are also believed to be of importance due to limited differentiation of the digestive system in the larvae. In addition, the content of some minerals is believed to be crucial.

The main objective of this study was to examine whether there were differences in biochemical composition between zooplankton and two intensive feeds (A and B) that could, at least in part, explain the increased growth when feeding on zooplankton at Lofilab AS (Steine, Norway). Natural rotifers are deficient or low in several nutritional components required for fish and thus they are normally enriched prior to feeding. A secondary aim was to compare the biochemical composition of the rotifers prior to and after enrichment. Three commercial enrichment media were also analysed and two underutilised marine sources of nutrients (krill and calanus) were included.

The results showed large differences in biochemical composition between zooplankton and the intensive feeds, in particular in components of typical marine origin. The sum of the most typical marine fatty acids, EPA+ DHA, in zooplankton was 38.5% of the total lipid fraction, corresponding to 3.1% of the diet. In the intensive feeds the EPA+DHA were 21.7% (A) and 20.5% (B) respectively, corresponding to 2.6% and 2.4% of the diets. The relative amount of FAA in zooplankton was 15.7%, while the equivalent amounts in the intensive feeds were 10.4% and 12.1% in feed A and B respectively. Zooplankton was rich in taurine, 8.4 mg/g dry weight (DW), while the two intensive feeds were almost devoid of it, reflecting the composition of the enrichment media. Except for phosphorus, zooplankton was richer in all of the minerals analysed. The main effect of the enrichment of rotifers was an increase of the amount of EPA and DHA in two out of three enrichment protocols. The existing enrichment protocols were ineffective if the target was to increase the content of the other nutritional components. The biochemical compositions of krill and calanus were more similar to zooplankton, as the content of taurine and EPA+DHA was higher than in the commercial enrichment media, thus supporting the idea of utilising these marine resources as new and improved enrichment media.

Keywords: cod larvae, zooplankton, intensive feeds, HUFA, taurine

SAMMENDRAG / NORWEGIAN SUMMARY

Høy dødelighet, langsom vekst og utviklingsmessige avvik under larvestadiet er utfordringer relatert til oppdrett av marine arter. Flere studier har vist at fôring av larvene med deres naturlige fôrkilde, dyreplankton, forbedrer disse faktorene betydelig sammenlignet med fôring med intensivfôr, som rotatorier. Den ernæringsmessige kvaliteten av fôret spiller sannsynligvis en avgjørende rolle. Komponenter antatt å ha innflytelse er for eksempel de typiske marine fettsyrene eicosapentaensyre (EPA) og docosaheksaensyre (DHA). Et høyt innhold av frie aminosyrer (FAA) og peptider er også tiltrodd viktighet på grunn av et lite differensiert fordøyelsessystem i larvene. I tillegg er innholdet av enkelte mineraler sentralt.

Hovedmålet med dette studiet var å undersøke hvorvidt det fantes forskjeller i biokjemisk sammensetning mellom dyreplankton og to intensivfôr (A og B) som kunne, i alle fall delvis, bidra til å forklare den økte veksten sett ved fôring med dyreplankton ved Lofilab AS (Steine, Norge). Siden rotatorier inneholder lite eller ingenting av flere ernæringsmessig viktige komponenter for fisk, blir de som regel anriket før bruk. Et annet delmål i dette studiet var derfor å sammenligne biokjemisk sammensetning av rotatoriene før og etter anrikning. Tre kommersielle anrikningsmedier ble også analysert og to hittil lite utnyttede marine kilder til næringsstoffer (krill og calanus) ble inkludert.

Resultatene viste at det var flere forskjeller i biokjemisk sammensetning mellom dyreplankton og intensivfôrene, spesielt i typisk marine komponenter. Summen av de mest typiske marine fettsyrene, EPA+DHA, utgjorde 38,5% av lipidene i dyreplankton, tilsvarende 3,1% av dietten. I intensivfôrene var denne summen henholdsvis 21,7% (A) og 20,5% (B), noe som tilsvarer 2,6% og 2,4% av dietten. Den relative mengden av FAA i dyreplankton var 15,7%, mens tilsvarende mengder i intensivfôrene var henholdsvis 10,4% (A) og 12,1% (B). Taurininnholdet var høyt i dyreplankton, 8,4 mg/g tørrstoff, mens mengden av taurin var neglisjerbar i intensivfôrene. Dyreplankton inneholdt også mer av alle de undersøkte mineralene, med unntak av fosfor. Anrikning av rotatoriene førte til et økt nivå av EPA og DHA i to av tre anrikningsprotokoller, men dersom målet var å øke innholdet av andre næringsstoffer, var de eksisterende protokollene ineffektive. Den biokjemiske sammensetningen av krill og calanus var mer lik sammensetningen av dyreplankton, ettersom innholdet av taurin og EPA+DHA var høyere enn de kommersielle anrikningsmediene, noe som støtter ideen om en mulig utnyttelse av disse råstoffene i nye og forbedrede anrikningsmedier.

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INTRODUCTION

Fish and other seafood are traditionally regarded as healthy food. The healthy aspects of seafood have focused on their favourable fatty acid composition, with low levels of saturated fat and high levels of the long-chain polyunsaturated omega-3 fatty acids (PUFAs). Several large studies have documented that there is a strong correlation between fish intake and reduced risk of cardiovascular disease. The reason for this is believed to be the high level of PUFAs (Bucher *et al.*, 2002, He *et al.*, 2004, Wang *et al.*, 2006). However, the advantages of eating fish are not limited to fat content. Fish is also rich in high quality proteins i.e. it contains all of the essential amino acids with high bioavailability (Friedman, 1996). In addition it is a source of important vitamins and minerals, such as vitamin D and B_{12}, selenium and iodine (Bourre and Paquotte, 2008a, Bourre and Paquotte, 2008b).

Aquaculture has become a more important part of the world's total production of fish and seafood, with a market share rising from 27% in 2000 to 36% in 2006 (FAO, 2008). The amount of fish produced by traditional fisheries has remained fairly stable during the same period. A continued growth in the aquaculture sector is expected and may be necessary to meet the global demand for seafood. To ensure the benefit of a high intake of fish, it is important to optimise knowledge of factors influencing the survival, growth and development of fish in aquaculture. The biochemical composition of fish muscle is dependent on diet and hence, it is important to make sure that the nutritional quality of fish flesh in farmed fish is maintained.

Aquaculture is an important industry in Norway. Since the middle of the 1980's production has steadily grown from 1000 tonnes per year to 800000 tonnes per year. Atlantic salmon (*Salmo salar*) is the most abundant species (approximately 85% of the total production), followed by rainbow trout (*Oncorhynchus mykiss*). Since the 1990's, several attempts have been made to farm marine species, such as Atlantic cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*) and spotted wolffish (*Anarhichas minor*). The production of farmed cod in Norway increased from 200-500 tonnes per year from 1992-2001 to approximately 11000 tonnes in 2006 (Statistics Norway, 2008). However, farming of cod and other marine species has several challenges when compared to the farming of salmon. Mortality during the larval stage is very high; the survival rate can be as low as 1% or even less. In wild populations, mortality is closely related to predation, changes in the physical

environment and access to and quality of food (Mountain *et al.*, 2008), but even in aquaculture where these factors can be controlled, a low survival rate makes it difficult to make farming of marine species profitable. Other challenges related to aquaculture of marine species other than salmon are linked to a higher feed factor i.e. the use of more feed per kg fish produced. Market competition with wild caught fish will also be a bigger challenge for the marine species than for salmon. The sum of all these factors makes it difficult to make production of farmed marine fish profitable.

Most marine fish hatch from small, pelagic eggs and their digestive system is usually less developed than freshwater larvae at the same stage (Rønnestad *et al.*, 2007). Generally the development of fish can be divided into four different stages; embryonic, larval, juvenile and adult, as illustrated in figure 1. In the embryonic phase (from fertilisation to the opening of the oesophagus), the energy demands of the individual have to be met by the vitelline reserves present in the egg at the moment of fertilisation. The vitelline reserves consist mainly of yolk proteins, but in some species there is also a lipid globule present. The larval phase begins when the oesophagus opens. At this stage, the digestive system in most marine species is not highly differentiated. The intestine is just a short, straight tube with no loops or evaginations and there is no defined stomach. The intestinal mucosa is thin and lacks the folds that are characteristic of juveniles and adults. The liver and pancreas are single lobes. Hence, the larvae are dependent on easily digestible live feed during the larval stage (Gatesoupe *et al.*, 1999).

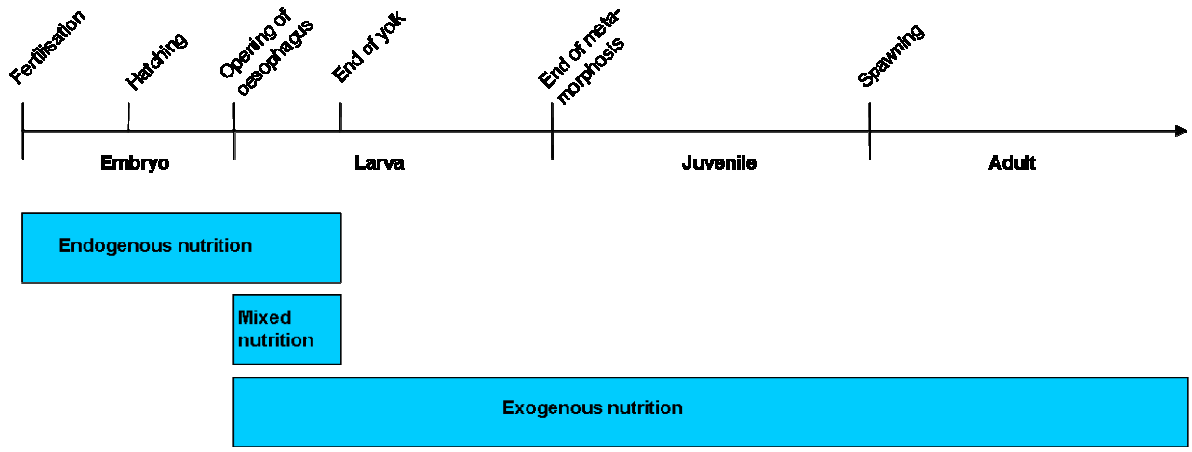

Figure 1. Development of fish from fertilisation to adult and types of nutrition during the different stages (Gatesoupe *et al.*, 1999)

Although the intestine has a simple structure, it is possible to detect activity of digestive enzymes at the earliest stages of larval development. Lipase and phospholipase A2 are the main lipolytic enzymes studied in fish to date (Infante and Cahu, 2007). Both enzymes are secreted by the hepatopancreas in response to the presence of their substrates. Lipase is activated by bile-salts and functions as a catalyst of the hydrolysis of carboxy-ester bonds in triacylglycerols (TAGs), cholesterol esters and fat-soluble vitamin esters. The activity of bile-salt activated lipase has been detected from the opening of the mouth (Murray *et al.*, 2003, Srivastava *et al.*, 2002). Phospholipase A2 catalyses the hydrolysis of the sn-2 fatty acyl chain of phospholipids (PLs) to yield fatty acids and lysophospholipids. This enzyme has been detected a few days after opening of the mouth (Infante and Cahu, 2001). The proteolytic enzymes are located in the stomach (pepsin), in the pancreas (trypsin, chymotrypsin and elastase) and in the intestine (membranous and cytosolic enzymes). As marine larvae lack a functional stomach the presence of pepsin is not detected at this stage of development. Activity of the pancreatic enzymes is detected from the moment of first feeding (Cara *et al.*, 2003). Secretion of the pancreatic enzymes increases during the first three weeks of larval life (Infante & Cahu, 2001). This process is controlled by the level of cholecystokinin (CCK) (Kurokawa *et al.*, 2000), which in turn is dependent on the level of dietary protein and degree of hydrolysation in the diet (Cahu *et al.*, 2004). The dominant intestinal enzymes during the first weeks are the cytosolic enzymes, mainly di- and tripeptidases, located in the immature enterocytes. Maturation of the enterocytes leads to a decrease in the activity of these enzymes and an increase in several brush-border enzymes such as alkaline phosphatase and aminopetidase N.

There are three main methods of larval rearing, intensive, semi-intensive and extensive. All methods are based on live feeds in the form of simple, unicellular organisms. Feeding starts approximately 3 days post hatch and lasts until 20-25 days post hatch, after which the larvae are weaned onto formulated feed (Nora A. Rist, Lofilab AS, personal communication). In intensive larval rearing the commonest feed sources are rotifers (*Brachionus* sp.) and brine shrimp (*Artemia* sp.). For these organisms, standardised protocols for cost-effective mass production exist (Conceicao *et al.*, 2009). Semi-intensive and extensive larval rearing, however, are based on copepods and other natural zooplankton organisms, which are the diet for most species in nature. In extensive rearing, the larvae are reared in closed seawater ponds, which usually have been fertilised to improve production of phytoplankton. This, in turn can give rise to an increase in the amount of zooplankton. This method is the one closest

to the natural conditions of marine fish, but is not commonly used because it is difficult to make profitable. In semi-extensive rearing the production of plankton is still preformed in closed seawater ponds. The plankton are then filtered and concentrated before they are transferred to the rearing tanks where the larvae are kept. The semi-intensive and extensive rearing methods are more dependent on space, temperature and light conditions than the intensive rearing method and have so far been more challenging and expensive. However, several studies have shown that there is higher growth and survival rate (Rajkumar and Vasagam, 2006), lower frequency of physical deviations such as skeletal deformities (Imsland *et al.*, 2006), pigmentation and eye migration errors (Hamre *et al.*, 2002) in marine larvae that have been fed zooplankton than in those fed intensive feed like rotifers. An example of growth curves for larvae fed zooplankton vs. larvae fed regular intensive feed is shown in figure 2. The experiments, from which these data were collected, were performed at the rearing site of Lofilab AS (Steine, Norway) and include zooplankton from this location and a standard intensive feed.

