Challenges related to delivery of water soluble nutrients to marine fish larvae

Evaluation of changes in nutritional quality due to production process and leaching from larval diets -with emphasis on protein quality

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Dissertation for the degree philosophiae doctor (PhD)

University of Bergen, Norway

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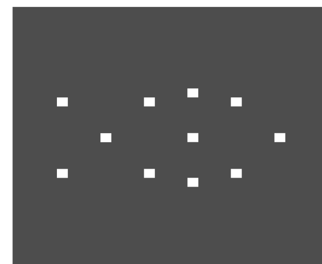

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Preface and Acknowledgements

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List of papers

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Paper II: **Andreas Nordgreen, Sigurd Tonheim and Kristin Hamre (submitted).** Effect of heat treatment and leaching on the protein quality of fish larval feed with increasing concentration of hydrolyzed protein. *Aquaculture nutrition*.

Paper III: **Sigurd Tonheim, Andreas Nordgreen, Ingmar Høgøy, Kristin Hamre and Ivar Rønnestad (2007).** *In vitro* digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and ingredients. *Aquaculture* 262, 426-435

Paper IV: **Andreas Nordgreen, Kristin Hamre and Chris Langdon (accepted).** Development of lipid microbeads for delivery of lipid and water-soluble materials to *Artemia*. *Aquaculture*.

In the following chapters these four papers are referred to in the text by their roman numerals.

List of abbreviations

AA	Amino acids
ARA	Arachidonic acid
D	Dalton
DHA	Docosohexaenoic acid
dw	Dry weight
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAA	Free amino acids
LSB	Lipid spray beads
LWC	Lipid walled capsules
MP	Marine phospholipids
N	Nitrogen
OTC	Oxytetracycline
PL	Phospholipid
SDS	Sodium dodecyl sulphate
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

Abstract

Marine fish larvae fed formulated diets have suppressed growth and survival compared to larvae fed live feed for the first weeks. Live feed is successfully used in the aquaculture industry, although there are difficulties delivering controllable concentrations of several nutritional compounds. In research, the use of formulated diets is therefore essential to accomplish proper nutritional dose response studies. The focus of this work was to study the properties of a protein cross-linked diet and a heat coagulated diet used for nutritional studies of marine fish larvae. The ability to deliver water soluble nutrients and changes in protein quality due to production processes and exposure to leaching was emphasized. A pancreatic protein *in vitro* digestibility method, simulating stomachless fish larvae, was used to investigate the digestibility of various live feeds and feed ingredients. The same *in vitro* model was also used to study the effect of the production processes and the effect of increased inclusion of hydrolyzed protein in compound diets.

Both diets showed substantial changes in protein quality due to the production process and exposure to leaching. The protein cross-linked capsules had a nearly complete loss of water-soluble nitrogen (N) during cross-linking and the following washing steps and more than 90 % loss of other water-soluble micronutrients. The protein cross-linking led to a 25 % reduction in *in vitro* protein digestibility. A large fraction of the soluble N in the feed ingredients was made insoluble by heat denaturation during production of the heat coagulated diet, but the concentrations of peptides and free amino acids (FAA) were not influenced. However, after exposure to leaching for 6 min most of the soluble N fraction was lost and there were no significant difference in concentrations of soluble N between diets with increasing concentration of hydrolyzed protein ranging from 0 % to 45 % of total protein. There were no significant differences in *in vitro* digestibility between the four diets with

increasing concentration of hydrolyzed protein. However, the leached diets showed significantly reduced digestibility compared to the diets that had not been exposed to leaching. In conclusion, neither the protein cross-linked nor the heat coagulated diet may be suitable for the delivery of water-soluble nutrients to marine fish larvae.

The protein *in vitro* digestibility of the protein cross-linked diet, heat coagulated diets and a commercial larval diet (53 – 73 %) were lower than frozen live feed (84 – 87 %). The digestibility of the soluble N fractions was similar for the marine meals and the live feed and higher than the respective insoluble protein fractions. However, the live feed contained 54-67 % soluble N in comparison to the marine meals that only contained 11-17 % soluble N.

In the search for other possible diets to deliver soluble nutrients, lipid spray beads (LSB) were investigated. LSB, as part of complex particles, has been an interesting candidate for delivering soluble nutrients with a high retention efficiency, although the fatty acid profile of the LSB have made them inappropriate when delivered in large quantities. LSB with an improved fatty acid profile were developed by inclusion of high concentrations of phospholipids. Due to the hydrophilic behavior of phospholipids, the LSB dispersed in water and could therefore be used to deliver water soluble micronutrients to live feed. The riboflavin content of *Artemia* was increased from 55 ± 0.6 mg kg^{-1} (dw) to 329 ± 62 mg kg^{-1} (dw) after 1 h enrichment. There is still a severe leaching rate of highly soluble nutrients and the LSB might therefore not be able to deliver nutrients needed in large quantities such as FAA and peptides. However, for nutrients needed in small quantities, the LSB seems to be a promising tool.

1 Introduction

1.1 Biological background

Although there can be large differences in reproductive strategy between the different fish species there are still many similarities. One of the main choices of reproduction strategy is the choice between producing a large number of offspring with a high mortality or to put more energy into fewer offspring and increasing the chances for each individual offspring to reach maturity. For instance Atlantic Cod (*Gadus morhua*) spawn 2.5 – 9 million eggs with a diameter of 1.2 - 1.6 mm (Scott and Scott, 1988), while Atlantic salmon (*Salmo salar*) spawn only 10 000 - 15 000 eggs which are approximately 100 times bigger (5-7 mm) (Pethon, 1998). Although species as A. salmon, rainbow trout (*Oncorhynchus mykiss*) and cat fish (*Anarhichas minor*) produce large eggs, most fish species of commercial interest produce large number of small pelagic eggs.

A natural consequence of producing small eggs is the hatching of small fish larvae which are "the smallest independently functional vertebrates" (Segner *et al*., 1994). Fish larvae are sensitive to mechanical disturbance, have a small mouth opening which reduce the choice of food at exogenous feeding (Marte, 2003) and poor swimming abilities with most species being nearly planktonic at an early stage (Moser *et al*., 1984; Leis, 2006). Most of these species also have a digestive system that is not fully differentiated and lack a functional stomach at start of exogenous feeding. In addition, these larvae have a low supply of endogenous nutrients and therefore a need for early exogenous feeding. The salmon fry for example, have a yolk sack that lasts for 5-6 weeks (Pethon, 1985) compared to cod that has to start exogenous feeding within 4-5 days after hatching. So instead of being provided with high quality endogenous nutrients the larvae have to ingest prey to go through a rapid and complex biological development.

1.2 The digestive system of fish larvae

Dependent on the development of the digestive system, teleosts can roughly be divided into two groups called altrical and precocial (Rønnestad and Hamre, 2001). Precocial species have a digestive system that is differentiated into a fully functional system, with all organs functional as found in adult fish, before start of exogenous feeding. These species can either have a long embryonic phase where the development occurs in the egg or a long yolk sack stage (free embryo), also referred to as intermediated development (Balon, 1999). Altrical fish larvae have a digestive system that is not fully differentiated at start of exogenous feeding and lack several of the functional organs found at later stages. The gastrointestinal tract is separated into four regions, the oesophagus, foregut, midgut and hindgut . The altricial larvae lack a functional sphincter that can close off the stomach region (Rønnestad *et al.*, 2000) and have not developed gastric glands producing hydrochloric acid (HCl) and pepsinogen (Luizi *et al.*, 1999; Kjørsvik *et al.*, 2004). They therefore lack an acidic environment which denatures and unfold proteins and leaves the peptide bonds more open for further enzymatic cleavage by pancreatic enzymes. In addition there is secretion of pepsinogen into the stomach which is activated by HCl into its active form called pepsin. Pepsin pre-digests the protein making it more soluble and accessible for further pancreatic digestion. *In vitro* digestion with digestive extracts from the stomach of sea bream (*Sparus aurata*) indicated that 35 % of the protein in fish meal and 20 % of the protein casein was digested (Fernandez-Diaz *et al.*, 2001). The development of a fully functional stomach occurs during metamorphosis for most species (Luizi *et al.*, 1999; Ribeiro *et al.*, 1999a; Elbal *et al.*, 2004). However, there are species which have shown a further development after metamorphosis (Luizi *et al.*, 1999; Ribeiro *et al.*, 1999b; Kjørsvik *et al.*, 2004).

So from start of exogenous feeding until metamorphosis, the fish larvae's digestion is fully dependent on digestion performed by pancreatic enzymes and brush border bond enzymes (Kjørsvik, 2004; Hoehne-Reitan and Kjørsvik, 2002; Kvåle et. al., 2007a). Although most pancreatic enzymes such as trypsin, chymotrypsin, lipases and amylases are present at first feeding (Ribeiro *et al*., 1999b; Krogdahl, 2001; Cara *et al*., 2003; Kjørsvik *et al*., 2004), the enzyme activities are low for most fish larvae (Cousin *et al*., 1987; Munillamoran and Stark 1989; Gawlicka *et al*., 2000; Hoehne-Reitan *et al*., 2001; Kvåle *et al*., 2007a). However, there is an improved capacity in both pancreatic digestion (Nolting *et al*., 1999; Cahu *et al*., 2004) and digestion performed by the brush border-membrane bound enzymes as the digestive tract matures (Kvåle *et al*., 2007a). The capacity for protein digestion may to some extent also depend on feed quantity and quality (Zambonino Infante and Cahu 1994; Peres *et al*., 1996). Sole (*Solea senegalensis*) larvae showed a more than ten fold increase in trypsin activity from day 2 after hatching to day 15 after hatching (Ribeiro *et al*., 1999b) and both cod and halibut have shown a three to five fold increase in the specific activity of brush border-membrane bound proteases towards the end of metamorphosis (Kvåle *et al*., 2007a). This emphasizes that the low protein digestion capacity at early larval stages is not only influenced by the lack of a functional stomach, but also significantly affected by an immature pancreatic and intestinal digestion. As suggested by Tonheim (2004), there might be a larger variability in protein digestibility between different protein sources in altrical larvae than in precocial larvae, because the protein is not denaturized and the intestinal proteolytic enzymes have to attack natively folded proteins which have different molecule forms.

Marine fish larvae have shown a pinocytotic absorption and digestion of macro-proteins by enterocytes in the hind gut (Watanabe 1982; 1984; Kishida *et al*., 1998), and the ability for intracellular digestion is reduced at the end of metamorphosis for both smelt (Hypomesus

olidus) (Watanabe, 1982) and halibut (Luizi *et al*., 1999). It has therefore been suggested that this pinocytotic uptake compensates for the reduced ability to digest protein extracellularly (Govoni *et al*., 1986). However, intracellular digestion of horseradish peroxidase took from 10 to 24 h in smelt (Watanabe, 1982) and lipovitellin from *Artemia* seemed to be intracellularly digested by Striped bass (*Morone saxatilis*) within 4 to 12 h (Kishida *et al*., 1998). Due to this slow intracellular digestion it has been questioned if the pinocytotic uptake of proteins contributes significantly to protein nutrition in fish larvae (Rønnestad *et al*., 2003).