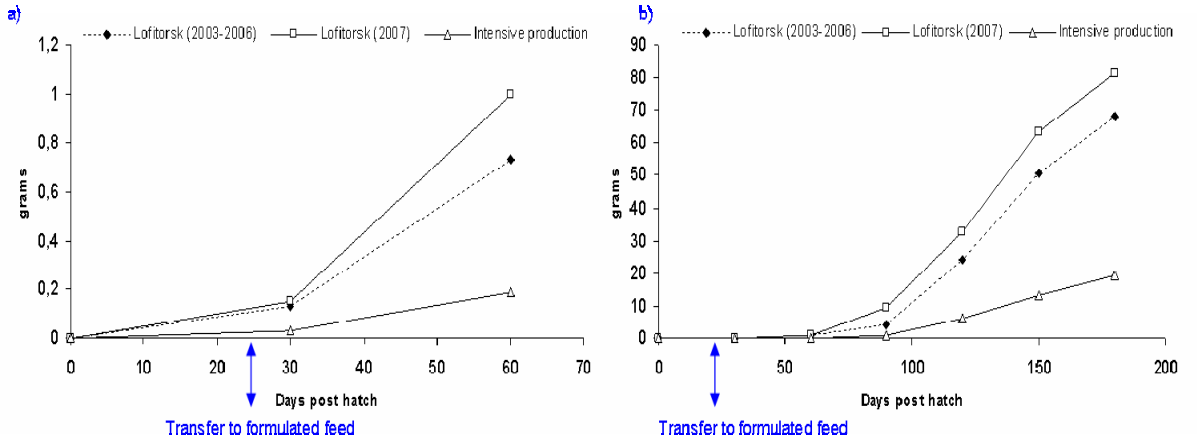

Figure 2: Growth curves of cod larvae the first 60 days (a) and the first 180 days (b) post hatch. Larvae are fed zooplankton or standard intensive feed (rotifers) from approximately day 3 post hatch to day 25 post hatch, after which they are weaned onto formulated feed (Lofitorsk AS, 2008)

There are multiple factors that can explain these facts, but nutritional quality of the different live prey is probably the most significant single factor (Cahu *et al.*, 2003a). Both rotifers and artemia are deficient in important nutritional components, such as the highly unsaturated fatty acids (HUFAs) eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3). Other limiting nutrients are some amino acids, vitamins and minerals (Conceicao *et al.*, 2009). The solution to this problem has therefore been to enrich the rotifers and artemia before feeding them to the fish. Traditionally the main focus has been to optimise the fatty acid profile of the rotifers. Numerous studies have been performed and published on this

research field, with focus on growth, survival rate and fatty acid profile in the fish (Garcia *et al.*, 2008a, Garcia *et al.*, 2008b, Garcia *et al.*, 2008c, Park *et al.*, 2006), but because of the high specific growth rate in the larval period the composition of amino acids in the enriched rotifers and artemia may be just as important (Aragao *et al.*, 2004). Another way of enhancing the nutritional value of rotifers and artemia is co-feeding with some species of microalgae. They can act as feed for other live feed, but also be added directly into the larvae tanks as, so-called "green water". The microalgae have several beneficial properties as they seem to be able to provide nutrients directly to the larvae (Moffatt, 1981) and to protect nutritional quality in the live prey (Makridis and Olsen, 1999). Addition of microalgae also improves the water quality as the visual contrast in the water is enhanced (Naas *et al.*, 1992, Naas *et al.*, 1996). The microbial diversity both in the water and in the gut of larvae is also positively affected (Skjermo and Vadstein, 1999, Nicolas *et al.*, 1989, Reitan *et al.*, 1997, Olsen *et al.*, 2000).

The exact nutritional requirements of cod and cod larvae have yet to be defined, but the National Research Council (NRC, 1993) have given an overview of the nutritional requirements of adult fish and juveniles. All fish larvae need the fatty acids linoleic acid (LA, C18:2, n-6) and α-linolenic acid (ALA, C18:3, n-3). These fatty acids are essential and can not be synthesised *de novo*. As most animals, marine larvae have a limited ability for chain elongation and desaturation of the C18 fatty acids and hence, HUFAs must be supplied via the feed. The most abundant HUFAs in fish are EPA and DHA. The availability of HUFAs affects the viscosity and permeability of the membranes and hence the activity of the membrane-bound enzymes and transport proteins. The specific need for DHA is more defined than the need for EPA. Tissues with an active metabolism, such as the nervous system including the brain and the eye, are very dependent on high levels of DHA (Mourente *et al.*, 1991, Furuita *et al.*, 1998). Elevated levels of bone abnormalities associated with low levels of DHA have also been observed (Roo *et al.*, 2009). Prostaglandins, thromboxans and leucotriens are derivatives of fatty acids containing 20 C-atoms (C20:4, n-6, 20:3, n-6 and 20:5, n-3). The formation of these compounds is limited, but important for fish as well as for humans even if their significance is not known in detail. A high content of HUFAs in the feed is not enough *per se*. The digestibility and utilisation of lipids vary with the presence of digestive enzymes. Cahu *et al.* (2003b) showed that the response of lipase was not linear with respect to the presence of TAGs, but levelled off at approximately 200g TAG/kg diet. The response of phospholipase A2, however, was highly dependent on the presence of its

substrate. Studies comparing different ratios of TAGs vs. PLs in the feed show that there are large differences in growth, survival and gut development between the groups, feed rich in PLs being more favourable (Cahu *et al.*, 2003b, Wold *et al.*, 2009, Wold *et al.*, 2007). Histological changes in the gut have also been observed in larvae fed diets deficient in PLs. They tend to form fat droplets in the enterocytes of the intestine (Dragnes, 2001, Fontagné *et al.*, 1998), the height of the mucosal epithelium is increased and the total liver volume and the size of the hepatocytes are decreased (Fontagné *et al.*, 1998).

In the larval stage the specific growth rate is very high. Thus, the larvae have a much higher need for high quality proteins than juveniles and adult fish. Amino acids (aa) are building blocks for proteins. Traditionally the amino acids are classified as either essential or non-essential. Essential amino acids are those which can not be synthesised to an adequate level by the animals and comprise the same nine aa's for fish and humans; histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp) and valine (Val). In addition, arginine (Arg) is regarded as essential for most fish due to the lack of a functional urea cycle (Mambrini and Guillaume, 1999). The term conditionally essential amino acids has also become common during recent years. These are amino acids that in certain periods have to be provided by the feed because the utilisation rate is higher than the rate of synthesis. In fish, these are recognised as cysteine (Cys), glutamine (Gln), hydroxyproline (Hyp), proline (Pro) and taurine (Tau) (Li *et al.*, 2009). Besides protein synthesis and growth the amino acids have several important functions in the body. These include cell signalling, osmoregulation, endocrine/metabolic response, antioxidative defense, metamorphosis and ammonia removal (Bouckenooghe *et al.*, 2006).

Prior to development of a functional stomach the larvae seem to utilise free amino acids better than peptides and proteins (Kvåle *et al.*, 2007, Rønnestad *et al.*, 1999). Experiments using pre-hydrolysed protein revealed that the limiting factor is low proteolytic activity and not absorption capacity (Tonheim *et al.*, 2005).

Minerals are important for many of the natural processes in fish, including formation of the skeletal structure, electron transfer, regulation of acid-base balance and osmoregulation. In addition, they are important components of hormones and enzymes and they serve as catalysts in enzymatic reactions. In order to maintain osmotic balance, marine fish have an uptake of

several minerals via the gills, skin and mouth. It is therefore difficult to determine specific requirements for the different minerals (Kaushik, 1999).

As the current most common enrichment media for rotifers may not be optimal for ensuring the nutritional requirements for marine fish larvae, a possible improvement of these may be to make use of other marine resources, such as krill and calanus. During recent years interest in utilisation of krill has been growing, both as raw material for aquaculture feed and as a nutritional supplement for humans. Krill is a common name for several species of small shrimp-like crustaceans, such as Antarctic krill (*Euphausia superba*) and north-Pacific krill (*Euphausia pacifica*). Further north in the north-Atlantic *Meganyctophanes Norvegica* is the dominant krill species. Predicting the availability of krill is difficult, but the FAO suggest that the biomass of krill in the Antarctic is between 125-720 million tonnes. The amount in the Barents Sea is estimated to be around 50 million tonnes. Comparing these numbers with the world's total available fish resources, around 100 million tonnes, one can see that the potential is great (Lekang and Gutierrez, 2007).

Calanus (*Calanus finmarchicus*) is another under-utilised source of marine nutrients. Calanus is a zooplankton, somewhat smaller than krill. Their size is approximately 3 mm and their main food source is phytoplankton. A challenge, however, when it comes to utilisation of the lipids in calanus, is that they mainly store lipids as wax esters. Wax esters are neutral lipids which differ from TAGs in that the fatty acids are linked to a long-chain fatty alcohol instead of to glycerol. The wax ester is therefore more hydrophobic than TAG and hence, uptake in an aqueous environment such as the intestinal lumen may be difficult (Oxley *et al.*, 2009). However, utilisation of wax esters as an energy source during the early larval stage has been described in several species, such as yellowtail kingfish (*Seriola lalandi*) (Hilton *et al.*, 2008), common dentex (*Dentex dentex* Linnaeus 1758) (Gimenez *et al.*, 2008) and white seabream (*Diplodus sargus*) (Cejas *et al.*, 2004).

Aims of this study

Low survival rate, slow growth and developmental abnormalities during the larval stage are challenges related to aquaculture of marine species. Several studies have shown that feeding the larvae their natural feed source, zooplankton, improves these factors significantly

compared to feeding them intensive feeds like rotifers. The nutritional quality of the feed is likely to play a crucial role.

The main objective of this study was to examine possible differences in biochemical composition between zooplankton and intensive feeds. Natural rotifers are deficient or low in several nutritional components required for cod larvae and thus they are normally enriched prior to feeding. A secondary aim was to compare the biochemical composition of the rotifers prior to and after enrichment. Three commercial enrichment media were also analysed and two underutilised marine sources of nutrients (krill and calanus) were included. To obtain knowledge on these matters, the following specific tasks were framed:

1. To compare the biochemical composition of zooplankton collected in a semi-intensive larval rearing farm with two mixtures of rotifers collected in an intensive larval rearing farm.
2. To examine the biochemical composition of rotifers and the effect of enrichment.
3. To examine the biochemical composition of different enrichment media used in the production of rotifers for marine larvae.
4. To examine whether the under-utilised biomass (krill and calanus) from the North Atlantic Ocean could be used as enrichment media for marine larvae.

MATERIALS AND METHODS

Raw materials

Zooplankton / Copepods

The zooplankton/copepods used in this experiment were collected twice at Lofilab AS (Steine, Norway); June 30th and September 8th 2008. As the amount of material collected on June 30th was not sufficient for all analyses, it was necessary to perform another collection on September 8th. A characterisation of the copepods performed in the spring of 2008 revealed that the most numerous of the adult copepods were *Eurythemora affinis*, *Calanus finmarchicus* and *Microsetella norvegica*. There were also a large number of nauplii and copepodites present, but these could not be characterised because of their very general appearance.

The production of zooplankton/copepods followed a standard procedure at Lofilab AS. This included fertilisation of closed seawater ponds, monitoring of temperature, salinity and oxygen saturation of the water. The aim of fertilisation is to keep the oxygen saturation in the water at a little over 100% and the typical frequency of fertilisation is 4-5 times per season. In 2008 the fertiliser used was NPK 21-4-10 and 21-3-8 during the spring season and NPK 21-4-10 and 18-3-15 during the autumn season (Felleskjøpet, Norway). Oxygen saturation and water temperature on September 8th were 121% and 10.6°C respectively (not measured on June 30th). As the rearing tanks are located outdoors the amount of light is solely dependent on the time of year. Day lengths on the two collection days were 24h on June 30th and 14h on September 8th, respectively.

Seawater from the closed ponds was filtrated through several filters with pore sizes ranging from 50 to 1000 μm, after which the different fractions of zooplankton/copepods were fed continuously to the rearing tanks containing the cod larvae, starting with the smallest fractions. Samples for analyses were collected from the outlet of the filtering unit, rinsed with fresh seawater and frozen immediately (Nora A. Rist and Espen Vang, Lofilab AS, personal communication).

Rotifers

Rotifers were collected at Troms Marin Yngel AS (TMY AS, Tromsø, Norway) in October 2008. Their standard procedure is to keep rotifers in large tanks and given 0.3 g of DHA-enriched *Chlorella* (Chlorella Industry Co. Ltd., Tokyo, Japan) per million rotifers per day as maintenance feed. Prior to enrichment, the rotifers are rinsed and transferred to enrichment tanks where they are enriched either with Multigain from Dana Feed AS (Horsens, Denmark) (medium A), Origreen from Trouw France SA (Fontaine les Vervins, France) (medium B) or the DHA-enriched freshwater algae *Chlorella* (medium C). Multigain and Origreen are given at a dose of 0.2 g per million rotifers while Chlorella is given at a dose of 0.5 mL per million rotifers. After enrichment, lasting for 2 hours, the rotifers are rinsed again. The standard diet given to the cod larvae is a 50/50 mixture of Multigain-enriched or Origreen-enriched rotifers and *Chlorella*-enriched rotifers. The larvae are fed four times daily i.e. every 6 hours. A flow-chart of production is shown in figure 3. (Thor Arne Hangstad and Kristin Skar, TMY AS, personal communication).

Samples were collected before enrichment (RU), after each of the three enrichment treatments (RM, RO and RC, respectively) and after preparation of the final products (MMC and MOC, respectively). All samples were filtered to remove seawater and subsequently frozen. In addition, samples of the enrichment media were also collected. As not all enrichment procedures are performed every day the samples used in these experiments were collected over several days in October 2008. Rotifers enriched with medium A and C and the 50/50 mixture of these two rotifers were collected on October 10th. Natural rotifers and rotifers enriched with medium B were collected on October 14th. The 50/50 mixture of rotifers enriched with medium B and C was collected on October 17th.