The absence of a stomach also reduces the capability to store food and the larva is therefore more dependent on continuous feeding than adult fish. Several species have reduced ingestion rate after metamorphosis due to increased conversion efficiency and storage capacity of the feed (Kjørsvik 2004). It is suggested that the sphincter between the midgut and hindgut has a stomach like function and releases controlled amounts of food for final digestion in the hindgut (Rønnestad *et al*., 2003). However, there is a rapid passage of feed through the digestive system of marine fish larvae compared to adult fish (see review by Gonovi *et. al*.1986). The evacuation time is affected by ingestion rate (Laurence 1977; Canino and Bailey, 1995) and continuously feeding larvae have more rapid evacuation than portion fed larvae (Canino and Bailey 1995). An evacuation time of more than 50 % of ingested protein within 4 h after feeding a single meal to Atlantic halibut has been reported (Tonheim *et al*., 2005).The rapid evacuation time emphasizes the importance of strict demands of the nutritional availability and quality of the ingested feed.

Altrical species can as juveniles and adults either be strictly herbivore or carnivore, with great diversity in anatomy and physiology of the digestive system. However, independent of the adoption of the digestive system for adult fish, most altrical species are strictly carnivore and

feed on zooplankton with similar nutritional quality. It is therefore suggested that they have similar nutritional requirements until metamorphosis and a fully developed digestive system is accomplished. In comparison to precocial species that may be fed compound diets from first-feeding, most altrical species are dependent on live feed such as zooplankton, rotifers or *Artemia* for a shorter or longer period to get good growth and survival (see review by Kolkovski, 2001). The period that the use of live feed is necessary is highly species dependent and correlates with time for maturation of the digestive system (Hoehne-Reitan *et al*., 2001; Yùfera *et al*., 2004).

1.3 Replacement of live feed with compound diets

Copepods are the main natural food for marine fish larvae and have shown to give increased growth, survival and a reduction in malformations compared to the use of rotifers (*Brachionus sp*) (Toledo *et al*., 1999; Rajkumar and Vasagam 2006) and *Artemia* (Naess *et al*., 1995; McEvoy *et al*., 1998; Naess and Lie, 1998; Shields *et al*., 1999). However, copepods may not be cultured in large quantities. They have to be filtered from fertilized lagoons in order to be harvested in sufficient amounts, and the production is strictly season dependent. Harvesting of copepods is therefore not optional for hatcheries that have a year-round production and rotifers and *Artemia* are therefore used by most hatcheries. Rotifers and *Artemia* are deficient in several essential nutrients compared to the natural food for marine fish larvae (Van der Meeren 2003) (table 1), which is suggested to be the reason for reduced larval performance. However, it is to some extent possible to change/improve the nutritional content by feeding/enriching the live feed with nutrients found to be deficient (Rainuzzo *et al*., 1994; Merchie *et al*., 1995a; Harel *et al*., 1999; Dhert *et al*., 2001; Monroig *et al*., 2003; Moren *et al*., 2006b). An extensive amount of work has been undertaken to develop enrichment protocols to change/improve the nutritional content of rotifers and *Artemia* (Watanabe *et al*.,

1983; Nichols *et al*., 1989; Rainuzzo *et al*., 1989; Olsen *et al*., 1993). This has led to improved larval performance for most investigated species (Olivotto *et al*., 2006; Park *et al*., 2006).

Table 1: Concentration of selected nutrients in different live feed (Van der Meeren, 2003)

	Polar lipids	**EPA+DHA**	**FAA**	**Taurine**	**Astaxanthine**
	% of tot lipid	G kg^{-1} (dw)	g kg^{-1} (dw)	g kg^{-1} (dw)	µg g^{-1} (dw)
Copepods	57.1	51.8	56.1	10.5	627
Rotifers	39.8	19.4	16.6	0.4	24
Artemia 1	15.4	18.4	33.7	8.2	ND
Artemia 2	17.4	29.2	27.5	7.3	ND

The rotifers (Brachionus plicatilis) were enriched with Rotimac and Isochrysis sp.

Artemia 1 was enriched with DHA-Selco for 1 day.

Artemia 2 was 3 days old and enriched with DHA- Selco and Algamac 2000.

However, the use of live feed for nutritional studies has major drawbacks due to limited possibilities in delivering different and controllable concentration of nutrients. Although it is possible to increase and to some degree control the concentration of essential fatty acids in both *Artemia* (Takeuchi *et al*., 1992; McEvoy *et al*., 1996; Evjemo and Olsen 1997; Narciso *et al*., 1999; Sorgeloos *et al*., 2001) and rotifers (Dhert *et al*., 2001; Castell *et al*., 2003), there are severe problems with rapid lipid metabolism and, thereby, an uncontrollable change in the nutrients investigated (Olsen *et al*., 1993; Evjemo *et al*., 1997). Although live feed has a significant higher concentration of polar lipids than reported used in most compound diets, it has to be stressed that *Artemi*a (17.8 % of tot lipid) and rotifers (39.8 % of tot lipid) have significantly lower levels of polar lipids than copepods (57.1 % of tot lipid) (Meeren, 2003). Attempts to enrich *Artemi*a with PL and thereby change the lipid class composition significantly have not been successful (Rainuzzo *et al*., 1994; Harel *et al*., 1999). Except for vitamin C (Merchie *et al*., 1995ab, 1996ab, 1997ab), the enrichment of *Artemia* and rotifers with water-soluble micronutrients or its fat soluble derivate is not thoroughly investigated.

The enrichment is commonly achieved by either direct addition of micronutrients to the culture water or by adding the soluble nutrients to lipid emulsions fed to live feed cultures. The current enrichment methods may not be optimal due to low uptake efficiencies and a large amount of micronutrients is therefore required (Hamre, unpublished results). However, for scientific purposes the enrichment by addition of selenium to the water has increased the content 4 fold in rotifers (Mollan and Hamre, unpublished). The crude protein amino acid (AA) profile of rotifers (Srivastava *et al*., 2006) and *Artemia* (Aragao *et al*., 2004b) is difficult to modify, but the concentration of free amino acids (FAA) and the AA profile of the FAA is significantly affected by enrichment media (Aragao *et al*., 2004b). The FAA concentration of rotifers constitute for less than 6 % of the crude protein content (Aragao *et al*., 2004a; Srivastava *et al*., 2006) and a change in the FAA profile would most likely not affect the crude AA profile. It is also questionable if changes in both FAA concentration and AA profile can be controlled to such an extent that dose response studies can be accomplished.

The replacement of live feed with formulated feeds prior to onset of gastric development has been thoroughly investigated during the last 30 years (see review by Teshima *et al.,* 2000; Langdon, 2003), but most fish larvae still need live feed for the first period. However, there has been an increased success in early weaning of fish larvae (Cahu *et al*., 1998; Baskerville-Bridges and Kling 2000a; 2000b; Hoehne-Reitan *et al*., 2001; Cahu *et al*., 2003; Cahu *et al*., 2004). Especially sea bass was fed solely on a formulated diet with specific growth rate and survival rates of 7-11% and 35-70 (%), respectively (Cahu *et al.,* 1998; 2003; 2004). Although solely feeding of compound diets from start feeding leads to reduced larval performance, co-feeding from an early stage may improve the larvae nutrition with increased growth and survival compared to feeding either type of feed (Champigneulle 1988; Walford *et al*., 1991b; Salhi *et al*., 1994; Qin *et al*., 1997). As suggested by Pedersen and Hjelmeland

(1988) and Le Ruyet *et al*. (1993) this could be due to the additional enzymes from the live feed or more likely due to the additional supply of crude energy and essential nutrients not delivered in sufficient concentration with rotifers and *Artemi* However, the most important benefit of co-feeding is the improved weaning success and shortening of the weaning period (se review by Rosenlund *et al*., 1997).

Commercially, the choice between formulated feed versus live feed is related to financial factors such as growth rate, survival, and rate of deformities. For scientific purposes, in addition to good growth and survival, it is important to have a good control over the nutritional composition of the feed and have the opportunity to manipulate the composition in a controlled manner, so that proper dose response studies may be carried out. It is difficult to use live feed for dose response studies and formulated diets are therefore required. To accomplish proper dose response studies it is essential to have compound diets that deliver controllable concentration and quality of the nutrients we want to investigate. To increase the nutritional knowledge of marine fish larvae at early stages it is of great importance to establish procedures to estimate fish larvae requirements for different nutrients. It therefore have to be an increased open source knowledge around the problems involved in the production and usage of formulated feed for nutritional studies of marine fish larvae.

1.4 Effect of formulated feeds on the performance of marine fish larvae

Stomachless fish larvae fed formulated diets have suppressed growth and survival compared to larvae fed live feed (Cahu and Zambonino Infante, 2001; Kolkovski, 2001). It is important to emphasis that although they lack a functional stomach (Govoni *et al*., 1986; Pittman *et al*., 1990; Segner *et al*., 1994), have a low digestive enzyme activity (Cousin *et al*., 1987; Kvåle *et al*, 2007a) and a low ability to digest complex protein (Tonheim *et al*., 2004), marine fish

larvae have the potential for a very rapid growth rate (Houde, 1989; Kamler *et al*., 1992; Conceicao *et al*., 1997; Otterlei *et al*., 1999). Growth rates exceeding 25 % day^{-1} have been reported for cod (*Gadus morhua*) (Otterlei *et al*., 1999) and indicates an efficient utilization of the nutrients in live prey. This early and rapid growth is mainly protein deposition (Houlihan *et al*., 1995) and both high quality and quantity dietary protein is therefore required.

In addition to a high protein deposition, a high proportion of the energy needed for metabolic energy is derived from AA; 60 % of the energy metabolized by Atlantic halibut during the first month of exogenous feeding came from AA (Rønnestad and Naas, 1993). Tube feeding of larval Atlantic Halibut (*Hippoglossus hippoglossus*) showed that there was a higher absorption efficiency of hydrolyzed protein compared to intact soluble protein (Tonheim *et al*., 2005). Marine fish larvae have an increased capability to digest complex protein towards the end of metamorphosis when the digestive tract is more differentiated. This seems to coincide with the increased acceptance of compound diets which usually contain complex insoluble protein. Although the developmental stages of the digestive system occurs in the same order (Kjørsvik *et al*., 2004), there can be large differences in time of development between species.

It is suggested that the high concentration of water soluble N 54 ± 2 %, 56.9 ± 0.8 % and 54 ± 0.4 % of total N in *Artemia*, Rotifers and copepods respectively (Carvalho *et al*., 2003; Srivastava *et al*., 2006; Tonheim *et al*., 2007) with high concentration of low molecular weight N (Carvalho *et al*., 2003; Tonheim *et al*., 2007), may explain the high growth and survival of larvae fed live feed compared to formulated feed, which usually have much lower concentrations of soluble N. Carvalho *et al*., (2003) showed that nearly 90 % of the soluble N in rotifers and *Artemia* was less than 500 D in size. To mimic the N pool of live feed and

obtain improved growth and survival, FAA or hydrolyzed protein has been added to formulate feeds for early stage larvae (Carvalho *et al*., 1997; Cahu *et al*., 1999; Kvåle *et al*., 2002; Carvalho *et al*., 2004). Although the results are varying and not consistent, inclusion of small concentrations of hydrolyzed protein in the diets usually improves survival and growth (Zambonino Infante *et al*., 1997; Cahu *et al*., 1999; Carvalho *et al*., 2004), but inclusion of higher concentrations have led to negative performance of several species (*Sparus aurata* L., (Kolkovski and Tandler 2000); *Cyprinus carpio*, (Carvalho *et al*., 1997; Carvalho *et al*., 2004); *Dicentrarchus labrax*, (Cahu *et al*., 1999); *Hippoglossus hippoglossus* (Kvåle *et al*., 2002, 2007b)). However, it is a contradiction that formulated diets with high levels of hydrolysed protein, which actually is lower than the levels of low molecular weight nitrogenous compounds found in live feed, may lead to decreased larval performance. Supplementation of 40 % pepsin hydrolysed protein to a heat coagulated diet improved survival rates in cod (*Gadus morhua*), compared to lower levels of supplementation (Kvåle *et al*., 2007b). In contradiction, Atlantic Halibut (*Hippoglossus hippoglossus*) fed the same diets had a decline in survival rate with increase in supplementation of hydrolyzed protein (Kvåle *et al*., 2002, 2007b). It is suggested that this difference in survival is due to halibut's slow feeding behavior (Stoss *et al*., 2004), and therefore increased loss of soluble nitrogenous compounds proportional to the inclusion level of hydrolyzed protein. As shown for marine fish larvae, the larvae of common carp have an improved survival with increased concentration of soluble and hydrolyzed protein in the diet (Carvalho *et al*., 2004). However two weeks after start of exogenous feeding there was no beneficial effect, indicating an improved pancreatic protein digestion capacity as shown for marine species (Tonheim *et al*., 2004; 2005).

There is little available knowledge concerning the availability of the lipid delivered through compound diets compared to live feed. Except for problems regarding per-oxidation (Lopez Albors *et al*., 1995; Tocher *et al*., 2002, 2003; Fontagne *et al*., 2006), no quality changes during the production process or storage has been reported for compound diets for marine fish larvae. Loss of lipid during feeding has not been reported and is presumed to not be a problem. However, change in lipid quality during production of compound diets and changes in digestibility dependent on lipid source needs further investigation. As discussed by Evjemo *et al*. (1997) and reviewed by Coutteau *et al*., (1997) phospholipids (PL) have beneficial affect on several species of marine fish larvae. Soya lecithin is widely used in formulated diets in small amounts and has shown to give good results for several species of marine fish larvae (Kanazawa *et al*., 1983ab) However, if the PL content is considered to be of the similar amount as found in copepods, the fatty acid profile of the PL has to be considered. Turbot larvae fed with high concentration of Soya PL had a significant lower growth rate than the marine phospholipids (MP) fed larvae and appeared to have swollen enterocytes (Leifson *et al*., 2003b). No reports have been found on problems delivering formulated diets with high concentration of PL. Due to the difficulties with modifying the PL content of *Artemia* and rotifers, the use of formulated feed is therefore required to further investigate effect of increasing the PL concentration.

Little is known about the recommended concentrations of micronutrients for the different species at the larval stage. The lack of knowledge is both due to the focus on lipid and protein nutrition and the technical difficulties in enrichment of live feed with water soluble nutrients. As for FAA and peptides, an extensively leaching of all water soluble micronutrients would probably occur in a short period of time (Marchetti *et al*., 1999; Langdon 2003; Yùfera *et al*., 2003), but the loss of micronutrients from different formulated diets has not been investigated

thoroughly. Since all vitamins are per definition essential, deficiency in only one of the vitamins may have severe effects. A major difference between micronutrients and macronutrients are the low concentrations needed. It will be difficult to compensate for a 60 % loss of nitrogenous compounds during feeding, while micronutrients may be compensated for by including a larger concentration in the diet.

Marine fish larvae have the ability to digest carbohydrates from an early stage (Kjørsvik *et al*., 2004), but there are indications that the enzyme activity may be most evident the first days of start-feeding when the larvae ingest micro algae (Kjørsvik *et al*., 2004). However, the low concentration of glycogen in copepods (0.5 % dw) (Hamre *et al*., 2002) suggests that the need for carbohydrate is very low. This is confirmed by (Hjertnes, 1991; Hamre *et al*., 2003) showing a decreased growth rate in halibut larvae using 7.5 % extruded wheat (Hamre *et al*., 2003). Carbohydrates are used as binders in several formulated diets and should therefore be considered.

Although dependent on parameters such as size, texture, taste and binder (Carr *et al*., 1996; Kolkovski *et al*., 1997a; Guthrie *et al*., 2000), most investigated fish larvae ingest formulated diets from first feeding (Guthrie *et al*., 2000; Clack, 2006; Seiliez *et al*., 2006). Without co-feeding of live feed, the larvae will show depressed growth or death. Juvenile flounder (*Paralichthys Olivaceus*) (Seikai *et al*., 1997), Larval red sea bream (Teshima *et al*., 2000), blue spotted goby (*Asterropteryx semipunctata*) (Clack, 2006) have been reported to eat more formulated feed (dw) than live feed and still have a suppressed growth compared to fish fed live feed. Red sea bream larvae at day 25 have shown intake of a micro bound diet (MBD) to be 10-20 times higher on a dry weight basis compared to rotifers and 6 - 8 times compared to *Artemia* at day 34 (Teshima *et al*., 2000). Dependent on the water content, formulated feed

can withhold up to 25 times the gross energy content on a wet weight (ww) basis compared to live feed (Rosenlund *et al*., 1997). Considering the high energy content and the reports on high ingestion rates, it is unlikely that the depressed growth and survival of larvae fed formulated diets, compared to larvae fed live feed, is due to lack of crude energy in the diet. Rather, the difference is probably a question of nutrient quality and availability.

1.5 Technical properties of the feed particles

There are great technological challenges producing a formulated diet particle of high nutritional quality small enough to be eaten by fish larvae with a length as short as 2 mm. It might be technical rather than nutritional challenges that are the main limiting factor for an early introduction of compound diets in large scale production systems (Rosenlund *et al*., 1997).

1.5.1 Particle size

An important factor for understanding the problems of producing micro diets is related to the ratio between surface area and size of the particle (Table 2). The smaller the particle the larger the ratio of surface compared to the weight of the particle (Table 2). For instance halving the diameter of a particle a larva has to eat eight times as many particles to get the same amount of nutrients. This indicates the importance of adjusting the particle size to the size of the larva. A common problem for several types of micro-diets is the large distribution in size of the particles. Depending on the technology used, this problem seams to increase with decreased particle size. It may be difficult to control survival and growth if considerable amount of the particles are unavailable for the larvae or the feed is in a size range to small for the larvae to ingest sufficient amounts of feed. There is different terminology to describe particle size and

the variation of particle size. Generally, particle size distribution is reported as “number %” or “volume %”. “Number %” is the relative number of particles of each size fraction and the “volume %” is the distribution of volume of particles within each size range (Lobeira, 2002). These two ways to describe size distribution will often give a completely different picture (Figure 1). In feeding trials the most common way to describe particle size distribution is to either give the mean particle size ± standard deviation or give the size range of the sieve used for the different fractions.

Table 2: Relationship between particle size, particle volume and particle surface. The table is a summary of table by (Vilstrup 2001) (Page 87)

Particle size **(µm)**	Particles g^{-1*}	Surface area g^{-1} **(mm^2 g^{-1})***
70	5 568 103	85714
90	2619834	66667
120	1105243	50000
160	466274	37500
250	122231	24
500	15279	12

* Particle density is assumed to be 1 g/cm^3

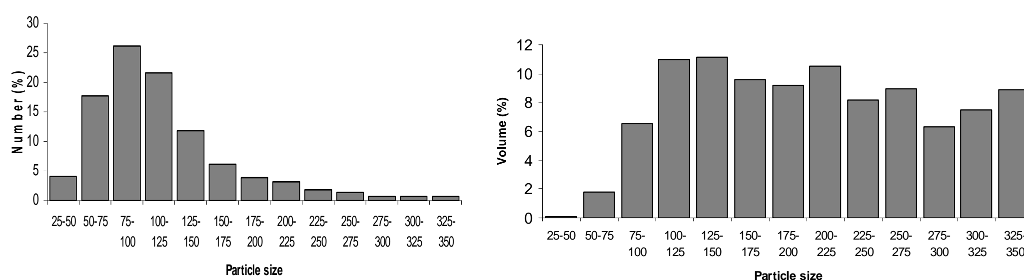


Figure 1: Distribution of particle size of protein cross-linked capsules measured as numbers of particles (number %) and as volume (volume %) of feed in each size category, respectively. The mean size of the feed batch evaluated was 116 ± 66 µm (number %).

Rehydration of formulated feed can lead to a significant increase in particle size (Yùfera *et al*., 2005) and should be considered when choosing size fraction. The increase in particle size by rehydration is both dependent on binder, feed ingredients, particle size and if the feed is

dispersed in fresh or saltwater (Heelan and Corrigan 1997; 1998; Shu *et al*., 2001; Zhang *et al*., 2004). A freeze dried alginate bound diet (Fernandez-Diaz *et al*., 2004, Yùfera and Fernandez-Diaz 2005) had an increase in diameter from 15 % - 100 % dependent on particle size (Yùfera, 2005) with the largest increase in size for particles smaller than 200 µm. It is therefore important to know the swelling properties of the diet so the proper particle size can be chosen.

1.5.2 Particle size of feed ingredients

Not only the nutritional quality, but also the particle size of the feed ingredients is of importance when producing larval feed. To obtain an even distribution of all non-soluble nutrients in each individual feed particle, these particles must be several times smaller than the diet actually produced. Micronutrients are needed in very small quantities and some may be toxic in larger concentrations. An even distribution of micronutrients in most larval diets may be accomplished by dissolving the micronutrients in either water or fish oil, dependent on solubility, and mix this solution thoroughly into the feed mixture. On the other hand, particle size of the micronutrients has to be considered carefully for lipid spray beads (LSB) that do not contain water (Önal and Langdon 2004ab).