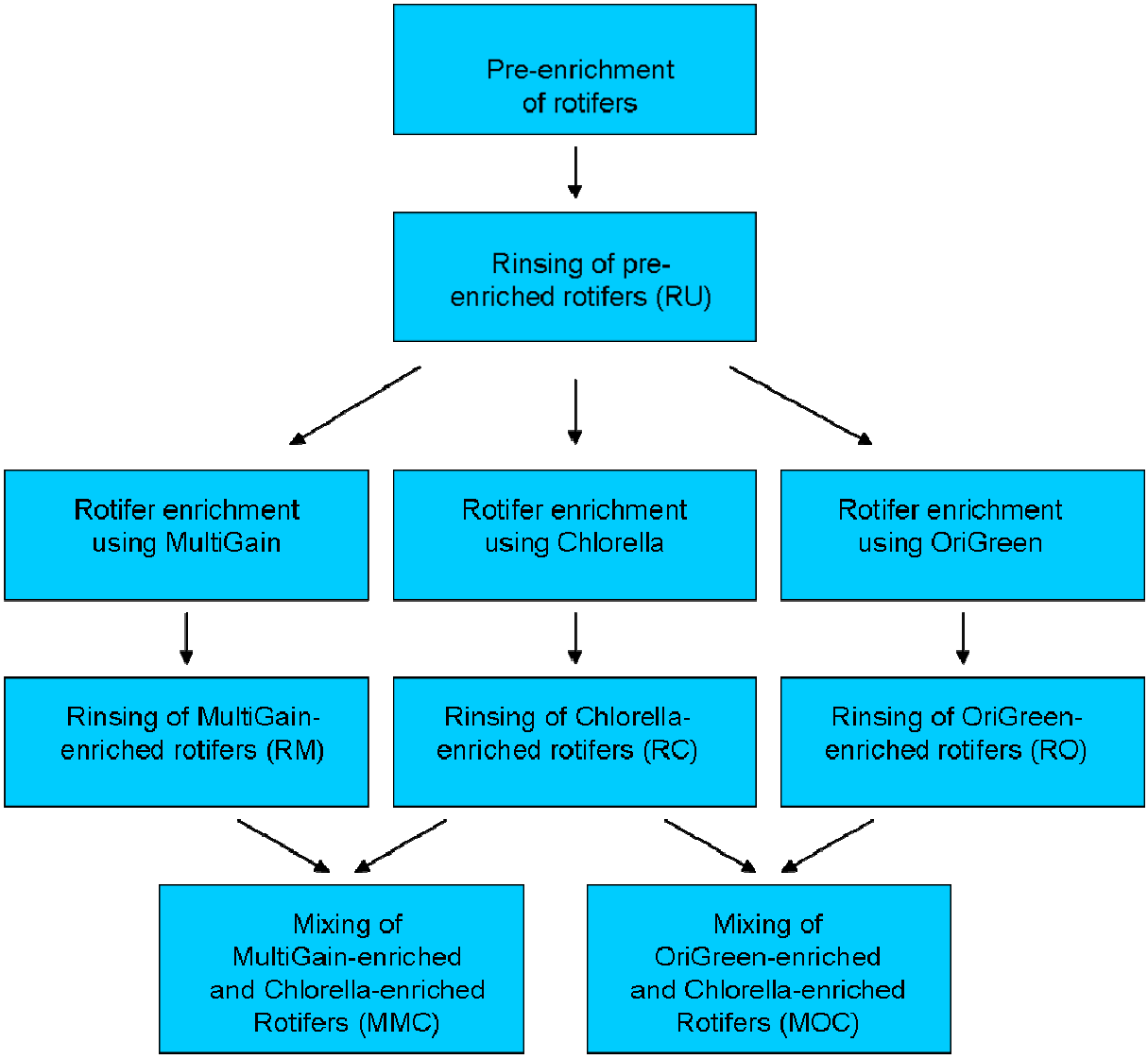

Figure 3. Flow chart of rotifer production at Troms Marin Yngel AS.

Krill

Northern krill (*Meganyctophanes norvegica*) was caught in the Barents Sea in September 2007 and frozen immediately. The samples were kindly provided by Professor Kurt Tande, University of Tromsø. Prior to analyses the krill was thawed and homogenised. One sample was homogenised together with the drip and one sample was homogenised without the drip. After homogenisation the samples were re-frozen and kept frozen until freeze-drying.

Calanus

Samples of freeze-dried calanus (*Calanus finmarchicus*) and calanus meal were kindly provided by Calanus AS (Tromsø, Norway).

Experimental design

Samples were subjected to proximate analysis (water, protein, lipids and ash), fatty acid composition, phospholipid analysis and free- and total amino acid analysis. For comparison purposes, all raw materials were freeze-dried prior to chemical analyses using a Vir-Tis Genesis 35EL freeze dryer (SP Industries, Gardiner, NY, USA). All raw materials were stored frozen (-55°C) until freeze-drying and all analyses were performed within one month of freeze-drying.

All analyses except the mineral analyses were performed at the Norwegian College of Fishery Science during the period November 2008 to November 2009. Mineral analyses were performed at NIFES (Bergen, Norway) in October/November 2009.

All reagents used in these analyses were of analytical grade. Chloroform and methanol were purchased from BDH (Poole, Dorset, UK). All other solvents and chemicals were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

Statistics

All results are expressed as a mean of three parallels ± standard deviation (SD). As the sample size of each collection was rather small a Levene's test of homogeneity of variance was performed and in most cases this test revealed that the variances were not equal. One-way analysis of variation (ANOVA) has equal variances as a qualification hence, a statistical analysis of this material would not give a "trust-worthy" picture of the situation. The sampling of copepods/zooplankton and rotifers was performed only once during a production cycle and must be seen as a "snapshot" of the production.

Analyses

Proximate composition; protein, water and ash

Protein content was determined using the Kjeldahl method, AOAC 981.10 (Horwitz, 2004). In short: 1 g of sample, 1 Kjeltec catalyst tablet and 10 mL H_2SO_4 were put into a Kjeldahl tube and digested for two hours at 420°C. The product was then made basic with 30% (w/v) NaOH, prior to distillation into 0.1 M HCl and titration against 0.25 M NaOH. The factor used to convert nitrogen into crude protein was 6.25.

Water content was determined with a modified version of AOAC 925.04 (Horwitz, 2004). Ten grams of sample was dried at 105°C until constant weight. Water content of the samples was determined gravimetrically.

Ash content was analysed using a modified version of AOAC 938.08 (Horwitz, 2004). The water- and fat-free sample was combusted at 500°C for 12 hours and ash content was determined gravimetrically.

Lipids

Extraction of lipids

Lipids were extracted according to Folch *et al.* (1957). Briefly, 1 g of sample was mixed with 20 mL of chloroform:methanol (2:1, v/v) and mixed thoroughly for 30 minutes. After filtration 4 mL of 0.9% (w/v) NaCl was added and the mixture was centrifuged at 4°C at 2000 rpm for 10 min. The upper methanol/water phase was removed and the lower lipid/chloroform phase was evaporated using a rotavapor (Heidolph Laborota 4000 (Heidolph Instruments GmbH, Schwabach, Germany) with a Büchi Vacuum Controller B-721 (Büchi Labortechnik AG, Flawil, Switzerland)). Fat content was determined gravimetrically.

Solid phase extraction (SPE)

The lipid classes in the extracted oils were isolated by solid phase extraction using Mega Bond Elut (500 mg) aminopropyl SPE columns from Varian Inc. (Middelburg, The Netherlands) on a Visiprep vacuum manifold (Supelco, Bellafonte, PA, USA) according to

Kaluzny *et al.* (1985). The column was pre-conditioned with heptane. Oil solutions (10-20 mg/ml in chloroform/methanol 2:1, v/v) were applied to the column. The eluents A (Chloroform: isopropanol (2:1, v/v)), B (2% acetic acid in diethyl ether (v/v)) and C (methanol) were added successively eluting neutral lipids (NL), free fatty acids (FFA) and phospholipids (PL) respectively. Each fraction was evaporated using a rotavapor (Heidolph Laborota 4000 (Heidolph Instruments GmbH, Schwabach, Germany) with a Büchi Vacuum Controller B-721 (Büchi Labortechnik AG, Flawil, Switzerland)) at 100-300 mbar and 40°C. The purity of the fractions from the SPE was examined using thin layer chromatography (TLC). Samples were diluted to approximately 25 mg/ml in chloroform and applied onto HP-TLC plates (Silica gel 60, 10x10 cm, Merck, Darmstadt, Germany). The plates were placed in elution trays with a saturated atmosphere of heptane / diethylether / acetic acid (70/30/1 v/v/v) and removed when the mobile phase was 1cm from the top of the plate. The plates were then sprayed with 10% cupric sulphate in 8% phosphoric acid as described in Vaghela & Kilara (1995) The plates were air-dried for 10 minutes and spots were developed by heating the plates to 180°C. The lipid classes were identified by comparison to known standards, 16-0A and 18-5A (NuChec Prep. Inc., Elysian, MN, USA).

Phospholipids

Determination of phospholipids were performed according to Stewart (1980). Approximately 10 mg of the oil samples were diluted to 25 ml in chloroform. Chloroform was added to aliquots of 250 µl, 500 µl and 1000 µl of the oil dilutions to a total volume of 2 ml of chloroform. 2 ml of ammonium ferrothiocyanate solution was added and the samples were mixed thoroughly. The upper phase was removed and the lower phase was measured spectrophotometrically at λ488 nm and compared to a standard curve made using egg lecithin (BDH, Poole, Dorset, UK).

Fatty acid composition

Analysis of fatty acid composition was performed on the phospholipid fraction from the SPE and on the total lipid fraction. Each fraction was redissolved to a concentration of approximately 10 mg/mL in chloroform:methanol (2:1, v/v). The samples were methylated according to Stoffel *et al.* (1959), with minor adjustments. A volume of 0.1 mL of this solution was mixed with 0.9 mL of chloroform and 2 mL of 2% (v/v) H_2SO_4 in methanol and

boiled for one hour. The fatty acid methyl esters were then extracted by adding equal amounts of heptane and 5% (w/v) NaCl. The upper heptane phase was transferred into a new tube and evaporated under N_2 in an N-EVAP (Organomation Assoc. Inc., Berlin, Germany). The fat was redissolved in 0.1 mL heptane and transferred into GC vials.

Gas chromatography was performed using an Agilent 6890N equipped with a 7683 B auto injector and a flame ionization detector (FID) (Agilent Technologies Inc., Santa Clara, CA, USA). Helium was used as the carrier gas. The column used was a Varian CP7419 capillary column (50 m x 250 µm x 0.25 µm nominal) (Varian Inc., Middelburg, The Netherlands). Injector and detector temperatures were 240^{o}C and 250^{o}C respectively. A predefined temperature programme was used to ensure the best possible separation of the fatty acids (50 ^{o}C for two minutes, then 10^{o}C per min to 150^{o}C followed by 2^{o}C per min to 205^{o}C and finally 15^{o}C per min until 255^{o}C and stabilization for 10 minutes). The fatty acids were identified by comparison to the fatty acid standards 1895, 1893, 1891, PUFA no 1 and PUFA no 3 from Sigma (Sigma Chemicals Co, St. Louis, MO, USA) and fatty acid standard 68D from NuChek (NuChec Prep. Inc., Elysian, MN, USA).

Amino acids

Free amino acids (FAAs) were extracted by homogenising approximately 0.2 g freeze-dried sample with 9 mL distilled water and 1 mL of 20 mM norleucine, which served as an internal standard. The sample was homogenised at 20,000 rpm using an Ultra Turrax T25 basic (IKA Werke GmbH, Staufen, Germany) for 15 seconds. 1 mL of 35% sulfosalicylic acid (SSA) was added for removal of proteins and peptides followed by another 15 seconds of homogenisation. After centrifugation at 20,000g at 4ºC for 10 minutes the supernatant was diluted 1:5 with a lithium citrate buffer, pH 2.2.

Total amino acids (TAAs) were extracted by homogenising approximately 50 mg of freeze-dried material with 0.7 mL of distilled water, 0.5 mL of 20 mM norleucine and 1.2 mL of concentrated hydrochloric acid. The homogenate was flushed with nitrogen gas for 10 seconds before hydrolysis at 110°C for 20-24h (van der Meeren *et al.*, 2008). Prior to analysis, 100 µl of the hydrolysate was evaporated to dryness under nitrogen gas and redissolved in 1 mL of a lithium citrate buffer, pH 2.2.

As tryptophan is completely destroyed during acidic hydrolysis, an alkaline hydrolysis was performed for determination of this amino acid, according to Levine (1982). Approximately 50 mg of freeze-dried material was homogenised with 0.5 mL of 20 mM norleucine, 75 µl of thiodiglycol and 1.9 mL of 4.2 M NaOH. Thiodiglycol served as a protector against oxidation. The homogenate was flushed with nitrogen gas for 10 seconds before hydrolysis at 110°C for 20-24h. After hydrolysis 0.5 mL of the solution was transferred to an eppendorf tube, pH-adjusted to approximately pH 2 using 3 M HCl and finally centrifuged at 20,000g at 4°C for 10 minutes. One hundred µl of the supernatant was evaporated under nitrogen gas to dryness and redissolved in 1 mL of a lithium citrate buffer, pH 2.2.

All amino acid samples were analysed by chromatographic separation on an ion exchange column, using lithium citrate buffers of different pH and ionic strength and a pre-defined temperature programme as described in Spackman *et al.* (1958). Ultra violet detection of the amino acids was possible after a reaction with ninhydrin resulting in a blue complex with a light optimum at 570 nm for most amino acids and a yellow complex with a light optimum at 470 nm for proline and hydroxyproline. The analysis was performed using a Biochrom 30 amino acid analyser (Biochrom Co, Cambridge, UK) and the UV signals were analysed by Chromeleon software (Dionex, Sunnyvale, CA, USA) and compared to A9906 physiological amino acids (Sigma Chemicals Co, St. Louis, MO, USA).

Minerals

Calcium, iron, magnesium, phosphorus, manganese, copper, zinc and selenium were analysed. Analyses were performed according to method 186 of the Nordic Committee of Food Analyses (2007). Samples were added to concentrated HNO_3 and hydrogenperoxide (30%, v/v) and digested in a microwave oven. Quantitative ICP-MS was used for determination of the elements and calculation of the concentrations was based on individual standard curves. Scandium was used as an internal standard for Ca, Fe, Mg and P, whilst Rhodium was used as an internal standard for Mn, Cu, Zn and Se.

Fluoride

Analysis was performed according to Malde *et al.* (2001) The samples were dried and combusted at 525°C using NaOH as a combustion aid. The samples were then neutralised and the concentration of fluoride was determined using an ion-selective electrode.

RESULTS

Proximate composition

Analyses of the proximate composition of all raw materials were performed. The lipid, protein and ash content showed large variations within sample groups. This may be due to variation in water content and an uneven distribution of suspended material. Hence, it was decided to freeze-dry all materials prior to analysis and that all comparisons be performed on a dry weight basis (DW).

Results presented in table 1 showed that zooplankton had the highest water content before freeze-drying, with a mean of 90.6% while enrichment medium B had the lowest at 5.2%. All rotifers, including the intensive feed mixtures had a water content of 85-90%. After freeze-drying the water content of the different raw materials was more equal, ranging from 1.2% in enrichment medium C to 11% in rotifers enriched with medium B. Lipid content of zooplankton and the different rotifers or live feeds was quite equal, ranging from 8.3-13.3%. The enrichment media were high in lipids with values that ranged from 19.2 to 38.9%. Krill were also rich in lipids at 30-40%, while the lipid content in the calanus samples was 12.4% in the calanus meal and 19.9% in the freeze-dried whole calanus. Protein content of the three different enrichment media was 30.9% in medium A, 50.8% in medium B and 67.8% in medium C. In rotifers the protein content did not change significantly after enrichment at 55-57%. The protein contents of zooplankton and krill was quite similar to that of rotifers, whilst the protein content of calanus samples was somewhat higher at around 70%. The ash content, reflecting the mineral content, varied from 8.2% in enrichment medium C to 32% in zooplankton.

Table 1: Proximate composition of zooplankton, two intensive feeds, natural rotifers and rotifers enriched with enrichment media A, B and C, enrichment media A, B and C, krill and calanus.

	Water before freeze drying *(g /100 g WW[a])*	***Water after freeze drying*** *(g/100 g DW[b])*	***Lipids**** *(g/100 g DW[c])*	***Protein*** *(g/100 g DW[c])*	***Ash*** *(g/100 g DW[c])*
Live feeds					
Zooplankton	90.6 ± 0.2	1.7 ± 0.1	8.3 ± 0.4	56.0 ± 2.2	32.0 ± 0.4
Intensive feed A	86.4 ± 0.1	5.5 ± 0.3	12.1 ± 0.8	62.0 ± 3.0	16.7 ± 0.6
Intensive feed B	89.8 ± 0.0	5.0 ± 0.4	11.8 ± 1.7	65.6 ± 6.1	20.4 ± 0.0
Rotifers					
Natural rotifers	87.1 ± 0.2	10.8 ± 0.8	10.7 ± 1.5	55.0 ± 2.7	19.9 ± 0.5
Rotifers + A	86.1 ± 0.3	2.6 ± 0.1	13.3 ± 1.0	55.1 ± 2.2	15.7 ± 0.2
Rotifers + B	87.3 ± 0.1	11.0 ± 0.9	12.1 ± 1.4	57.3 ± 1.5	19.9 ± 0.6
Rotifers + C	85.8 ± 0.1	2.2 ± 0.1	8.7 ± 0.3	55.0 ± 2.2	13.5 ± 0.6
Enrichment media					
Medium A	8.9 ± 0.1	7.8 ± 0.6	38.9 ± 3.0	30.9 ± 2.9	9.8 ± 0.4
Medium B	5.2 ± 0.2	1.8 ± 0.3	26.9 ± 0.6	50.8 ± 2.4	11.3 ± 0.5
Medium C	88.3 ± 0.3	1.2 ± 0.1	19.2 ± 1.6	67.8 ± 3.0	8.2 ± 0.2
Krill incl. drip	87.7 ± 0.2	6.9 ± 0.7	30.6 ± 2.9	50.2 ± 2.5	23.1 ± 0.5
Krill excl. drip	78.6 ± 0.8	4.0 ± 0.2	40.8 ± 0.6	54.0 ± 2.5	13.3 ± 0.4
Freeze-dried calanus	12.8 ± 0.2	7.9 ± 0.5	19.9 ± 0.2	69.3 ± 2.4	12.7 ± 0.2
Calanus meal	9.6 ± 0.0	4.0 ± 0.6	12.4 ± 0.2	71.8 ± 3.9	14.4 ± 0.4

a: Weight expressed as g/100 g of the original wet weight (WW) samples
b: Weight expressed as g/100 g of the freeze-dried material
c: Calculated weight, with respect to the residual water in the sample after freeze-drying.
*: Lipid extraction performed by the Folch method
**: Protein content determined by the Kjeldahl method of determination of total nitrogen

Lipids

Fatty acid composition – total oil

Fatty acid composition of all raw materials was analysed after extraction of total lipid by Folch's method (figures 4-7). The dominant saturated fatty acid in all samples was palmitic acid (C16:0), except for the calanus samples where myristic acid (C14:0) was at approximately the same level. The fatty acid profile of zooplankton was significantly different from that of the two intensive feeds. In zooplankton the HUFAs were the dominant fatty acids at 13.8% EPA and 24.7% DHA respectively. Among the shorter PUFAs the stearidonic acid (SDA, C18:4, n3) was dominant, counting for 10.7%. The level of the other PUFAs was minor. The two intensive feeds however, were very rich in LA at around 21-22%. EPA was around 6% and DHA was 14-15% in both feeds.

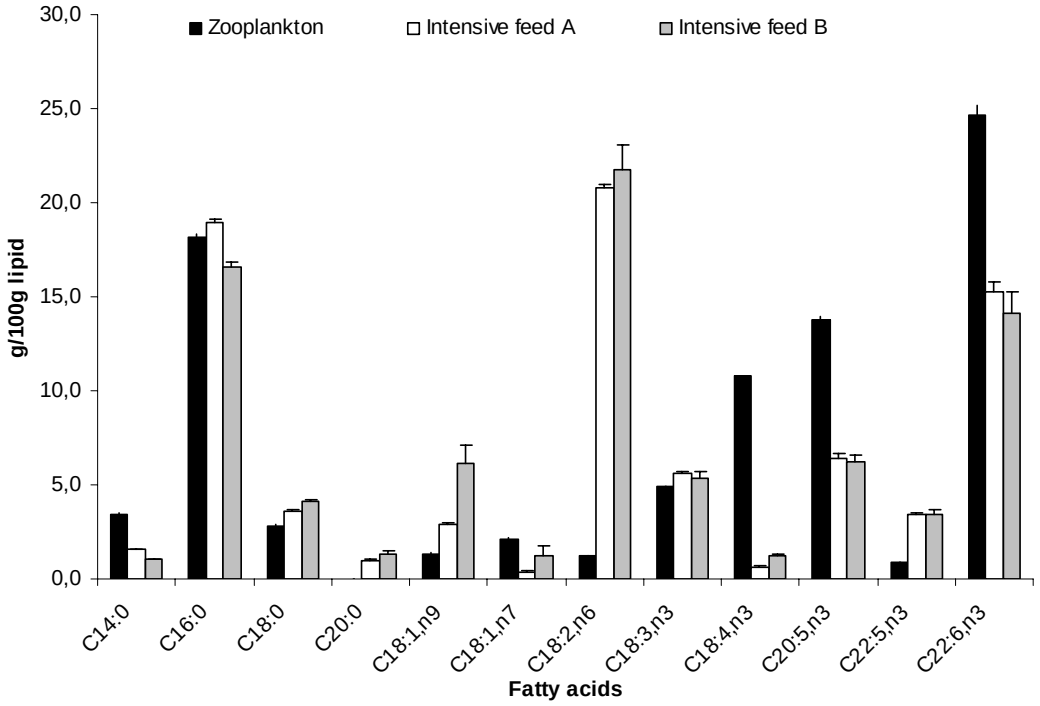

Figure 4: Main fatty acids in zooplankton and two different intensive feeds. Values are expressed as mean ± SD (n = 3) and in g/100 g lipid

All of the rotifers had high levels of LA, ranging from 14.9% in rotifers enriched with medium A to 19.9% in rotifers enriched with medium C. The concentration of ALA was highest in the rotifers enriched with medium C at 5.1% and lowest in the rotifers enriched with medium B at 3.2%. After enrichment, EPA levels ranged from 3.1% in the rotifers

enriched with medium B to 6.0% in the rotifers enriched with medium A. Both natural rotifers and the rotifers enriched with medium B had a DHA level of around 6%, while the level was around 9.5% in the other two enriched rotifers.

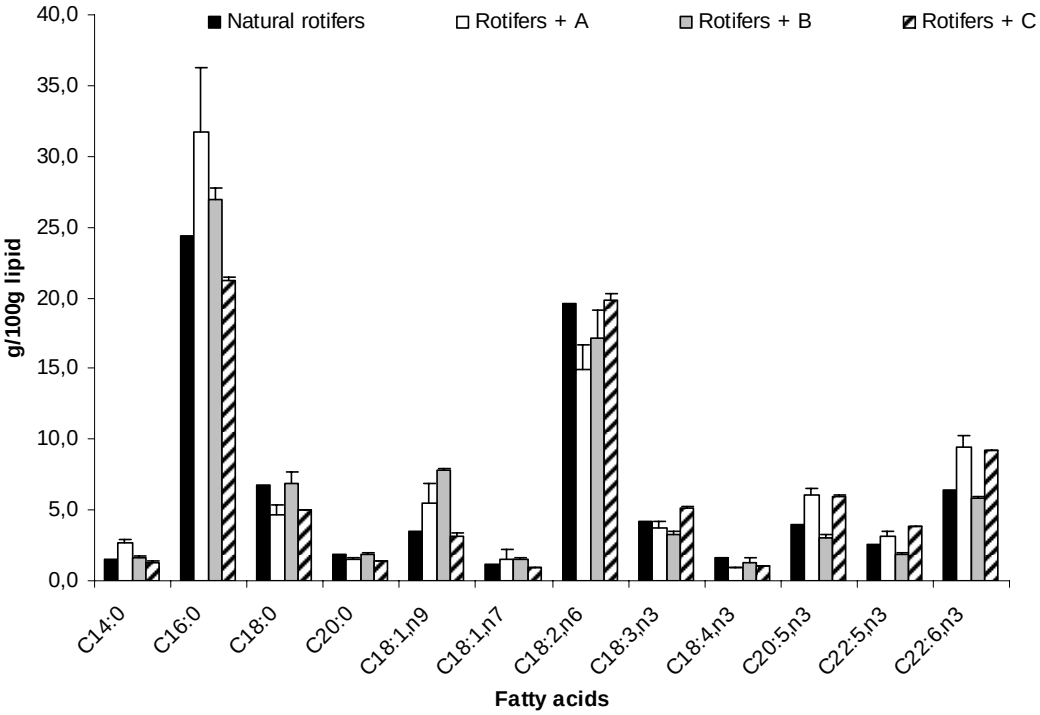

Figure 5: Main fatty acids in natural rotifers and in rotifers after enrichment with medium A, B and C. Values are expressed as mean ± SD (n = 3) and in g/100 g lipid.

Among the monounsaturated fatty acids, oleic acid (C18:1, n9) was dominant in all enrichment media and the highest level among them was in medium B at 11%. The dominant PUFAs were LA and DHA in all three enrichment media.

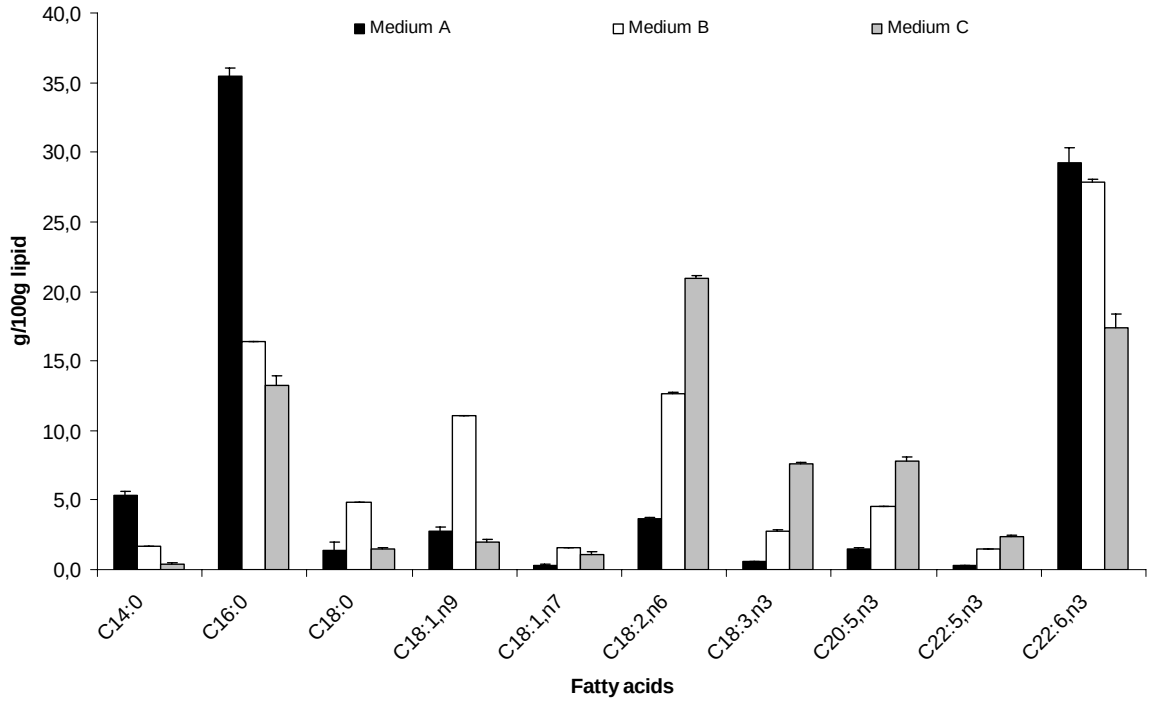

Figure 6: Main fatty acids in enrichment media A, B and C. Values are expressed as mean ± SD (n = 3) and in g/100 g lipid.

Krill was very rich in oleic acid and EPA, at around 20% and 13% respectively. The n3 fatty acids SDA and DHA were also quite high at around 5 and 6% respectively. In the calanus samples both EPA and DHA concentrations were around 10-11%. The dominant fatty acid in these samples was SDA at 15-16%.