1.5.3 Sinking rate

Several of the marine aquaculture species are flatfish that spend most of their time after metamorphosis on the bottom (halibut, turbot, sole and Japanese flounder). However, pre-metamorphosis all marine fish larvae are in the water column and are slow swimmers. This means that all diets used at this stage should have a low sinking rate and a good distribution throughout the tank for the larvae to be able to capture the feed. Due to the high surface

tension of water, some particles will not penetrate the water surface, but stay on top of the water column and thus be unavailable for the larvae and cause environmental problems in the tank (Rønnestad and Hamre 2001; Leifson *et al*., 2003a). To avoid this problem, some hatcheries are pre-hydrating the feed in water before feeding it to the larvae. A particle's sinking rate is dependent on size, structure and density (Vilstrup, 2001), and chemical interactions between the water and the surface of the particle may influence particles of small size. A particle that is sinking too fast will be less catchable for the larvae, and thus excessive feeding is needed to increase feed availability. However, feed with neutral buoyancy may stay too long in the water and loose a large fraction of the water-soluble nutrients. In both cases this may lead to environmental implications and commercial feed producers therefore recommend increased water flow when formulated feed is used instead of live feed.

1.5.4 Feeding response

Most marine fish larvae have undeveloped eyes at start feeding (Blaxter and Staines, 1970) and may therefore not be able to adapt to light intensity and quality outside the larvae's optimal range. It is suggested that this is one of the reasons that feeding incident and thereby survival of fish larvae may be highly effected by light intensity (Downing and Litvak, 2001), light quality (Downing and Litvak, 2001), tank colour (Downing and Litvak, 1999; Clack, 2006) and feed colour (Ostrowski, 1989; Denson and Smith, 1996). In individual feeding studies all the mentioned parameters have shown to have significant affects on the amount of feed ingested and larval performance, but one optimal setting for the different parameters has not been found. The use of black or dark tanks has in several studies shown improved growth compared to white tanks. It is suggested that this is because a black tank will have less light reflection than a white tank and will therefore give a better contrast and increase the possibility for the larvae to target the feed. Nevertheless, larval haddock (*Melangogrammus*

aegelfinus) had increased growth in white tanks compared to black tanks at low light intensity (Downing and Litvak, 1999). It is suggested that there is cumulative effect between background colours and light intensity and that each background has its own optimum light intensity level (Clack, 2006).

The colour of the feed particle has been shown to affect the feeding efficiency (Dendrinos *et al*., 1984; Clack, 2006). Sole (*Solea solea*) fed both natural *Artemia* and black stained *Artemia* had approximately 4 times higher ingestion rate of black stained *Artemia* compared to the natural coloured *Artemia*. (Dendrinos *et al*., 1984). Clack (2006) found an interaction between tank colour and colour of the feed particle on feeding incident. Blue spotted goby larvae (*Asterropteryx semipunctata*) fed white particles (zein particles) in a tank with black background had a 36 % feeding incident while larvae fed black particles in tanks with a white background had a 6 % feeding incident (Clack, 2006).

Taste also has a significant effect on feeding ratio of fish larvae and FAA, among other compounds, function as feed attractants (Kolkovski *et al*., 1997ab). According to Kolkovski *et al*. (1997a) the FAA: glycine, alanine, arginine and the ammonium base betaine (Knutsen, 1992; Kolkovski *et al*., 1997a) have stronger stimulatory effects on the feeding incident of sea bream compared to other FAA. Krill hydrolysate is a good attractant for several species (Kolkovski *et al*., 2000) and a commercial diet coated with krill hydrolysate increased the ingestion rate in Yellow perch (*Perca flavescens*) with 24 %. The pospholipid phospatidylcholine (PC) also stimulated the feeding activity at an early larval stage of gilthead sea bream, however this effect lasted only the first 26 days (Koven *et al*., 1998). It is suggested by Koven *et al*. (1998) that the choline trimethyl group of the PC acts as the attractant. This group is also found in the fish attractant betaine (Mackie and Mitchell, 1985).

1.6 The different formulated diets

Several different formulation concepts have been investigated as potential for fish larval diets. These can mainly be put into two categories; microbound particles and microencapsulated particles. Different types of microbound diets are widely used for marine fish larvae (see review by Langdon, 2003). These particles consist of a uniform matrix throughout each particle without a distinct surrounding wall (Langdon 2003; Önal and Langdon 2005a). The matrix is bound by either carbohydrate or protein binders (gelatine, zein, alginate, carboxymehtyl-cellulose, soluble fish protein, chitosan, carrageenan etc.; (Le Ruyet et al., 1993; López-Alvarado et al., 1994; Baskerville-Bridges and Kling, 2000a; Guthrie et al., 2000; Önal and Langdon, 2000; Garcia-Ortega et al., 2001; Hamre et al., 2001; Yùfera et al., 2002; Høgøy, 2005; Önal and Langdon, 2005a). These binders create a non- or low soluble matrix in the feed particle which holds the nutrients within the particle. The different binders need different treatments (heating, cooling, drying, or chemical cross-linking) to produce the binding matrix. Agar, carragenan and alginate are polysaccharides obtained from algae. Diets made with agar and carragenan need to be heated to over 85 °C due to a high melting point and gelling when the feed solution is cooled down. On the other hand, alginate bound particles can be produced at low temperatures by gelling with calcium chloride or other di or trivalent ions. Chitosan is derived from chitin, a polysaccharide and the structural part of exoskeleton of crustaceans. Chitosan is soluble in acidic solutions and is cross-linked in alkaline solutions.

The binder used also affects the properties of the particles (Le Ruyet *et al*., 1993; López-Alvarado *et al*., 1994; Guthrie *et al*., 2000). Weak binders might give to particles that are highly digestible. On the other hand they may easily disintegrate in the water, making them less available for ingestion by the fish larvae. Binders that are favourable with regard to

minimising particle disintegration and nutrient leaching may be unfavourable with regard to digestibility in the fish larvae (Le Ruyet *et al*., 1993; Guthrie *et al*., 2000). The concentration of binder might also affect the digestibility and should be considered. Different fish species may respond differently to various binders (Partridge and Southgate, 1999) and should be considered. For instance Halibut larvae have a low tolerance for carbohydrates in the diet (Hjertnes, 1991; Hamre *et al*., 2003), indicating that formulated diets containing a high level of digestible carbohydrates as a binding material should be considered carefully. Generally, microbound feeds may be produced on a large scale at relatively low production costs.

The binder used may affect feeding incidence: Guthrie *et al*., (2000) found that Walley (*Stizosedion Vitreum*) had similar feeding incident at first feeding of alginate-bound particles (68 ± 2 %), agglomerated particles (TIC Gums) (65 ± 6 %), zein-bound particles (69 ± 7 %), microextruded/maurmurmuriced particles (TIC Gums) (71 ± 8 %) and *Artemia* (71 ± 6 %), while MBD diets bound with carboxymethylcellulose (27 ± 0.07%), starch (21 ± 10 %) and Carragennan (20 ± 0.8 %) had less than one third of the feeding incident at start feeding. Interestingly the Walley larvae that initiated start feeding did not have a significant difference in the concentration of feed ingested, suggesting that larvae that initiate feeding consume similar amounts of feed independent on the binder used (Guthrie *et al*., 2000). MBD have shown different *in vitro* protein digestibility dependent on the binder (Garcia-Ortega et al., 2000a). Though it is not known to what degree the different binders are digested or if the digestive enzymes penetrate the feed particles and digests the different nutrients within the ingested particles.

The different diets can be produced by using a spray nozzle (Önal and Langdon, 2005ab), agglomerating technology (Guthrie et al., 2000), emulsion technology (Jones et al., 1974;

Langdon, 1989; Yùfera et al., 1999,2005) or by crushing and sieving particles to wanted size (Guthrie et al., 2000; Hamre et al., 2001). Crushing and sieving is the most common method in nutritional studies conducted on altrical fish larvae. The use of spraying to produce MBD particles is beneficial for production of particles within the smallest size range, but not for larger particles. As a consequence, it may be difficult to accomplish a complete feeding trial from start feeding through metamorphosis without change in feed type. Depending on the spraying system and set up used, the particles produced may be within such a narrow size range that sieving is unnecessary. The particle size and range is dependent on type of nozzle, spraying pressure and viscosity of the feed solution sprayed. It may therefore be difficult to reproduce a batch of feed with the same particle size.

While microbound particles have a uniform matrix through the whole particles, microencapsulated particles have an insoluble membrane surrounding a core matrix (Figure 2). The concept of microencapsulation is often used to reduce or control leaching of soluble components from the core matrix, a strategy that has been proven effective, dependent on the encapsulation technique used (López-Alvarado *et al.*, 1994). Chitin/alginate, lipid and protein encapsulated particles can be produced by spraying particles into a bath or through a vapour that contains a cross-linking agent or by using emulsifying technology (López-Alvarado *et al.*, 1994; Ozkizilcik and Chu 1996; Önal and Langdon 2000; Yùfera *et al.*, 2000; Høgøy 2005).

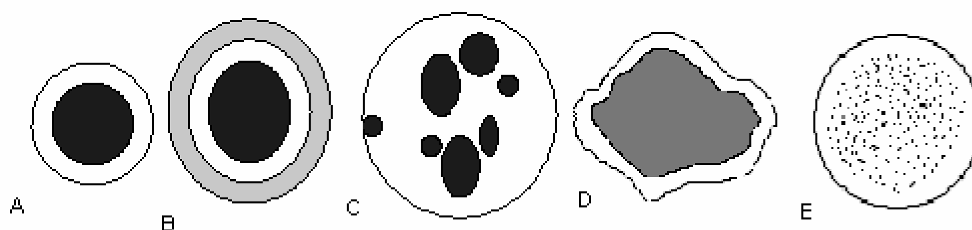


Figure 2: Different types of microcapsules that are made for marine fish larvae. A) Microcapsule containing a single membrane (Protein walled particles: Jones *et al.*, 1974; Langdon, 1989; Yufera *et al.*, 1999) B) Microcapsules containing a double membrane (Protein walled particles coated with lipid; López-Alvarado *et al.*, 1994) C) Multi- core microcapsule (Protein walled capsules containing LSB; Ozkizilcik and Chu, 1996) D) Coated microcapsules (microbound diets coated with lipid; López-Alvarado *et al.*, 1994) E) Matrix particle (lipid particles; Buchal and Langdon, 1998, Önal and Langdon, 2004ab)

One of the best described encapsulated diets used for larval feeding is a protein encapsulated diet that is made with an emulsifying technology (Jones *et al*., 1974; Jones 1980; Hayworth 1983; Langdon 1989; López-Alvarado *et al*., 1994; Ozkizilcik and Chu 1996; Önal and Langdon 2000; Yùfera *et al*., 2000; Høgøy 2005). An aqueous dietary solution is emulsified with an organic solvent. The small emulsified aqueous droplets containing the dietary nutrients and the wall forming protein are exposed to a chemical cross-linking agent. The chemical cross-linker polymerises the water soluble proteins at the interface between water and the organic solvent phase, resulting in a stable capsule of cross-linked protein that envelopes each particle. Although there have been promising results with the use of the protein encapsulated diet for feeding marine fish larvae, most of the feeding trials are preliminary and few feeding studies have investigated growth and survival over a longer time period. Sea bass (*Lates calcarifer*) larvae fed the protein encapsulated diet from first feeding were all dead at day 10, and larvae fed microcapsules together with rotifers for 5 days and than microcapsules alone for 1 week had a mean survival rate of 2.4% (Walford *et al*., 1991a). Fernández-Díaz and Yúfera (1995) discovered that by dispersing the protein cross linked particles in a gelatin solution, the digestibility of the particles increased. The use of this diet gave good growth and survival for sea bream (*Sparus aurata* L) larvae after only feeding rotifers for the first 4 days (Yùfera *et al*., 2000). The technique is well suited for small scale laboratory preparation and no expensive equipment is necessary.

Production of capsules with chitin, alginate or both is relatively easy and cheap and is well documented in the medical literature. However, most of the alginate bound particles are not truly microencapsulated diets with a protective membrane of alginate, but microbound diets with a porous gel (Yùfera and Fernandez-Diaz, 2005). A well studied particle consists of a microbound alginate bead coated with a thin membrane of chitin. The particles may be

produced by spraying an aqueous dietary solution containing alginate into a solution of chitosan and calcium chloride (Gaserod et al., 1998; Gaserod et al., 1999; Yùfera et al., 2000; Vandenberg and De La Noue 2001; Vandenberg et al., 2001). There are reports on the use of chitin capsules as a formulated diet for marine fish larvae (Høgøy 2005), and it is not yet known to what degree fish larvae are able to digest the chitin. Production processes and leaching properties of chitin/alginate capsules are well described for medical purposes (Chen *et al*., 1996; Huguet and Dellacherie 1996; Huguet *et al*., 1996; Bartkowiak and Hunkeler 1999; Shu *et al*., 2001; Vandenberg and De La Noue 2001; Vandenberg *et al*., 2001; Chen *et al*., 2002; Shu and Zhu 2002), but are not investigated thoroughly for use in larval rearing.

Lipid based particles can not be used as a complete diet due to a high lipid: core ratio and clumping during feeding due to the hydrophobic behavior of the particles (Önal and Langdon, 2005). Nevertheless, they have interesting properties for larval rearing purposes. In lipid based particles, water soluble nutrients are encapsulated within a lipid matrix, and the technologies used are based on the lipids ability to solidify at low temperature, either in a cooled water bath for lipid walled capsules (LWC) López-Alvarado *et al.,* 1994) or in cooled air for lipid spray beads (LSB) Buchal and Langdon, 1998). LSB can deliver a higher payload than LWC (Buchal and Langdon 1998). Studies have shown that up to 21 % glycine can be incorporated within a LSB (Önal and Langdon 2004b), while only a 6 % FAA payload has been achieved with LWC (López-Alvarado *et al*., 1994). The lower incorporation efficiency of LWC is presumably due to loss of FAA due to leaching in the water bath. To obtain a satisfactory low leaching, the amount of saturated fatty acids (FA) with a high melting point is important (López-Alvarado *et al*., 1994; Buchal and Langdon 1998). The problem that has to be solved is to make stable capsules with lipids that are digestible and have a satisfactory nutritional value. Japanese flounder younger than 20 days did not manage to digest tripalmitin

lipid walled capsules (López-Alvarado *et al*., 1994). A similar type of capsule was broken down at an earlier stage when oils with a lower melting point were used, resulting in increased leaching of the entrapped water soluble component (Table 3; López-Alvarado *et al.,* 1994; Buchal and Langdon, 1998). LSB containing menhaden stearine was broken down by a 3 days old clown fish (Önal and Langdon, 2004b). Menhaden stearine has an acceptable technical quality and a higher content of n-3 fatty acids than lipids that have been used previously for production of lipid based particles (Önal and Langdon 2004b), and would be an interesting candidate for further investigations.

1.7 Quality changes during manufacturing of larval diets

During the production of formulated diets there may be a severe loss of nutrients and change in the nutritional quality due to heating, chemical interactions or exposure to leaching (Gabaudan 1980; Garcia-Ortega *et al*., 2000b; Yùfera *et al*., 2002; Yùfera *et al*., 2003; Önal and Langdon 2005b). Diets produced using a spray nozzle to form beads in a aqueous solution, such as the chitosan and alginate bound particles, may have significant loss of nutrients during the stay in the chemical solution, due to exposure to leaching (Huguet and Dellacherie 1996; Vandenberg and De La Noue 2001; Vandenberg et al., 2001). Nonetheless, this may be solved by saturating the aqueous cross-linking and washing solution with the soluble compounds which are encapsulated (Ostberg and Graffner, 1992). This is described in the medical literature for the encapsulation of small concentrations of one active compound (Ostberg *et al*., 1992, 1994). On the other hand, larval feed contain a complex blend of soluble compounds which might give both technical and financial challenges. Diets produced with either heating or cross-linking of proteins will have a significant decrease in water soluble protein due to denaturation and/or polymerisation (Boye *et al*., 1997; Garcia-Ortega *et al*., 2000b), and this may oppose with larval preferences (Carvalho *et al*., 2004). The use of

chitosan and/or alginate binding is a “mild” procedure that allows the protein within the feed particle to maintain native properties (Leonard *et al*., 2004). In addition, this method is well suited to encapsulate and deliver live cells (Schwinger *et al*., 2002, 2004), bioactive ingredients (Polk *et al*., 1994) or fish vaccines (Polk *et al*., 1994). On the other hand, there is often high protein diffusion both during production (Wheatley *et al*., 1991; Rilling *et al*., 1997; Vandenberg and De La Noue 2001; Vandenberg *et al*., 2001) and feeding (López-Alvarado *et al*., 1994). Change in lipid quality of larval diets has not been thoroughly investigated, but oxidation during the production process might be a severe problem for several of the diets and should therefore be investigated. Fish larvae may be more sensitive to oxidised lipids than juvenile or adult fish (Fontagne *et al*., 2006) and diets containing oxidised lipids had a severe affect on survival and deformities of sturgeon larvae (Fontagne *et al*., 2006). If a high proportion of oxidized lipid is present during the production process, this might affect the protein digestibility due to complexes made between the protein and the oxidized fatty acids (Murray *et al*., 1977; Ufodike and Matty 1983; Sullivan and Reigh 1995; Chong *et al*., 2002). As the nutritional value of the feed ingredients may change severely during production, the production method and its impact on the ingredients that are being used must be considered carefully in order to obtain the wanted nutritional composition of the final diet.

1.8 Nutrient leaching from formulated larval diets

Loss of water soluble compounds are severe in almost all described formulated diets for marine fish larvae (López-Alvarado *et al*., 1994; Hamre 2006; Kvåle *et al*., 2006) and > 90% loss of FAA in less than 2 min has been observed (Table 3; López-Alvarado *et al.,* 1994). The high leaching rate from most micro feeds is a result of rapid hydration and short diffusion distance within the small particles. In the protein encapsulated diets, there is a significant

higher leaching rate of FAA from the diets produced according to the method of Langdon (1989) and Ozkizilcik and Chu (1996) (50 - 60 % in 2 min; Ozkizilcik and Chu 1996; López-Alvarado *et al.,* 1994) compared to the method of Fernández-Díaz and Yúfera (1995) (8 % in 5 min; Yúfera *et al*., 2002). Although LWC and LSB can deliver significant amounts of FAA, small peptides and micronutrients in a controlled manner (Table 3; Önal and Langdon, 2004a; b; 2005), these particles are not capable of delivering a complete diet. In addition the high melting point of the lipids needed to efficiently prevent leaching. Which may oppose with the nutritional requirements of fish larvae (Buchal and Langdon, 1998, López-Alvarado *et al.,* 1994).

Table 3: Loss of FAA after exposure to leaching for 2 min from selected formulated diets for marine fish larvae (López-Alvarado *et al*., 1994).

Particle type	Leaching (%)
Microbound carragenan	85 ± 7
Microbound alginate	81 ± 2
Microbound zein	91 ± 2
Protein encapsulated	59 ± 1
Protein encapsulated and lipid coated	39 ± 2
Lipid walled (tripalmitin + triolein)	47 ± 9
Lipid walled (tripalmitin)	4 ± 2

To our knowledge, there is no available formulated diet that can deliver controlled amounts of low molecular weight nitrogenous compounds and micronutrients in a single digestible diet with the appropriate proportions of macro and micronutrients. In addition to a reduced nutritional quality, leaching may affect the water quality by increased bacterial growth (Rønnestad and Hamre 2001) and high ammonia levels (Baskerville-Bridges and Kling, 2000c) and thereby reduced survival and loss of appetite (Rosenlund *et al*., 1997). An important aspect when performing nutritional studies is the masking effect of the water quality on larval performance (Rosenlund *et al*., 1997)

Leaching of protein increases with decreasing molecular size of the amino acid source (protein < hydrolysed protein < serine; Kvåle *et al.,* 2006). Though, in some diets the leaching of hydrolysed protein appears to at the same level as FAA. In two microbound diets, a heat coagulated and an agglomerated diet (Hoestmark 1992; Hamre *et al*., 2001), up to 54% and 98% of the hydrolysed protein, respectively, was lost from the diet particles within 5 min immersion in water (Kvåle *et al*., 2006). The leaching potential from the small feed particles (<1mm) seems to be reached within the first 1-5 min after immersion in water (López-Alvarado *et al*., 1994; Hamre, 2006; Kvåle *et al*., 2006), but increasing the particle size to the upper range of what is acceptable for the larvae, will reduce the nutrient loss (Kvåle *et al*., 2006). To obtain a better understanding of leaching properties, and to make it easier to compare different diets and diet recipes, leaching of protein should be measured and evaluated against the total content of water soluble protein in the diets and not only towards the crude protein content (N x 6.25). A diet with a low concentration of water soluble protein may seem to have a low leaching rate, even if there is a 100 % loss of the water soluble fraction.

Studies on chitosan/alginate capsules have shown that leaching of soluble protein is dependent on the isoelectric point of the protein, pH, interaction of the protein with the particle matrix, ion concentration of the matrix, ion concentration of the leaching medium, as well as the molecular size (Huguet *et al*., 1996; Gaserod *et al*., 1999). The leaching of neutral molecules, such as the carbohydrate dextran, is mostly correlated to the molecular size and to some extent the flexibility of the molecule (Huguet *et al*., 1996). Formulated diets might have a significant degree of leaching of water soluble carbohydrates as dextran (Wheatley *et al*. 1991; Chen *et al*. 1996; Gaserod *et al*. 1998; Brazel and Peppas, 1999), but no negative

effects have been recorded with loss of water soluble carbohydrates for marine fish larvae.