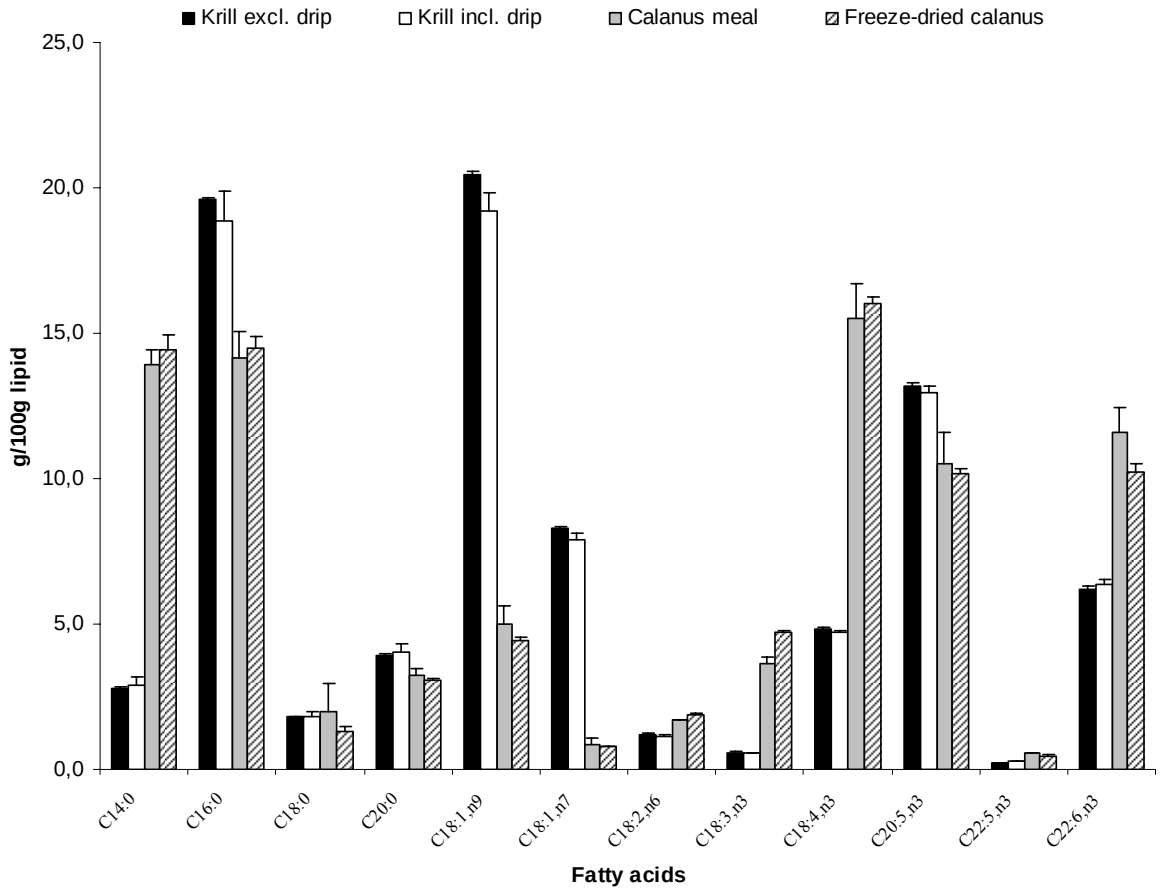

Figure 7: Main fatty acids of krill and calanus. Values are expressed as mean ± SD (n = 3) and in g/100 g lipid.

Solid phase extraction

In order to obtain a pure PL fraction from the different oils solid phase extraction was performed. The purity of the fractions was determined by thin layer chromatography (TLC) as illustrated in figure 8. Identification of the different lipid classes was performed by comparison of the samples to two known TLC standards, 18-5A and 16-0A (NuChec Prep. Inc., Elysian, MN, USA).

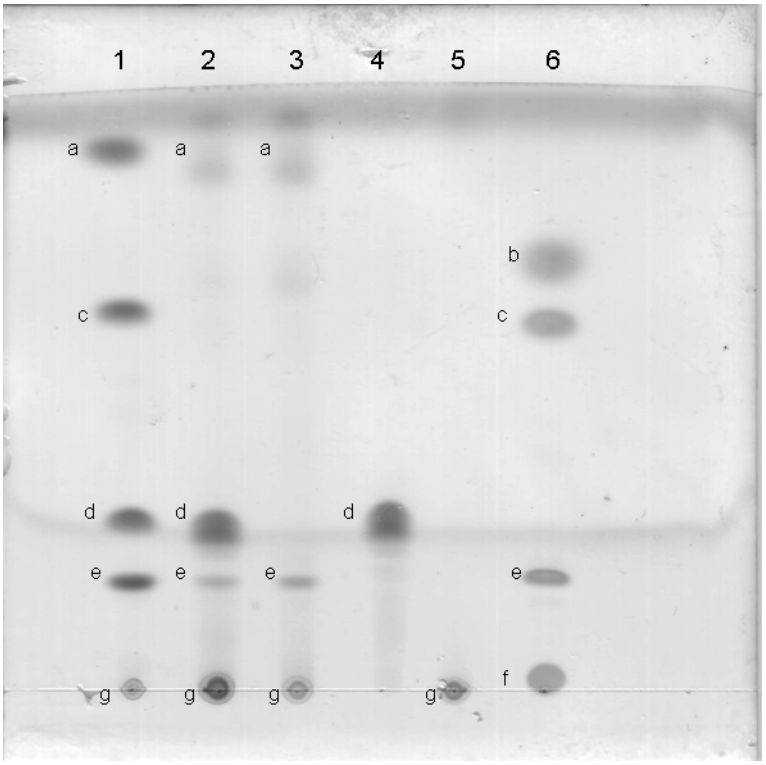

Figure 8: Thin layer chromatography plate of zooplankton before and after fractionation by solid phase extraction. Lane 1: TLC-standard 18-5A, lane 2: non-fractionated zooplankton oil, lane 3: Neutral lipid-fraction, lane 4: FFA-fraction, lane 5: Phospholipid fraction, lane 6: TLC-standard 16-0A. The spots marked in small letters are a: Wax ester, b: methylated fatty acid, c: TAG, d: FFA, e: cholesterol/diacylglycerol, f: monoacylglycerol, g: PL.

Phospholipids

The concentration of the PLs in the oils was determined using a method based on the formation of a phospholipid-ferrothiocyanate complex (tables 2-4). The PL concentration in zooplankton was 9.2%, whilst the intensive feeds had levels of 22.9% and 29.8% in A and B respectively.

Table 2: Phospholipid content of zooplankton and two different intensive feeds. Values are expressed as mean ± SD (n=3) and in g phospholipid/100g lipid.

	g PL/100 g lipid		
	Zooplankton	***Intensive feed A***	***Intensive feed B***
Phospholipids	9.2 ± 0.5	22.9 ± 1.5	29.8 ± 1.4

In natural rotifers the PL concentration was 10.6%. After the different enrichments the levels in the rotifers were 17.8%, 11.5% and 23.0% in rotifers enriched with medium A, B and C respectively.

Table 3: Phospholipid content of natural rotifers and rotifers after enrichment with medium A, B and C. Values are expressed as mean ± SD (n = 3) and in g phospholipid/100 g lipid.

	g PL/100 g lipid			
	Natural rotifers	***Rotifers + A***	***Rotifers + B***	***Rotifers + C***
Phospholipids	10.6 ± 0.8	17.8 ± 0.3	11.5 ± 0.8	23.0 ± 0.6

In the enrichment media the PL concentrations were 5-6%. Both krill samples had a PL concentration of around 26%, while the calanus samples were rather low in PLs at around 2%.

Table 4: Phospholipid content of enrichment media A, B and C, krill incl. and excl. drip, freeze-dried calanus and calanus meal. Values are expressed as mean ± SD (n = 3) and in g phospholipid/100 g lipid.

	g PL/100 g lipid						
	Medium A	***Medium B***	***Medium C***	***Krill excl. drip***	***Krill incl. drip***	***Freeze-dried calanus***	***Calanus meal***
Phospho-lipids	5.9 ± 1.4	5.9 ± 0.9	5.3 ± 0.6	26.6 ± 1.5	25.7 ± 0.6	2.6 ± 0.3	1.8 ± 0.2

The fatty acid composition was also determined in the purified phospholipid fractions of the different raw materials (tables 5-7). As in the total lipid fractions palmitic acid was the dominant saturated fatty acid in all of the samples.

The fatty acid profile of the PL fraction of zooplankton was significantly different from the PL fractions of intensive feeds (table 5). In zooplankton EPA and DHA concentrations were 8.5% and 32.5% respectively. In the intensive feeds these levels were rather low with EPA at 1.5-2% and DHA at 3-4%. In the intensive feeds the concentrations of palmitic acid were 35.5% and 25.6%, while it was 13.8% in zooplankton. The concentrations of LA were 15.5 and 17.2% in the two intensive feeds and 1.7% in zooplankton.

Table 5: Main fatty acids of the phospholipid fraction of zooplankton and two different intensive feeds. Values are expressed as g fatty acid/100 g phospholipid (n = 1).

	g fatty acid/100 g PL		
	Zooplankton	***Intensive feed A***	***Intensive feed B***
C14:0	3.5	3.7	1.6
C16:0	13.8	35.5	25.6
C16:1, n7	2.0	1.3	1.3
C18:0	3.9	5.2	5.4
C18:1, n9	6.9	4.5	7.2
C18:1, n7	1.5	0.9	1.5
C18:2, n6	1.7	15.5	17.2
C18:3, n3	1.7	2.3	2.1
C18:4, n3	4.1	2.2	3.9
C20:0	2.1	2.9	3.3
C20:5, n3	8.5	1.4	1.9
C22:5, n3	n.d	2.0	1.9
C22:6, n3	32.5	3.0	3.7

In the rotifer samples the concentration of stearate (C18:0) was also relatively high, ranging from 9-14%. The dominant PUFA was LA in all the rotifer samples, ranging from 13-16%. Among the HUFAs, EPA was dominant in all rotifer samples, ranging from 4-7%.

Table 6: Main fatty acids of the phospholipid fraction of natural rotifers and after enrichment with medium A, B and C. Values are expressed as g fatty acid/100 g phospholipid (n = 1).

	g fatty acid/100 g PL			
	Natural rotifers	***Rotifers + A***	***Rotifers + B***	***Rotifers + C***
C14:0	1.7	3.6	1.6	1.2
C16:0	18.6	22.7	15.8	17.9
C16:1, n7	1.3	1.7	1.0	0.9
C18:0	9.5	11.4	9.4	14.9
C18:1, n9	7.2	4.9	4.4	3.9
C18:1, n7	1.5	1.8	1.2	2.2
C18:2, n6	16.3	12.8	15.4	16.0
C18:3, n3	2.4	1.9	2.3	2.4
C18:4, n3	4.5	5.1	5.3	2.8
C20:0	4.3	3.6	4.8	3.0
C20:5, n3	5.0	5.0	4.4	7.2
C22:5, n3	1.5	1.2	1.3	1.6
C22:6, n3	2.6	2.3	2.2	2.6

In all of the enrichment media the main fatty acids were oleic acid and LA. The levels of HUFAs were low in all enrichment media, DHA in medium A being highest at 8%. In the PL fraction of the krill samples the SDA and DHA concentrations were slightly higher than the concentrations in the total lipid fractions. The oleic acid concentration was lower at 9-12%, while the EPA concentration was higher at 21-24%. The fatty acid profile of the total lipid

fraction of calanus (figure 7) showed a very high concentration of SDA (15.5-16%), whilst the PL fraction contained only 3.5% of this fatty acid (table 7). In addition to palmitic acid, the dominant fatty acids in this fraction of the calanus samples were oleic acid, EPA and DHA, accounting for 10-12%, 7.5-9% and 23-24% respectively.

Table 7: Fatty acid composition of the phospholipid fraction of enrichment media A, B and C, krill incl. and excl. drip, freeze-dried calanus and calanus meal. Values are expressed as g fatty acid/100 g phospholipid (n = 1).

	g fatty acid/100 g PL						
	Medium A	***Medium B***	***Medium C***	***Krill excl. drip***	***Krill incl. drip***	***Freeze-dried calanus***	***Calanus meal***
C14:0	0.9	0.6	0.8	1.5	1.0	1.0	1.3
C16:0	31.9	17.3	18.9	25.5	27.1	10.9	13.7
C16:1, n7	0.9	0.9	0.5	5.0	3.9	n.d.	0.6
C18:0	4.2	5.3	2.7	2.7	2.8	7.0	6.7
C18:1, n9	11.6	15.3	3.3	12.3	9.4	11.9	10.1
C18:1, n7	1.0	1.2	n.d.	7.1	7.0	n.d.	n.d.
C18:2, n6	22.1	35.6	25.8	1.3	1.3	2.5	2.1
C18:3, n3	3.2	8.9	7.3	0.8	0.8	1.4	1.2
C18:4, n3	n.d.	n.d.	n.d.	6.7	7.1	3.3	3.4
C20:0	n.d.	n.d.	n.d.	2.0	1.1	n.d.	0.6
C20:5, n3	0.7	n.d.	2.2	21.4	24.2	7.5	9.3
C22:5, n3	n.d	n.d.	0.7	n.d.	n.d.	n.d.	0.6
C22:6, n3	8.2	2.2	4.7	9.7	11.4	23.1	23.8

Amino acids

Free amino acids

The concentration of FAA was determined by ion-exchange chromatography followed by post-column derivatisation with ninhydrin. The main difference in the FAA between zooplankton and the two intensive feeds was the concentration of taurine, which was 8.4 mg/g DW in the zooplankton, whilst it was only 0.2 mg/g DW in the intensive feeds. Proline and glycine were also higher in zooplankton than in the intensive feeds, concentrations being 15.1 and 12.4 mg/g DW in zooplankton and 1.4-1.7 and 1.1-1.4 mg/g DW in the intensive feeds respectively. Leucine and phenylalanine were higher in the intensive feeds than in zooplankton.