1.9 Strategies for reducing leaching from formulated diets

There is no diet at the present time that seems to be significantly superior to the others with regard to the compromise between digestibility and appropriate nutrient composition on one side, and leaching at the other (López-Alvarado *et al*., 1994). Nevertheless, microbound diets have been used in weaning of larval fish species with good results (Cahu *et al*., 1999; Baskerville-Bridges and Kling 2000a; 2000b; Hoehne-Reitan *et al*., 2001), despite the extensive leaching from such diets (López-Alvarado *et al.,* 1994; Hamre, 2006, Kvåle *et al.,* 2006). To reduce leaching from microbound or protein encapsulated diets, coating with lipid has been tried (Table 3; López-Alvarado *et al.,* 1994). Unfortunately, the lipids needed to reduce leaching have an unacceptable nutritional composition, and the total lipid level may become too high, as for the lipid walled capsules. All use of material to coat whole feed particles has to be considered carefully since coating material often has a low nutritional value. Loss of water soluble nutrients is in most cases diffusion driven, and the coating thickness needed to prevent or decrease leaching is presumed to be independent of particle size. If a 10 µm film coat is required to reduce the leaching to a minimum, this film coat will be necessary if the capsule has a diameter of 70 µm or 500 µm. For instance, a 10 µm film coat constitutes over 53 % of a capsule with a diameter of 70 µm but only 11 % if the diameter is 500 µm (Table 4).

Table 4: Coating material required to provide a 10 µm film coat for particles for selected sizes of feed particles. The density of film coat and core are assumed to be equal. The table is based on (Vilstrup, 2001) page 87.

Particle size (µm)	% coating material (w/w) required to provide a 10 µm film coat (%)
70	52,9
90	45,2
120	37
160	29,8
250	20,6
500	11,1

Even though lipid based particles can not deliver a complete larval diet, these particles are still interesting candidates for incorporation into microbound particles to form a complex feed (Villamar and Langdon 1993; Ozkizilcik and Chu 1996; Önal and Langdon 2005ab). There are, however, technical challenges with incorporating LSB into complex particles due to leaching or melting of the LSB during the prod. process (Önal and Langdon, 2005). A zein bound complex diet with promising properties has been developed to deliver FAA and peptides to marine organisms (Önal and Langdon 2005). Zein complex particles are produced by blending feed ingredients and LSB into a solution of zein and ethanol. Zein is alcohol soluble and will act as the binder when the feed is spray dried. Most FAA and peptides have a very low solubility in ethanol so there will be a very low leaching of FAA from the LSB during the feed production. Clack (2006) reported glycine retention of 96 % after 10 min immersion in 90 % ethanol. To our knowledge, no studies have investigated the loss of ethanol soluble nutrients during the production process.

Liposomes are lipid vesicles mainly made of a PL membrane enveloping a core with nutrients dissolved in water. They have been proven successful carriers for water soluble nutrients (New, 1990) and give a good retention of the incorporated nutrient (Monroig et al., 2003). Unfortunately, the documented payload of water soluble nutrients in liposomes (Touraki et al., 1995) is low compared to the payload obtained with LSB (Önal and Langdon 2004b). As for LSB, liposomes can not be used as a complete diet due to the high lipid content, but liposomes have proved to disperse freely and can be fed directly to both *Artemia* (Hontoria *et al*., 1994; Touraki *et al*., 1995; Tonheim *et al*., 2000; Monroig *et al*., 2003; Monroig *et al*., 2006) and fish larvae (Koven *et al*., 1999, 2001). The use of liposomes to enrich *Artemia* with water soluble nutrients such as FAA (Tonheim *et al*., 2000), antibiotics (Touraki *et al*., 1995) and lipid (Ozkizilcik and Chu 1994; McEvoy *et al*., 1996; Monroig *et al*., 2003; Monroig *et*

al., 2006) has been describes quite thoroughly. Most investigated liposomes for delivering of nutrients to *Artemia* or fish larvae have been produced with phospholipids containing low concentration of poly unsaturated fatty acids (PUFA) originating either from Soya or egg yolk (Hontoria *et al*., 1994; Touraki *et al*., 1995; Koven *et al*., 1999; Tonheim *et al*., 2000). However, liposomes made with phospholipids from marine sources containing a large concentration of n-3 PUFA have been produced and successfully fed *Artemia* (McEvoy et al., 1996; Monroig et al., 2003; Monroig et al., 2006). The use of n-3 PUFA have shown to increase the leaching rate severely (Monroig et al., 2003), but with the inclusion of cholesterol the liposomes could withhold the encapsulated soluble compounds long enough to enrich *Artemia* (Monroig et al., 2003). The problems with liposomes are presumed to be the high cost of production and low particle stability.

2 Aim and objectives for the study

To investigate effects of production process of formulated larval diets and leaching during feeding of the fish larvae, on quality, concentration and digestibility of nutrients, with emphasis on protein and free amino acids.

- To investigate how the cross-linked protein walled capsule technique affects the concentration and quality of both macro and micronutrients and *in vitro* protein digestibility of the diet.

- To investigate the changes in protein quality of a heat coagulated diet with increasing levels of hydrolyzed protein, due to processing and leaching by exposure to water. Investigate the *in vitro* digestibility on the respective diets before and after exposure of leaching.

- To investigate the concentration of water soluble protein of selected live feed and protein sources used in larval compound diets and the *in vitro* digestibility of water soluble and water insoluble protein fractions.

- To evaluate the effects of additions of phospholipids on the characteristics of LSB and investigate the use of these LSB as a tool for delivery of micronutrients to marine fish larvae through enrichment of *Artemia*.

3 Methodological considerations

3.1 Ingredients

The feed ingredients used in the present studies are commercially available and presumed to be products with relatively small nutritional variance between production batches. The pepsin hydrolyzed cod fillet (Paper II and III) was made according to the method described by Kvåle *et al*., (2002). The complete hydrolyzed solution with both the soluble and remaining insoluble fraction was included in the different diets (Paper III) and *in vitro* digested (Paper II). The fact that the hydrolyzed protein contained a significant concentration of insoluble protein should be considered when comparing the results in Paper II and III with other studies.

3.2 The formulated diets

The production of the protein cross-linked diet (Paper I and II), the protein heat coagulated diet (Paper III) and the LSB (Paper IV) were produced in close collaboration with the institutions that have developed the respective diets. It is assumed that diets produced in the present studies are similar to previous studies on the respective diets. The production method of the commercial larval diet Minipro^{TM} (Paper II) is described by Høgøy (2005), though it is not known if the production process for the investigated diet deviates from the described technology. Due to commercial interests, the type and concentration of the different feed ingredients are unknown.

The cross-linked protein capsules investigated in paper I and II used Na-caseinate as the cross-linking protein. In previous studies, acid precipitated casein has been used as the cross-linking protein (Yùfera *et al.,* 1999, 2000, 2002, 2003; Chu and Ozkilcik 1999; Kvåle *et al.,*

2006). There were no technical complications using Na-caseinate and it has previously been successfully used to produce cross-linked protein microspheres (Millar, 1991; Heelan and Corrigan, 1997; Corrigan and Heelan, 2001). However, the change in cross-linking protein should be considered, since there may be differences in leaching rate and swelling properties. The four heat coagulated diets were produced prior to planning of the work performed in paper III, and samples of the raw ingredient mixture could therefore not be investigated. The concentrations of soluble N and TCA soluble N in the raw ingredients were therefore calculated. The analysed protein content of the individual feed ingredients did not deviate more than 4.3 % from the analysed crude protein content (Table 5), indicating that the calculated values are within acceptable limits.

Table 5: Calculated crude protein (N x 6.25) and analysed crude protein in four heat coagulated diets with increased concentration of pepsin hydrolyzed protein (n=3). The inclusion levels ranged from 0 % to 45 % pepsin hydrolyzed protein of total protein.

Diet	0	15	30	45
Calculated concentration	72	72	70	68
Analysed concentration	69.4 ± 0.3	68.6 ± 0.1	67.7 ± 0.4	66.9 ± 0.3

3.3 Analysis of nitrogenous compounds

3.3.1 Analysis of crude protein

Crude protein (N x6.25), soluble N and TCA soluble N (Paper I, II and III) were analysed by total combustion using a nitrogen analyser (Leco FP-528, St. Joseph, MI). Crude protein was calculated as N x 6.25 (Jones, 1931) which is based on an amino acids profile equivalent to 16 % N and insignificant amounts of non-protein N. However, this may be unsuitable for some protein sources (Sosulski and Imafidon, 1990). Analysis of the N content from recovered amino acids has shown a significant variation in crude protein conversion factor (SaloVaananen and Koivistoinen 1996). However, the concentration of non-protein N (FAA

not included) in the fish based ingredients (Paper I, II, III) are presumed to be low (Ruiz-Capillas *et al*., 2002; Lapa-Guimaraes *et al*., 2005; Karthikeyan *et al*., 2006, 2007) and SaloVaananen and Koivistoinen (1996) reported a non-protein N content (FAA not included) to range from 1.1 % to 4.8 % (of tot. N) in 26 different species of fish. Based on these reports, the use of N x 6.25 is presumed reliable since the relative differences between diets and protein fractions analysed will be similar.

The concentration and solubility of the different non-protein N compounds is poorly described for live feed. Both *Artemia* and copepods (Paper II) contain chitin in the exoskeleton (Cauchie *et al*., 1997; Brandt and Raben, 1919-22). *Artemia* has 5.6 to 21 mg g^{-1} (dw) chitin (Cauchie *et al*., 1997) or for 1.5 - 3.3 % of the total protein content (calculated from chitin content by Cauchie *et al*., 1997). Considering that 33 % of the crude protein in newly hatched *Artemia* (Paper II) is insoluble, the chitin will constitute for 4.5 - 10 % of the N in the non soluble fraction. The copepod *Calanus finmarchius* is reported to have 44 - 50 mg g^{-1} (dw) chitin (Brandt and Raben 1919) and might therefore lead to a higher overestimation of insoluble protein than showed for *Artemia*. No references on the concentration of the soluble non-protein N fraction was found for *Artemia* and copepods, but according Carvalho *et al*. (2003) the molecular weight distribution of *Artemia* and rotifers where similar. Srivastava *et al*. (2006) found a nitrogen to protein conversion rate of 4.46 for the crude fraction and 3.52 for the soluble fraction of rotifers, although the AA profile of rotifers has a crude AA profile equivalent to 15.96 % N and a soluble AA profile equivalent to 16.2 % N (calculated from AA profile by Srivastava *et al*., 2006). This indicates that approximately 28 % of crude N in rotifers and approximately 43 % of the soluble N is non-protein N. The TCA soluble fraction is presumed to have an even higher non-protein N content since the soluble non-protein N is not presumed to be precipitated by TCA. This uneven and unknown

distribution makes it difficult to evaluate the N fraction solely based on N analyses. Based on these data, the use of nitrogen to protein conversion is not considered reliable for investigating protein content in the different fractions of *Artemia* or copepods (Paper II).

3.3.2 Analysis of soluble nitrogen

When analysing the concentration of soluble N (paper I, II and III) the diets and ingredients were dispersed in phosphate buffer 80 mM (pH 8) and the insoluble protein fraction was precipitated by centrifugation. The solubility of protein is affected by ionic strength and pH (Stefansson and Hultin, 1994) and medium should be considered when comparing these results with other studies. The phosphate buffer was used to be able to relate the solubility of the different ingredients (Paper II) and diets (Paper II and III) to differences in protein *in vitro* digestibility (Paper II and III). Kvåle *et al.*, (2007b) investigated the dietary protein solubility by estimating the amount of free α-amino groups in the same four diets as investigated in paper III (Figure 3). Although there are significant methodical differences, the two methods show a similar trend of soluble protein. Indicating that the used for calculating soluble proteinic compounds based on soluble N may be reliable.

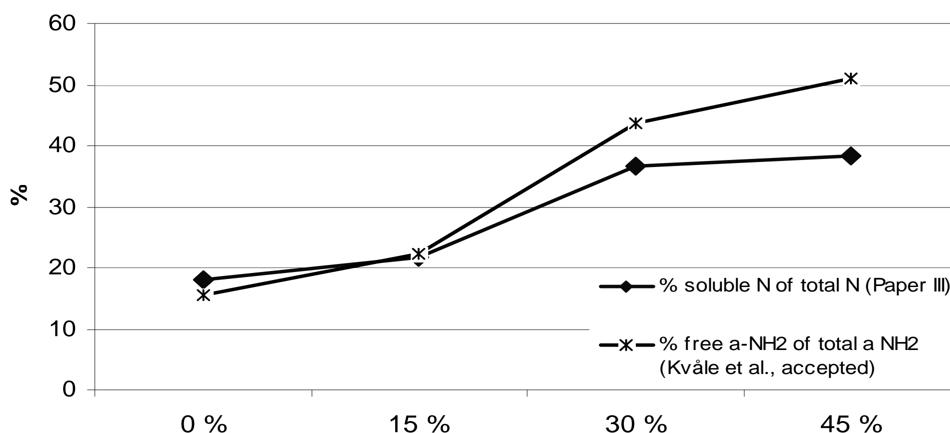


Figure 3: Concentration of water soluble proteinic compounds measured as soluble N and free a-NH2 in heat coagulated diets with increasing concentration of pepsin hydrolyzed cod fillet (Paper III; Kvåle et al., 2007b)

3.3.3 Analysis of TCA soluble nitrogen

Precipitation with TCA was used (Greenberg and Shipe 1979; Yvon *et al*., 1989) to analyse the concentration of low molecular weight nitrogenous compounds (Paper I, II and III). As for the analysis of soluble N, the different diets and feed ingredients were dispersed in phosphate buffer. Due to the high water solubility of most low molecular weight nitrogenous compounds, the choice of buffer solution is presumably of less importance. The average molecular weight of peptides soluble in 10 % TCA was between 330 to 380 D (Greenberg and Shipe, 1979). The molecular size fraction of the TCA soluble N was not analysed, so the fractions of FAA and peptides in the diets and feed ingredients are unknown (Paper I, II and III). Previous studies on feed, feed ingredients and live feed have focused on FAA concentrations and to a lesser extent on low molecular weight N. This has to be considered when comparing the present results with previous work.

3.4 Leaching experiments

Leaching studies are usually performed in small water volumes, mostly in a higher concentration of feed per volume compared to feed distributed in a fish tank. To make the conditions more similar to tank feeding, it is of importance to have a higher water to feed ratio than the saturation level of the investigated nutrients. Investigated leaching for the LSB (Paper IV), two highly soluble compounds with known solubility were used (glycine and OTC). For glycine and OTC the initial concentration was 520 and 2600, respectively, times lower than the saturation level at 100 % loss of the incorporated compounds. For the heat coagulated diet (Paper III), the source of soluble N is a complex mixture of different proteins, peptides and FAA and the true solubility of these compounds are therefore unknown. The

high loss of TCA soluble N during leaching trials (paper III) indicates that the saturation level was not reached. In tank feeding the particles are falling freely through the water column, while in leaching trials the tubes are agitated to keep the feed particle in suspension (Paper III and IV). Although the agitation of the leaching tubes was done carefully, this might affect the leaching rate compared to tank feeding.

Several types of leaching media have been used to study formulated diets (Table 6). Although most of the investigated diets are meant for marine fish larvae, the leaching trials are often performed in distilled water (Paper IV) or buffers (Paper III) due to methodical considerations. However, the medium should be considered carefully when comparing leaching rates of different diets as done by (López-Alvarado *et al*., 1994). The pH and ionic strength of different leaching media may affect the swelling of particles (Heelan and Corrigan, 1998; Beaulieu *et al*., 2002) and swelling of particles is shown to severely affect the leaching rate (Heelan and Corrigan 1997; Lee and Rosenberg 2001; Shu *et al*., 2001; Zhang *et al*., 2004). The ionic strength and pH also interferes with the interaction between the protein and matrix (Shu and Zhu 2002; Zhang *et al*., 2004). This is especially the case for alginate and chitin capsules that have large variability in loss of soluble compounds dependent on leaching media (Ostberg *et al*., 1994; Shu and Zhu 2002; Zhang *et al*., 2004). However, analysing the leaching rate of one specific type of formulated diet with different levels of a soluble compound (paper III), the choice of leaching medium will presumably not affect the relative differences in leaching rate.

Table 6: Selected parameters for investigating leaching of water soluble compounds from compound diets for fish larvae.

Compound analysed	Analytical method	Leaching medium	Ref
FAA	HPLC	0.5 M borate buffer (pH 8.5)	Lopez- Alvarado *et al*., 1994
^{14}C-labelled N	Liquid scintillation	3 % NaCl	Kvåle *et al*., 2006
FAA	HPLC	Distilled water	Yùfera *et al*., 2002
Riboflavin	Spectrophotometer	Water	Önal and Langdon, 2004,2005
Poly R (dye)	Spectrophotometer	Distilled water	Önal and Langdon, 2000
^{14}C-labelled protein	Liquid scintillation	Sea water	Partridge and Southgate, 1999
OTC.HCl	Spectrophotometer	Sea water	Buchal and Langdon, 1998
Riboflavin	Spectrophotometer	Sea water	Buchal and Langdon, 1998
OTC.HCL	Spectrophotometer	Sea water	Langdon and Buchal, 1998
OTC.HEM	Spectrophotometer	Sea water	Langdon and Buchal, 1998
Lysine	Ninhydrin	Deionized water	Ozkizilcik and Chu, 1996
^{3}H-Leucine	Liquid scintillation	Deionized water	Ozkizilcik and Chu, 1996
^{14}C-labelled protein	Liquid scintillation	Sea water	Langdon, 1989

3.4.1 Exposure of LSB to leaching

The leaching experiments with LSB were carried out based on a modified method described by Önal and Langdon (2004a). Preliminary studies showed leaching rates of glycine from LSB containing 100 % menhaden stearine similar to previous results (Önal and Langdon 2000), but there was a large increase in loss of glycine with increase in soy lecithin concentration. The large difference in leaching rate indicated that the increased dispersion of the LSB affected the leaching rate (Paper IV). This was also observed by Önal and Langdon (2004b) who showed that LSB with 5 % emulsifier had an improved dispersion, leading to an increased water contact of the particles and thereby a higher leaching rate than for LSB containing only triglycerides. To compare the leaching, of LSB with different hydrophobic properties it was necessary to use distilled water with 0.1 % Sodium dodecyl sulphate (SDS) to make the LSB more equally dispersible. Dispersion of the LSB containing 100 % menhaden stearine in 0.1 % SDS led to a 3 fold increase in loss of glycine while there was no

significant effect of SDS on leaching of dispersible LSB containing 40 % lecithin (Figure 4). This indicates that the difference in leaching rate was due to clumping of LSB with only neutral lipids during the leaching studies. Although there was a large increase in dispersion of the LSB containing 100 % menhaden stearine by using 0.1 % SDS, there was still some clumping during the leaching trials. This could explain the difference (not significant) in leaching between the LSB containing 0 and 40 % soy lecithin (Figure 4).

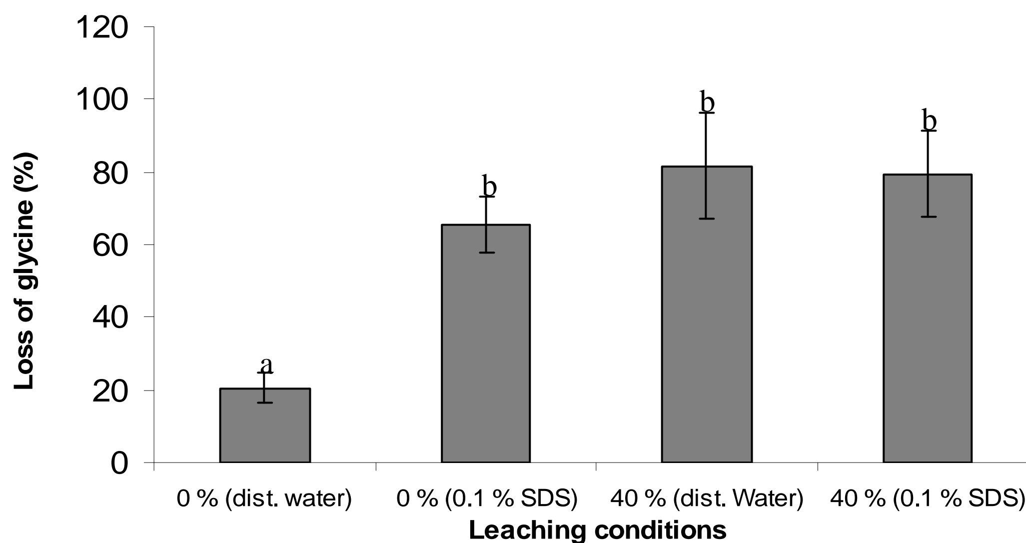


Figure 4: Percent loss of glycine after 10 min leaching of LSB in either distilled water or water containing 0.1 % SDS. LSB initially contained 16 % particulate glycine and either 0 % or 40 % w/w soy lecithin. Data are means of three replicates. Error bars represent standard deviations. LSB types sharing the same letter are not significantly different from each other (ANOVA, followed by Tukey HSD, $p < 0.05$).

As earlier reported, there is an increased loss of water soluble nutrients with a decrease in particle size (Kvåle *et al.*, 2006). In addition to lecithin's emulsifying properties, the increased inclusion of soy lecithin led to an increased viscosity of the molten lipid solution. Change in viscosity may affect the size of the particles and thereby the leaching rate. The effect of particle size on leaching from LSB produced with the described technology has not been investigated and should be investigated more thoroughly in later studies. The ninhydrine method (Doi *et al.*, 1981) was not affected by the addition of 0.1 % SDS. The leaching trial was not accomplished in saltwater due to a presumed effect on the ninhydrine method. Though, later investigations indicated that saltwater did not affect the ninhydrine method and

future studies may therefore be accomplished in saltwater.