Table 8: Free amino acid concentrations in zooplankton and two different intensive feeds. Values are expressed as mean ± SD (n = 3) and in mg aa/g DW. n.d. = not detected

	mg aa/g DW		
	Zooplankton	***Intensive feed A***	***Intensive feed B***
Essential amino acids			
Thr	0.9 ± 0.1	1.9 ± 0.1	1.6 ± 0.0
Val	1.3 ± 0.1	2.3 ± 0.1	1.5 ± 0.0
Met	0.6 ± 0.0	1.1 ± 0.1	1.0 ± 0.0
Ile	0.9 ± 0.0	1.9 ± 0.1	1.5 ± 0.1
Leu	1.8 ± 0.0	4.6 ± 0.3	3.5 ± 0.1
Phe	6.2 ± 0.2	13.0 ± 1.1	11.8 ± 0.4
Lys	3.7 ± 0.0	3.2 ± 0.2	2.5 ± 0.1
His	0.5 ± 0.0	1.1 ± 0.0	0.9 ± 0.0
Trp	n.d.	n.d.	n.d.
Arg	10.6 ± 0.2	6.2 ± 0.3	4.3 ± 0.2
Conditionally essential amino acids			
Tau	8.4 ± 0.5	0.2 ± 0.0	0.2 ± 0.0
Gln	1.0 ± 0.1	3.6 ± 0.3	3.1 ± 0.1
Pro	15.1 ± 0.7	1.4 ± 0.1	1.7 ± 0.2
Non-essential amino acids			
Asp	0.8 ± 0.0	1.5 ± 0.1	1.1 ± 0.1
Ser	0.9 ± 0.0	3.1 ± 0.2	3.0 ± 0.1
Asn	0.6 ± 0.0	2.7 ± 0.3	2.2 ± 0.1
Glu	3.6 ± 0.5	4.8 ± 0.3	3.7 ± 0.2
Gly	12.4 ± 0.5	1.1 ± 0.1	1.4 ± 0.0
Ala	4.2 ± 0.1	3.4 ± 0.2	2.3 ± 0.1
Tyr	1.6 ± 0.2	3.3 ± 0.2	3.0 ± 0.0
Orn	0.0 ± 0.0	0.7 ± 0.1	0.3 ± 0.0
Sum FAA	75.2 ± 2.8	50.8 ± 1.5	61.1 ± 4.0

Table 9: Free amino acid concentrations in natural rotifers and in rotifers after enrichment with medium A, B and C. Values are expressed as mean ± SD (n = 3) and in mg aa/g DW. n.d. = not detected

	mg aa/g DW			
	Natural rotifers	***Rotifers + A***	***Rotifers + B***	***Rotifers + C***
Essential amino acids				
Thr	8.9 ± 0.0	0.4 ± 0.0	7.6 ± 0.3	0.9 ± 0.0
Val	8.4 ± 0.1	0.5 ± 0.0	6.8 ± 0.2	1.4 ± 0.1
Met	5.3 ± 0.2	0.2 ± 0.0	4.2 ± 0.1	0.2 ± 0.0
Ile	10.4 ± 0.1	0.4 ± 0.0	8.1 ± 0.1	0.6 ± 0.1
Leu	20.0 ± 0.0	0.6 ± 0.0	16.9 ± 0.3	1.3 ± 0.1
Phe	60.3 ± 0.1	2.4 ± 0.1	51.8 ± 0.8	2.9 ± 0.1
Lys	15.3 ± 0.1	0.7 ± 0.1	13.2 ± 0.2	0.8 ± 0.0
His	4.3 ± 0.1	0.5 ± 0.0	4.0 ± 0.1	0.8 ± 0.0
Trp	3.6 ± 0.3	n.d.	3.2 ± 0.1	n.d.
Arg	17.2 ± 0.2	2.0 ± 0.1	16.0 ± 0.3	2.7 ± 0.0
Conditionally essential amino acids				
Tau	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Gln	12.0 ± 0.5	0.7 ± 0.1	10.7 ± 0.4	1.1 ± 0.1
Pro	10.8 ± 0.4	1.2 ± 0.0	9.4 ± 0.3	1.4 ± 0.0
Non-essential amino acids				
Asp	8.5 ± 0.1	0.3 ± 0.0	6.7 ± 0.2	0.3 ± 0.0
Ser	11.8 ± 0.1	0.7 ± 0.0	10.9 ± 0.1	1.0 ± 0.0
Asn	14.2 ± 0.3	0.6 ± 0.1	11.6 ± 0.1	0.7 ± 0.1
Glu	18.0 ± 0.3	1.9 ± 0.0	15.6 ± 0.3	2.4 ± 0.1
Gly	5.9 ± 0.1	0.8 ± 0.0	5.0 ± 0.1	0.9 ± 0.0
Ala	12.1 ± 0.0	0.8 ± 0.0	10.4 ± 0.2	1.0 ± 0.0
Tyr	11.5 ± 0.1	0.9 ± 0.1	10.9 ± 0.1	1.3 ± 0.0
Orn	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Sum FAA	260.9 ± 1.7	16.1 ± 0.5	225.1 ± 3.6	22.5 ± 0.6

The concentration of FAA in the three enrichment media was generally low, with some exceptions, such as glutamine and glutamate in medium A and alanine in medium C. The FAA fraction of the natural rotifers was high, phenylalanine being the dominant amino acid at 60.3 mg/g DW. The high FAA fraction was maintained in the rotifers enriched with medium B, while the rotifers enriched with the other two media were low in FAA. Taurine was not present in either of the enrichment media and was generally very low in all of the rotifers. The total FAA pool was relatively similar in the two calanus samples and in krill incl. drip. The krill excl. drip was approximately 40-50% lower in all FAAs. Arginine and glycine were higher in the calanus samples than in the krill incl. drip, 18 and 15 mg/g DW in the calanus and 7.5 and 7.0 mg/g DW respectively. Krill was richer in taurine and proline, 9 and 17 mg/g DW in krill and 6 and 5 mg/g DW in calanus respectively.

Table 10: Free amino acid concentrations in enrichment media A, B and C, krill incl. and excl. drip, calanus meal and freeze-dried calanus. Values are expressed as mean ± SD (n = 3) and in mg aa/g DW. n.d. = not detected

	mg aa/g DW						
	Medium A	***Medium B***	***Medium C***	***Krill excl. drip***	***Krill incl. drip***	***Calanus meal***	***Freeze-dried calanus***
Essential amino acids							
Thr	0.5 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	1.2 ± 0.0	2.4 ± 0.3	2.5 ± 0.2	2.4 ± 0.0
Val	0.5 ± 0.1	0.1 ± 0.0	0.4 ± 0.0	1.8 ± 0.1	3.4 ± 0.4	3.9 ± 0.1	3.8 ± 0.1
Met	n.d.	1.5 ± 0.2	0.2 ± 0.0	0.9 ± 0.0	1.9 ± 0.2	1.8 ± 0.0	1.5 ± 0.0
Ile	0.2 ± 0.0	n.d.	0.1 ± 0.0	1.4 ± 0.1	2.9 ± 0.3	2.8 ± 0.1	2.8 ± 0.1
Leu	0.3 ± 0.0	n.d.	0.2 ± 0.0	2.8 ± 0.1	5.2 ± 0.6	5.3 ± 0.1	5.1 ± 0.2
Phe	1.2 ± 0.0	n.d.	0.8 ± 0.2	9.2 ± 0.4	14.0 ± 1.4	10.4 ± 1.2	12.1 ± 0.4
Lys	0.3 ± 0.0	0.2 ± 0.0	1.6 ± 0.0	3.2 ± 0.1	6.0 ± 0.7	6.6 ± 0.2	6.3 ± 0.1
His	0.2 ± 0.0	1.0 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	n.d.	0.3 ± 0.0
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arg	1.2 ± 0.1	0.2 ± 0.0	1.6 ± 0.0	4.3 ± 0.2	7.5 ± 0.7	17.4 ± 0.2	18.0 ± 0.2
Conditionally essential amino acids							
Tau	n.d.	n.d.	n.d.	4.2 ± 0.1	9.1 ± 1.0	6.1 ± 0.0	6.3 ± 0.0
Gln	2.6 ± 0.1	n.d.	n.d.	1.0 ± 0.1	2.0 ± 0.3	1.4 ± 0.2	2.0 ± 0.0
Pro	n.d.	n.d.	n.d.	7.3 ± 0.2	16.8 ± 2.1	5.0 ± 0.1	5.2 ± 0.2
Non-essential amino acids							
Asp	0.5 ± 0.0	0.1 ± 0.0	0.5 ± 0.1	1.1 ± 0.1	2.1 ± 0.3	2.1 ± 0.0	1.8 ± 0.0
Ser	0.5 ± 0.0	n.d.	0.2 ± 0.0	1.3 ± 0.1	2.7 ± 0.3	2.7 ± 0.1	2.6 ± 0.1
Asn	0.6 ± 0.1	n.d.	n.d.	1.1 ± 0.1	2.2 ± 0.2	1.5 ± 0.1	1.6 ± 0.1
Glu	2.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	n.d.	n.d.	1.3 ± 0.1	1.8 ± 0.0
Gly	0.5 ± 0.0	0.2 ± 0.0	1.2 ± 0.0	3.3 ± 0.1	7.0 ± 0.8	14.8 ± 0.1	15.3 ± 0.0
Ala	2.0 ± 0.0	0.5 ± 0.0	6.2 ± 0.1	2.6 ± 0.1	5.5 ± 0.7	6.5 ± 0.0	6.3 ± 0.0
Tyr	0.3 ± 0.0	n.d.	n.d.	2.4 ± 0.1	2.7 ± 0.3	2.3 ± 0.1	3.1 ± 0.0
Orn	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.0	1.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
Sum FAA	14.8 ± 0.5	4.5 ± 0.4	15.3 ± 0.0	50.1 ± 1.6	95.0 ± 10.7	94.6 ± 2.2	98.3 ± 0.9

Total amino acids

Total amino acids were determined in the same manner as the FAA after acid hydrolysis of the peptide bonds of the peptides and proteins. For determination of tryptophan it was necessary to perform an alkaline hydrolysis. The TAA level was not very different between zooplankton and the two intensive feeds (table 11). Both of the intensive feeds contained more phenylalanine than did zooplankton. Taurine and proline were higher in the TAA pool of zooplankton than in the intensive feeds.

Table 11: Total amino acid concentrations in zooplankton and two different intensive feeds. Values are expressed as mean ± SD (n = 3) and in mg aa/g DW. n.d. = not detected

	mg aa/g DW		
	Zooplankton	***Intensive feed A***	***Intensive feed B***
Essential amino acids			
Thr	17.4 ± 1.4	18.2 ± 1.5	19.3 ± 0.3
Val	21.7 ± 2.0	24.3 ± 1.7	24.8 ± 0.6
Met	9.2 ± 0.8	7.7 ± 0.7	6.5 ± 1.0
Ile	18.3 ± 1.6	23.1 ± 1.7	23.5 ± 0.8
Leu	29.0 ± 2.5	35.1 ± 2.5	36.0 ± 1.5
Phe	79.0 ± 7.2	106.1 ± 8.0	107.3 ± 1.9
Lys	32.6 ± 2.8	35.0 ± 2.6	35.8 ± 1.0
His	8.4 ± 0.7	8.5 ± 0.6	9.1 ± 0.2
Arg	29.5 ± 3.0	26.5 ± 2.2	28.3 ± 0.6
Trp	4.9 ± 0.3	4.5 ± 0.4	4.6 ± 0.3
Conditionally essential amino acids			
Tau	7.7 ± 0.6	n.d.	n.d.
Glx*	57.5 ± 4.8	57.8 ± 4.7	60.1 ± 1.0
Pro	33.0 ± 1.4	25.9 ± 2.2	28.7 ± 0.5
Cys	4.0 ± 0.3	3.6 ± 0.4	3.8 ± 0.1
Non-essential amino acids			
Asx*	31.5 ± 3.0	35.4 ± 2.5	36.6 ± 1.1
Ser	16.3 ± 1.6	20.0 ± 1.9	21.9 ± 0.3
Gly	33.1 ± 3.1	20.9 ± 1.5	20.1 ± 0.6
Ala	28.9 ± 2.8	21.3 ± 1.8	25.0 ± 0.4
Tyr	14.7 ± 1.8	15.1 ± 1.1	12.6 ± 6.9
H-cys	0.2 ± 0.1	n.d.	n.d.
Orn	0.1 ± 0.0	0.3 ± 0.0	0.5 ± 0.1
Sum TAA	479.2 ± 41.6	489.4 ± 37.3	504.6 ± 17.6

* Asx and Glx are the sum of Asp+ Asn and Glu+Gln respectively, as Asn and Gln are present in their acidic forms after acid hydrolysis.

The TAA concentrations in natural rotifers and in rotifers after enrichments were quite similar.

Table 12: Total amino acid concentrations in natural rotifers and rotifers after enrichment with medium A, B and C. Values are expressed as mean ± SD (n = 3) and in mg aa/g DW. n.d. = not detected

	mg aa/g DW			
	Natural rotifers	***Rotifers + A***	***Rotifers + B***	***Rotifers + C***
Essential amino acids				
Thr	20.2 ± 0.5	17.7 ± 1.8	19.3 ± 0.9	18.0 ± 0.4
Val	27.1 ± 0.9	24.6 ± 2.3	26.3 ± 0.7	25.2 ± 1.1
Met	8.9 ± 0.3	8.0 ± 0.7	8.3 ± 0.2	8.1 ± 0.4
Ile	25.9 ± 1.0	23.5 ± 1.9	24.6 ± 0.5	23.5 ± 0.9
Leu	38.8 ± 1.0	35.3 ± 3.0	36.3 ± 0.7	35.9 ± 1.0
Phe	115.4 ± 3.4	105.0 ± 8.0	110.4 ± 4.1	105.9 ± 3.1
Lys	41.2 ± 0.9	35.0 ± 3.2	37.3 ± 1.4	35.6 ± 1.0
His	9.6 ± 0.1	8.6 ± 0.7	9.4 ± 0.3	9.1 ± 0.3
Arg	29.4 ± 0.9	26.3 ± 2.6	28.0 ± 0.7	26.7 ± 0.8
Trp	6.2 ± 0.7	4.3 ± 0.3	5.8 ± 0.4	5.2 ± 0.5
Conditionally essential amino acids				
Tau	n.d.	n.d.	n.d.	n.d.
Glx*	62.0 ± 1.4	56.3 ± 4.8	59.8 ± 1.4	56.8 ± 1.4
Pro	33.4 ± 1.1	26.7 ± 1.8	31.3 ± 1.3	26.6 ± 0.6
Cys	3.8 ± 0.3	3.5 ± 0.3	4.1 ± 0.1	3.4 ± 0.2
Non-essential amino acids				
Asx*	39.2 ± 0.9	35.4 ± 3.3	37.0 ± 1.2	35.2 ± 0.9
Ser	21.9 ± 0.6	18.7 ± 2.1	21.8 ± 0.6	19.3 ± 0.6
Gly	22.7 ± 0.6	20.2 ± 2.0	21.6 ± 0.8	20.6 ± 0.8
Ala	25.1 ± 0.7	20.9 ± 2.2	23.5 ± 0.8	21.5 ± 0.5
Tyr	17.7 ± 0.8	14.4 ± 1.1	17.4 ± 0.8	15.1 ± 0.4
H-cys	n.d.	n.d.	n.d.	n.d.
Orn	n.d.	n.d.	n.d.	0.4 ± 0.1
Sum TAA	548.5 ± 13.7	484.3 ± 41.6	522.2 ± 16.4	490.4 ± 14.3

* Asx and Glx are the sum of Asp+ Asn and Glu+Gln respectively, as Asn and Gln are present in their acid forms after acid hydrolysis.

The TAA concentration was significantly lower in the enrichment medium A than in the other two enrichment media. Enrichment medium C had a higher TAA level than enrichment medium B. In the krill and calanus samples the largest variations in TAA were observed, krill incl. drip being the lowest (395.7 ± 15.8 mg/g DW) and calanus meal being the highest (653.8 ± 28.5 mg/g DW). Tryptophan and proline concentrations were relatively similar in all samples. Otherwise, the differences in the single amino acids reflected the TAA.

Table 13: Total amino acid concentrations in enrichment media A, B and C, krill incl. and excl. drip, calanus meal and freeze-dried calanus. Values are expressed as mean ± SD (n = 3) and in mg aa/g DW. n.d. = not detected

	mg aa/g DW						
	Medium A	***Medium B***	***Medium C***	***Krill excl. drip***	***Krill incl. drip***	***Calanus meal***	***Freeze-dried calanus***
Essential amino acids							
Thr	5.4 ± 0.2	15.2 ± 0.7	21.0 ± 0.3	18.7 ± 0.8	15.0 ± 0.8	24.6 ± 1.2	20.7 ± 0.6
Val	7.2 ± 0.5	23.0 ± 1.0	30.7 ± 0.4	23.6 ± 1.2	19.9 ± 1.0	34.8 ± 1.6	29.2 ± 0.6
Met	2.0 ± 0.2	5.8 ± 0.4	7.8 ± 0.1	12.0 ± 0.8	9.4 ± 0.5	14.1 ± 0.6	11.8 ± 0.1
Ile	5.3 ± 0.4	20.5 ± 1.2	20.4 ± 0.1	24.3 ± 1.0	19.6 ± 1.0	27.8 ± 1.0	23.7 ± 0.8
Leu	8.6 ± 0.5	35.3 ± 2.1	44.9 ± 0.3	34.1 ± 1.4	26.9 ± 1.7	44.8 ± 1.6	38.2 ± 1.2
Phe	25.0 ± 1.2	110.3 ± 4.9	117.1 ± 1.4	94.7 ± 4.6	74.5 ± 3.8	108.3 ± 6.4	91.1 ± 3.7
Lys	6.5 ± 0.2	30.6 ± 1.4	44.2 ± 0.3	34.3 ± 1.6	27.1 ± 1.8	45.4 ± 1.8	40.1 ± 1.0
His	2.4 ± 0.2	9.5 ± 0.6	10.0 ± 0.1	8.4 ± 0.5	6.5 ± 0.4	12.5 ± 0.3	10.8 ± 0.3
Arg	12.6 ± 0.7	35.1 ± 1.7	34.3 ± 0.3	25.9 ± 1.4	22.4 ± 1.1	45.6 ± 1.7	39.8 ± 1.0
Trp	1.8 ± 0.1	5.4 ± 0.2	8.4 ± 0.2	4.0 ± 0.2	4.1 ± 0.4	4.6 ± 0.2	4.7 ± 0.4
Conditionally essential amino acids							
Tau	n.d.	n.d.	n.d.	5.0 ± 0.1	9.2 ± 0.3	7.2 ± 0.3	7.1 ± 0.3
Glx*	32.2 ± 1.3	69.9 ± 3.7	58.3 ± 0.6	55.2 ± 2.2	44.4 ± 2.7	80.2 ± 3.5	69.0 ± 1.8
Pro	6.4 ± 0.5	21.7 ± 0.8	29.0 ± 0.5	24.4 ± 1.0	28.7 ± 0.1	28.3 ± 0.9	24.5 ± 0.9
Cys	n.d.	2.7 ± 0.2	1.2 ± 0.1	3.9 ± 0.3	2.7 ± 0.4	5.6 ± 0.5	5.0 ± 0.2
Non-essential amino acids							
Asx*	10.6 ± 0.7	37.7 ± 1.7	34.3 ± 0.2	36.0 ± 1.6	28.9 ± 1.5	45.0 ± 2.0	37.7 ± 0.8
Ser	5.3 ± 0.3	18.8 ± 0.9	17.6 ± 0.3	15.7 ± 0.8	12.0 ± 0.7	20.8 ± 1.1	17.5 ± 0.4
Gly	7.3 ± 0.3	19.1 ± 0.9	31.4 ± 0.3	21.6 ± 1.1	21.4 ± 0.3	48.5 ± 2.1	44.1 ± 1.4
Ala	9.1 ± 0.5	20.4 ± 1.1	46.6 ± 0.6	24.8 ± 1.2	20.4 ± 1.0	45.8 ± 3.3	38.8 ± 1.7
Tyr	3.3 ± 0.1	13.8 ± 0.7	15.5 ± 0.2	1.9 ± 0.3	0.6 ± 0.2	8.8 ± 0.7	12.7 ± 1.0
H-cys	1.3 ± 0.1	n.d.	0.5 ± 0.1	n.d.	n.d.	n.d.	n.d.
Orn	0.6 ± 0.0	0.2 ± 0.0	n.d.	0.8 ± 0.1	1.5 ± 0.1	n.d.	0.2 ± 0.0
Sum TAA	152.3 ± 6.6	495.6 ± 23.8	573.3 ± 4.2	470.2 ± 21.2	395.7 ± 15.8	653.8 ± 28.5	568.0 ± 12.3

* Asx and Glx are the sum of Asp+ Asn and Glu+Gln respectively, as Asn and Gln are present in their acidic forms after acid hydrolysis.

Minerals

Mineral analyses were performed at NIFES in Bergen. Zooplankton had higher concentrations of all minerals than all of the rotifer samples and the enrichment media. Krill had higher concentrations of all minerals, except zinc, than the calanus samples. The selenium concentration was similar in krill and calanus samples. Fluoride was only determined in the krill samples.

Table 14: Macrominerals and fluoride in zooplankton and the two intensive feeds, in natural rotifers and rotifers after enrichment with medium A, B and C, enrichment media A, B and C, krill and calanus. Values are expressed as an average of two parallels and in mg/kg dry material. n.a. = not analysed.

	mg/kg DW				
	Ca	***Mg***	***P***	***Fe***	***F***
Zooplankton	17060	10290	8870	1550	n.a.
Intensive feed A	2350	4960	11010	150	n.a.
Intensive feed B	3080	6280	9770	210	n.a.
Natural rotifers	2420	5690	10370	140	n.a.
Rotifers + A	2430	5120	10360	140	n.a.
Rotifers + B	2580	5620	9920	140	n.a.
Rotifers + C	1930	4600	10830	150	n.a.
Medium A	1190	3170	3720	110	n.a.
Medium B	740	1140	6080	290	n.a.
Medium C	610	3330	14760	240	n.a.
Krill excl. drip	14410	6360	11590	220	1010
Krill incl. drip	11270	6730	9500	80	930
Freeze-dried calanus	1940	2810	7630	40	n.a.
Calanus meal	2550	3430	7990	70	n.a.

Table 15: Microminerals in zooplankton and the two intensive feeds, in natural rotifers and rotifers after enrichment with medium A, B and C, enrichment media A, B and C, krill and calanus. Values are expressed as an average of two parallels and in mg/kg dry material. n.a. = not analysed.

	mg/kg DW			
	Mn	***Cu***	***Zn***	***Se***
Zooplankton	62	15	210	1.5
Intensive feed A	9.7	5.0	51	<0.1
Intensive feed B	8.6	4.8	55	<0.1
Natural rotifers	9.3	3.7	46	<0.1
Rotifers + A	9.7	4.9	47	<0.1
Rotifers + B	8.4	3.5	46	<0.1
Rotifers + C	10	3.8	44	<0.1
Medium A	38	19	86	0.44
Medium B	11	2.5	36	0.15
Medium C	32	3.7	10	<0.1
Krill excl. drip	2.8	29	61	2.5
Krill incl. drip	2.3	29	54	2.3
Freeze-dried calanus	2.1	5.5	99	2.9
Calanus meal	2.3	6.8	120	3.2

GENERAL DISCUSSION

The nutritional quality of feeds is important in first feeding of marine species. This affects the survival rate, growth and development which are key challenges in production of marine larvae.

The main objective of this study was to examine and evaluate the biochemical composition and nutritional quality of different live feeds commonly used in the production of cod larvae in North Norway today.

Methodological considerations

The Folch's method based on the extraction of lipids using chloroform/methanol for determination of total lipids was chosen over the more common Soxhlet's method which is based on ether extraction of the lipids. This method was preferred due to its superiority regarding extraction of polar lipids which were expected to be high in several of the raw materials.

One of the widely used methods for protein determination in foodstuffs is the Kjeldahl method for determination of total nitrogen. Values for total nitrogen are converted to protein concentrations using a nitrogen-to-protein conversion factor usually set at 6.25. This factor assumes that the nitrogen content of proteins is 16%. However, there are two major pitfalls using this conversion factor. First, the nitrogen content of proteins is highly dependent on the amino acid composition of the proteins. Nitrogen content of the different amino acids is not equal. Secondly, not all nitrogen in foodstuffs is linked to proteins and amino acids. Molecules like urea, ammonia, nucleic acids and nitrates etc. contain nitrogen (Mariotti *et al.*, 2008). Recommendations from the FAO (2003) suggest that protein in foods should be reported as the sum of individual amino acid residues plus free amino acids whenever possible. In this study both the Kjeldahl method and the analysis of total amino acids were included and in general the protein values were higher when measured by the Kjeldahl method than by the TAA method.

The production of algae and hence, zooplankton, depends on specific light and temperature conditions and has traditionally been limited to the early summer in Norway. However, in

2008 Lofilab AS decided to make an attempt at producing a larval stock in early autumn as well. This attempt was successful, but resulted in a slightly lower yield than in a "normal" production cycle. Zooplankton included in this study was collected in the autumn of 2008. The nutritional quality of zooplankton in terms of biochemical composition may differ throughout the year. Seasonal variations are reported for instance by van der Meeren et al.(2008).

Major findings and discussion

Zooplankton vs. intensive feeds

The zooplankton collected at Lofilab AS contained approximately 8% lipids and 32% ash. Protein concentrations were 56% when analysed by the Kjeldahl method and 48% when reported as TAA. The lipid and protein concentrations found in this study were comparable to the ones presented for copepods and zooplankton collected at semi-intensive rearing sites in van der Meeren *et al.* (2008), while the ash concentration was somewhat higher. Lipid and protein content in both of the two intensive feeds considered in this study were higher than in the zooplankton, lipids being around 12% and protein being 62-65.6%, while the ash content was lower, 16.7-20%. The two different intensive feeds were 50/50 mixtures of rotifers enriched with medium A and C (feed A) and medium B and C (feed B), respectively. Their composition reflected the composition of the "single factors", except for the protein measured by the Kjeldahl method, which was higher than anticipated.

Fatty acid composition of the zooplankton in this study was quite similar to that described by van der Meeren *et al.* (2008). The major differences were that the levels of EPA and DHA were lower and the SDA content was higher in this study. This could be explained by a high fraction of calanoids in the zooplankton sample analysed in this study, as calanoids are well known to contain large amounts of SDA (Kattner and Hagen, 1995). The fatty acid compositions of the two intensive feeds reflected the fatty acid composition of the rotifers they were mixed from. Both intensive feeds were very high in LA (21 and 22%) while they were lower in EPA (6.4 and 6.2%, in feed A and B respectively) and DHA (15.3 and 14.1%, in feed A and B respectively) than zooplankton. The requirements of EPA+DHA for some marine species listed by the NRC (1993) range from 0.5-2.0% of the diet. The sum of EPA+ DHA in the zooplankton was 38.5% of the total lipid fraction, corresponding to 3.1% of the diet. For the two intensive feeds the EPA+DHA were 21.7% (feed A) and 20.5% (feed B)

respectively, corresponding to 2.6% and 2.4% of the diets. In the zooplankton sample from Lofilab AS the fatty acid composition of the PL fraction showed a higher concentration of DHA than in the total lipid (TL) fraction, while SDA and EPA levels were lower in this fraction, giving a sum of EPA+DHA of 41%. The fatty acid composition of the PL fraction of the two intensive feeds was, however, even more shifted towards palmitic acid and LA than the TL fraction, giving a sum of EPA+DHA of 4.4% and 5.6% in intensive feed A and B respectively. Several studies have suggested that a high content of HUFAs in the PL fraction is advantageous as the absorption of PLs seems to be higher than, for instance, TAGs (Wold *et al.*, 2009, Wold *et al.*, 2007, Cahu *et al.*, 2003b). Although the PL fraction was higher in both of the intensive feeds than in the zooplankton sample (23% in feed A, 30% in feed B and 9% in zooplankton), the EPA+DHA originating from this fraction were lower.

The specific growth rate during the larval stage is high and hence a sufficient intake of amino acids, both essential and non-essential, is crucial. Acid hydrolysis of proteins to peptides and free amino acids is normally catalysed by the proteolytic enzyme pepsin, located in the stomach. However, most marine larvae lack a defined stomach and hence protein digestion at this stage is dependent on pinocytotic uptake, followed by intracellular digestion in the hindgut. The rate of such protein hydrolysis is slow (Watanabe, 1984) and does not satisfy the metabolic requirements of rapidly growing fish (Rønnestad *et al.*, 2003). Rust *et al.* (1993) showed that the absorption of FAAs is more efficient than peptides and whole proteins. Thus, the concentration of FAAs should be high in the larval diet. Pre-hydrolysis of proteins prior to feeding them to larvae was also shown to be effective when it came to increasing the absorption of amino acids (Tonheim *et al.*, 2005).

As shown in table 11, the concentration and composition of the TAA pool of zooplankton and the intensive feeds were relatively similar. The FAA fraction of the zooplankton in this study was 75 mg/g DW which is 15.7% of the TAA pool. This corresponds well to previously reported levels of FAAs in zooplankton (van der Meeren *et al.*, 2008). The values for the two intensive feeds were 51 mg FAA/g DW in feed A and 61 mg FAA/g DW in feed B, which correspond to 10.4% and 12.1% of the TAA pools of feed A and feed B respectively. The main difference between the amino acid compositions of zooplankton and the intensive feeds was that the zooplankton was high in taurine, while both intensive feeds were almost devoid of it. Taurine (2-aminoethanesulfonic acid) is regarded as an amino acid, although it lacks a carboxyl group. It is also regarded as an exclusively free amino acid, as it is not bound to

proteins. Taurine is one of the most abundant FAAs in animal tissues and apart from some algae, it is absent in plants. Taurine is suggested to be a participant in many physiological processes, among them osmoregulation, immunomodulation and bile salt formation (Bouckenooghe *et al.*, 2006). Along with its precursors, cysteine and methionine, it is also an important factor in controlling oxidative stress reactions (Métayer *et al.*, 2008). Taurine is synthesised from cysteine which is derived from methionine. This synthesis occurs mainly in the liver via the cysteine sulfinate pathway. Taurine synthesis is species dependent, being high in rodents and low in humans. Human infants lack the ability to synthesise taurine and as a consequence it has been added to infant formula. Seafood is normally high in taurine, but some studies have shown that the synthesis rate is low during the first stages of development even in fish and shellfish and that supplementation via the feed for larvae and juveniles may be necessary (Kim *et al.*, 2005, Litaay *et al.*, 2001). Supplementation of taurine in fish feed has been reported to give increased specific growth rate, increased feed intake and to lower the feed conversion factor. In addition, the bile-salt activated lipase activity was increased, indicating that a high level of taurine in the feed could improve lipid metabolism by increasing the utilisation of TAGs (Chatzifotis *et al.*, 2008).

Minerals reported in this study included the macrominerals calsium (Ca), magnesium (Mg), phosphorus (P) and iron (Fe) and the microminerals manganese (Mn), copper (Cu), zinc (Zn) and selenium (Se). Minerals have a wide variety of functions in fish. An overview of the main functions, dietary requirements for some marine fish and symptoms of deficiency extracted from the NRC (1993) is shown in table 16. As shown in table 14 and 15 all minerals reported, except P, were higher in zooplankton than in both of the intensive feeds. Levels of Ca, Mg, Fe and Mn were all higher in zooplankton in this study than reported in Hamre *et al.* (2008), while P, Zn and Se contents were lower. The Cu level was within the same range. According to the overview in table 16, both zooplankton and the two intensive feeds contained sufficient amounts of all of the minerals analysed, with one exception. Neither of the two intensive feeds contained detectable concentrations of Se.

Table 16: An overview of main functions, reported dietary requirements for some marine species and symptoms of deficiency of some minerals (NRC, 1993).

	Main functions in fish	***Dietary requirement for marine fish***	***Symptoms of deficiency***
Calsium	• Development and maintenance of skeletal system	• Mainly uptake from environment • 0.34% (3400 mg/kg) diet	• Deficiency not detected in seawater species
Phosphorus	• Development and maintenance of skeletal system • Constituent of nucleic acids and cell membranes • Involved in all energy-producing cellular reactions	• 0.5 – 0.8% (5000 – 8000 mg/kg) diet	• Poor growth, feed efficiency and bone mineralisation • Increased activity of gluconeogenetic enzymes in the liver • Deformed head • Deformed vertebrae
Magnesium	• Essential co-factor in enzymatic reactions • Required in skeletal tissue metabolism • Osmoregulation • Neuromuscular transmission	• Can be met either from the diet or the water • 0.04-0.06% (400-600 mg/kg) diet	• Anorexia • Reduced growth • Lethargy • Calcinosis of the kidneys • Vertebrae deformity
Iron	• Cellular respiratory process • Electron transfer • Bound to proteins and enzymes	• 30-120 mg/kg diet	• Anaemia • Pale liver
Manganese	• Co-factor for activation of metal-enzyme complexes	• 2.4-13 mg/kg diet	• Reduced growth • Skeletal abnormalities • Reduced enzymatic activity
Copper	• Constituent of enzymes	• 3-5 mg/kg diet	• Reduced enzymatic activity
Zinc	• Constituent of enzymes	• Can be met either from the diet or the water • 15-30 mg/kg diet	• Growth suppression • Mortality • Lens cataracts • Erosion of fins and skin • Short-body dwarfism
Selenium	• Constituent in glutathione peroxidise • Antioxidant	• 0.15-0.38 mg/kg diet	• Growth depression • Reduced enzymatic activity

Enrichment of rotifers

Natural rotifers are deficient or low in several essential nutrients such as HUFAs, some amino acids, vitamins and minerals (Conceicao *et al.*, 2009). Improvement of the chemical composition of the rotifers can be accomplished by different enrichment regimes. In this study the proximate composition of un-enriched rotifers was 10.7% lipids, 19.9% ash and 55% protein. Protein content was independent of the analytical method used. The lipid content corresponded to previously reported analyses of un-enriched rotifers, while the protein content was higher (Park *et al.*, 2006). None of the enrichment procedures had significant effect on the total protein content of the rotifers. The total lipid content increased as a result of enrichment with medium A and B, while the total lipid content decreased after enrichment with medium C. Ash content was lower after enrichment with medium A and C, while it remained unchanged after enrichment with medium B. The main objective of most enrichment protocols has traditionally been to increase the content of HUFAs to cover the dietary requirements for marine larvae. The EPA+DHA in the un-enriched rotifers in this study were 10.4% of the total lipid content. After enrichment with medium A and C, the EPA+DHA levels were 15.5 and 15.1% respectively. Enrichment with medium B however, did not have an impact on the EPA+DHA level.

The share of FAAs in the un-enriched rotifers was 261 mg FAA/g DW or 47%. This share remained relatively high after enrichment with medium B whilst the FAA fraction after enrichment with medium A and C was reduced by approximately 90-95% compared to the un-enriched rotifers. The decrease in the FAA was evenly distributed i.e. none of the amino acids were more influenced than others. The relatively large difference between the FAA fractions of rotifers enriched with the different media could be due to the fact that the samples, due to production procedures, were not collected on the same day. Variations regarding enrichment procedures, temperature, light conditions and sampling procedures could contribute to such differences.

The total amount of amino acids in natural rotifers was approximately the same as in the zooplankton and the intensive feeds. After enrichment with medium A and C the TAA level was slightly reduced, while it remained stable after enrichment with medium B. The lower TAA level after enrichment with medium A and C can be explained by the corresponding reduction of FAAs in these groups.

The enrichment process did not influence the mineral contents to a great extent. The concentrations of Ca, Mg and P were slightly higher in the un-enriched rotifers in this study than reported for un-enriched rotifers by Hamre *et al.* (2008) while the level of the other analysed minerals were approximately in the same range.

Apart from an improved fatty acid composition after enrichment with medium A and C, these enrichment protocols did not seem to be sufficiently effective in enhancing nutrients other than the HUFAs. The chemical composition of rotifers after enrichment is dependent upon the composition of the enrichment media and probably on some physical factors, such as the length of enrichment, water temperature and light conditions. The duration of the rotifer enrichment used in the protocol of TMY was 2 hours. Other enrichment regimes have been reported to last for up to 24 hours (Ferreira *et al.*, 2008, Ferreira *et al.*, 2009, Garcia *et al.*, 2008b, Park *et al.*, 2006). There is a possibility that the effects of enrichment could be improved by increasing the duration of enrichment.

Enrichment media vs. krill and calanus

The proximate composition of medium A given by the producer was 17% crude protein, 44% lipids, 9% ash, 0.3% fiber and 0.8% phosphorus. Medium B was reported to contain 43% protein, 30% lipids, 12% ash, 1.5% fiber and 2.5% phosphorus. As shown in table 1, results in this study corresponded well with the data presented by the producers. Protein expressed as TAA was more similar to the producers' data than protein calculated from the Kjeldahl analysis. Medium C was the freshwater algae *Chlorella sp.* enriched with DHA. Chemical composition of this alga has been reported as within the range 3-4% (Dunstan *et al.*, 1992) and 13% (Tokusoglu and Ünal, 2003) lipids, 43-47% protein (Janczyk *et al.*, 2007, Tokusoglu and Ünal, 2003) and 6% ash (Tokusoglu and Ünal, 2003). *Chlorella* in this study showed higher concentrations of lipids and proteins whilst ash content was approximately at the same level.

Saether and Mohr (1987) reported the chemical composition of northern krill to be 13-29% lipids, 41-46% protein and 13% ash, while data from Suontama *et al.*(2007) were 59.1% protein, 18.2% lipids and 10.4% ash. Both lipid (30%) and ash (23%) content in the krill in this study were higher than the previously mentioned studies. Protein (50% when reported as Kjeldahl protein and 47% when reported as TAA respectively) levels were similar. The

biochemical composition of freeze-dried calanus in this study was 20% lipids and 13% ash. Protein contents were 69% and 57% when measured by the Kjeldahl method and as TAA respectively. The calanus meal was lower in lipids (12.4%), slightly higher in ash (14.4%) and higher in protein (72% when reported as Kjeldahl protein and 65% when reported as TAA respectively) than the freeze-dried whole animal.

The fatty acid compositions of the three enrichment media were quite different. In medium A palmitic acid and DHA counted for 65% of the total lipids. Medium B was also rich in DHA which accounted for 28% of the total lipids. The levels of oleic acid and LA were also quite high with 11 and 12.6% respectively. High levels of these fatty acids indicate a high content of plant oils in the medium. Medium C was very rich in LA which comprised 21% of the total lipids. Both ALA and EPA concentrations were higher in medium C than in the other two media, while DHA concentration was lower at 17%. Freshwater algae *Chlorella* are normally deficient in HUFAs (Kobayashi *et al.*, 2008, Petkov and Garcia, 2007), but *Chlorella* used as enrichment medium at TMY is enriched with DHA and as a consequence of this, the contents of this fatty acid is quite high. The contents of the other HUFAs may also be affected due to the fatty acid metabolism of the algae. The PL fraction of all three enrichment media was approximately 5% of the total lipid. The levels of EPA and DHA were significantly lower in this fraction compared to the TL fraction for all three enrichment media, while oleic acid and LA levels were higher.

The fatty acid composition of the TL fraction of krill showed that the main HUFA in this species is EPA, which was approximately twice as high as the content of DHA (13% EPA and 6% DHA respectively). The content of oleic acid was very high. This is in accordance with what has been described in Antarctic krill (*E. superba*)(Gigliotti *et al.*, 2008) and in *M. norvegica* collected from the north-eastern Atlantic and Kattegat (Virtue *et al.*, 2000). The amount of PL in krill was approximately 26%. The main fatty acid was EPA in this fraction as well as in the TL fraction. Both EPA and DHA were twice as high in the PL fraction as in the TL fraction, whilst the amount of oleic acid was almost half of that in the TL fraction (9-12% and 20% in the PL fraction and the TL fraction respectively). The main HUFAs in the TL fractions of the calanus samples were SDA (15-16%) followed by EPA (10%) and DHA (10-11%). The PL fractions of the calanus samples were small at 1.8-2.6%. In the PL fraction however, DHA was dominant at 23% while SDA was reduced to 3%. EPA was slightly reduced to 7.5-9%.

None of the enrichment media were high in FAAs (14.8mg/g DW in medium A, 4.5 mg/g DW in medium B and 15.3 mg/g DW in medium C). Due to the low TAA level in medium A the relative fraction of FAAs was almost 10%. In medium B and C the FAA fractions were 0.9% and 2.7%, respectively. The FAA fractions in the krill samples were 24% and 10.7% in krill with and without drip, respectively. The loss of small water-soluble molecules such as taurine, glycine and alanine, in thaw drip is well described (Larsen, 2007). The loss of FAAs was approximately 40-50% and was evenly distributed. TAA levels in krill ex. drip were higher than the TAA levels in krill incl. drip and the level of each amino acid reflected this relationship, except for taurine. The FAA concentration of calanus meal and freeze-dried whole calanus were 94.6 mg/g DW and 98.3 mg/g DW respectively. The relative FAA fractions were 14.5% and 17.3% respectively due to the higher TAA concentration in the calanus meal. The composition of the FAA pool was quite similar between the two calanus samples.

The mineral composition varied greatly between the three enrichment media, but medium A was generally richer in minerals except phosphorus and iron. Phosphorus was most abundant in medium C, whilst media B and C were quite similar in iron content. The only mineral reported in the ingredient list of the commercial enrichment media was P (0.8% in medium A and 2.5% in medium B, respectively). Results from these analyses showed lower contents in both media, 3720 mg/kg DW (0.4%) in medium A and 6080 mg/ kg DW (0.6%) in medium B. Krill was richer than all enrichment media in Ca, Mg, Cu and Se while it was poorer in Mn. Phosphorus was higher in medium C than in the krill samples whilst Fe and Zn were approximately in the same range as in the enrichment media. Calanus was generally lower in minerals than krill except for Zn. Levels of Mn and Se were approximately similar. Fluorine was analysed in the krill samples, because previous studies have reported a fluoride accumulation in fish bones and scales in fish fed krill-based diets when compared to fish meal diets (Yoshitomi *et al.*, 2006, Grave, 1981). The krill in this study had a higher content of fluorine than previously reported for Atlantic krill (Moren *et al.*, 2007).

Comparing the commercial enrichment media to krill and calanus, the most obvious difference is the taurine concentration, which is high in both krill and calanus, while the commercial enrichment media are devoid of it. Both krill and calanus contained high levels of HUFAs, especially in their PL fractions.

A possible way of enhancing the efficiency of rotifer enrichment could be to design an enrichment medium with a biochemical composition more similar to natural zooplankton than the existing enrichment media. The results from this study showed that both krill and calanus were more similar to zooplankton than the commercial enrichment media and hence, both of them could be good candidates for a new and improved enrichment medium. The high fluorine content of krill is a possible drawback when it comes to utilisation of this copepod. Several studies have been performed in order to determine the retention of fluoride in fish fed diets containing different levels of krill meal. Julshamn *et al.* (2004) found that there were no differences in the retention of fluoride in bone or muscle in Atlantic salmon fed diets containing between 0 and 30% krill meal. Moren *et al.* (2007) found no significant differences of fluoride content in bone or muscle in Atlantic salmon or Atlantic halibut fed diets containing from 0 to 60% krill meal. Yoshitomi *et al.* (2006) however, found that an increased content of krill meal in the diets of rainbow trout led to an increased level of fluoride in vertebral bone, but this effect was not detected in muscle.

CONCLUSION AND FURTHER WORK

When taking all the analyses performed in this study into account the nutritional quality of zooplankton is superior to that of the intensive feeds. The main constituents used to define the nutritional quality of fish feed are the contents of HUFAs, FAAs in general and especially taurine, in addition to some minerals. Although the intensive feeds contained sufficient amounts of HUFAs according to the estimated requirements, the levels were still lower than in zooplankton. The relative fraction of FAAs was also lower in the intensive feeds than in zooplankton. While zooplankton was rich in taurine, the intensive feeds were almost devoid of it. With the exception of phosphorus all of the minerals analysed were also lower in the intensive feeds than in the zooplankton. These differences in biochemical composition between zooplankton and the intensive feeds could, either separately or in combination, be a part of an explanation of the increased growth when feeding on zooplankton at Lofilab AS.

The enrichment process seemed to be sub-optimal using the current enrichment media, as only the EPA and DHA content was affected and only in two out of three enrichment protocols. None of the other parameters analysed was affected. Therefore, there seems to be a great potential for improvement of the enrichment media. The most important goal is to design an enrichment medium that is more similar to natural feed for marine larvae. The biochemical compositions of krill and calanus were more similar to zooplankton, as the content of taurine and EPA+DHA was higher than in the commercial enrichment media, thus supporting the idea of utilising these marine resources as new and improved enrichment media.

Further work

In order to rule out random errors due to small sample sizes, an expanded trial with multiple sample collections during complete production cycles, both in the semi-intensive and the intensive rearing sites, would be interesting. Documentation of the growth, development and biochemical composition of the larvae at corresponding stages would also be of interest. Another suggestion of further work to a follow-up of this study could be to make krill and calanus preparations suitable as enrichment media and thus design intensive feed more applicable to marine larvae.

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