3.5 Nutrient analysis

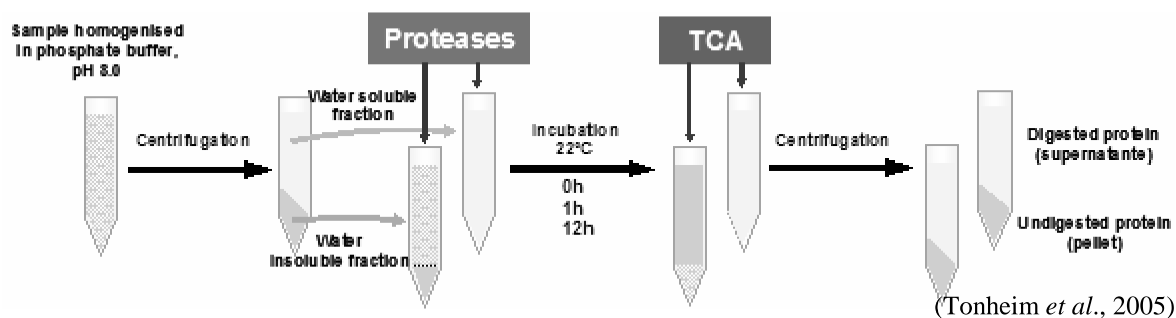
All the nutrient analyses such as micronutrients, crude protein, fatty acid profile, lipid class etc were done at NIFES according to accredited methods (NS-EN ISO/IEC17025). These methods are well established and are continuously being verified. Most analyses were accomplished with analytical replicates. Except for the *Artemia* analysed for riboflavin (Paper IV, see discussion below), all the analysed feed ingredients (Paper I, II and III) are included in matrixes for which the methods are authorized. The analysed results of all nutrients are therefore considered reliable.

3.5.1 The analysis of riboflavin in Artemia

Artemia is protected by a thick exoskeleton. It may be difficult to separate the nutrients from the complex matrix and this could affect the analytical results as for all matrixes analysed. In addition, riboflavin crystals have low solubility in water and a large fraction of the ingested riboflavin may be crystals embedded in a lipid matrix in the intestine of the *Artemia* (Paper IV). However, prior to the determination of riboflavin by high-performance liquid chromatography (HPLC) (Bronstad *et al.*, 2002), the freeze-dried samples were autoclaved at 121 ºC for 30 min in a solution of 0.1 M HCl. The samples were then pH adjusted to 4.0 and incubated over night at 37 ºC. The *Artemia* was completely dissolved, and any riboflavin still incorporated in the LSB would most likely be released by melting during the autoclaving at 121 ºC. The unfed control (Paper IV) had similar concentration of riboflavin as found by (Meeren, 2003). It is therefore believed that the method is reliable.

3.6 The *in vitro* digestibility method

In vitro studies may be performed with intestinal extracts (Jany, 1997; Nankervis and Southgate, 2006) or with commercially available proteases (Hsu *et al*., 1977; Saterlee *et al*., 1979). *In vitro* digestibility using intestinal extracts may give protein digestibility rates more similar to *in vivo* studies of the respective specie (Dong *et al*., 1993). However, there are several methodological difficulties. The extraction of sufficient amounts of intestinal enzymes from small fish larvae are difficult and therefore extract from juvenile or adult fish are used (Chong *et al*., 2002). Chong *et al*., (2002) demonstrated *in vitro* digestibility rates using three different protocols with commercial proteases. The data obtained, correlated with each other and with *in vivo* digestibility of juvenile discus. These results demonstrate that *in vitro* studies based on commercial enzymes may be used to evaluate the relative differences in protein digestibility of feed ingredients. The use of commercial enzymes may also give improved reproducibility compared to intestinal extract. The protocol used in paper II and III was a combination of two protocols (Hsu *et al*., 1977; Saterlee *et al*., 1979) tested by Chong *et al*., (2002) (Figure 5).



(Tonheim *et al*., 2005)

Figure 5: Method used to investigate protein *in vitro* digestibility of feed and feed ingredients in Paper II and III.

In a closed *in vitro* system, as used in this study, the pH could drop during the *in vitro* digestion leading to a decrease in enzyme activity. The magnitude of a pH drop is dependent on the amount of protein digested, the initial pH of the feed ingredient and the buffer capacity of the enzyme solution. The pH was not adjusted after the different feed samples and acidic

ingredients could therefore reduce the buffer capacity prior to the *in vitro* digestibility test. The initial low concentration of FAA and peptides in casein (Paper II) and high digestibility rates may indicates that buffer capacity was sufficient.

The *in vitro* digestion method was developed using casein as a reference protein. Casein is a highly digestible milk protein used as a reference protein in several *in vitro* digestibility studies (Gopalkrishnan and Prakash 2000). Several test trials of casein were performed in order to optimize the *in vitro* method. In addition, casein was used as reference protein during all the *in vitro* trials in Paper II. A reference protein with a known digestibility will give indications of suboptimal conditions during the digestion period. Unfortunately the heat coagulated diets (Paper III) were *in vitro* digested without casein as a reference protein. However, all the investigated diets used the same enzyme solution within the same day and the increased digestibility for all diets within 12 h (Paper III). The enzymes used in paper III were from the same batch as used in paper II, and all other parameters were similar. It is therefore presumed that the results are reliable and may be compared with the results obtained in paper II.

In vitro protein digestion, are usually investigated at one time interval (Hsu *et al*., 1977; Chong *et al*., 2002). In paper II and III, the protein digestibility was measured at different time points: 0 h, ½ h, 1h and 12 h. The experimental set up may give valuable information such as the time of complete digestion and change in relative difference in digestibility between feed ingredients at different time points (Lan and Pan, 1993; Paper II and III).

Based on the high concentration of non-protein N in live feed (chapter 3.3.1), the use of *in vitro* digestibility where the digested protein is described as TCA soluble N is not considered

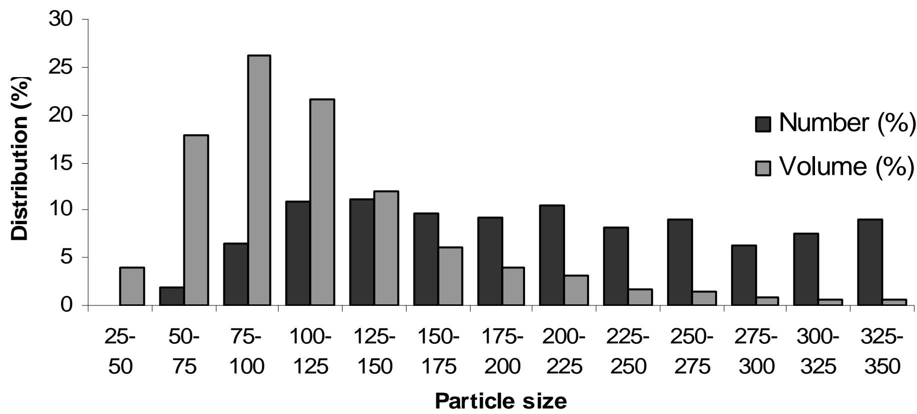
to be reliable. Live feed or other feed ingredients containing high concentrations of non-protein N should therefore be determined by increase in FAA or by detecting free amino groups (Lindner *et al*., 1995). The well established pH stat method should also be considered, although the method may not be suited for feed samples that have been partially hydrolyzed during their preparation (Dimes *et al*., 1994).

4 Discussion of results

4.1 Particle size

The protein cross-linked particles had a mean particle size of 114 ± 68µm (number %) (Paper I) similar to previous produced cross-linked diets (Yùfera *et al*., 1999). The data confirms the ability of the production method to produce stable particles within a size range optimal for start feeding of most marine fish larvae. The particle size was measured in dry form (Paper I), although Jayakrishnan *et al*. (1994) reported a 25 % increase in diameter (1 fold increase in volume) upon swelling in water. Dependent on the method used, the protein cross-linked diet has been produced in a size range from 6.06 ± 2.37 µm (Langdon 1989) to 171 ± 95 µm (Yùfera *et al*., 1995). The cross-linked particles are freeze dried and contain a porous matrix and have to saturate with water before they sink (Yùfera *et al*., 1999). This has been shown to be a rather rapid process for small particles (results not shown), however, it may be inappropriate to use freeze dried particles of a larger size since the time before hydration and sinking may be to long. It is therefore questionable if freeze dried particles of a larger size could be of practical use.

For the heat coagulated diet there was a good correlation between size distribution described as volume % and number % (Figure 6). However, for the protein cross-linked diet there was a large difference in size distribution described as number % or volume % (Figure 6). If fish larvae fed the respective cross-linked diet (Figure 6) could not ingest particles larger than 199 µm, 50 % of a diet with a mean particle size of 114 ± 68µm (number %) would not be ingested.



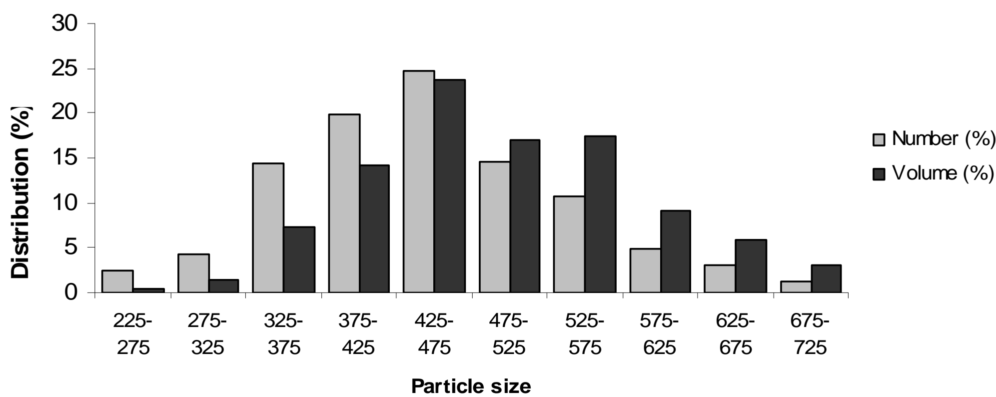


Figure 6ab: Particle size distribution measured for the protein cross-linked diet (a) and the heat coagulated diet (b) described as number % and volume % (Paper I and III).

4.2 Change in nutritional quality due to feed production.

For salmon and trout, most nutritional studies are performed on diets produced with similar production processes. In contrast, nutritional studies on marine fish larvae has been performed with numerous diets (Langdon, 2003), using different production processes. The same ingredient composition in different type of diets, have shown to have severe affect on larval performance (Barrows and Lellis, 2006). Feeding larval Walleye (*Sander vitreus*) with the same nutrient mix gave a survival rate of 49.1 ± 5.4 and 27.6 ± 9.9 dependent on the type of diet used (Barrows and Lellis 2006). The large changes in nutritional quality and protein digestibility due to the production process (Paper I, II, and III); stress the importance of investigating the effect of the respective production processes on the nutritional quality. This

is presumed to be of particular importance for the protein fraction, since soluble protein is highly susceptible to denaturation (paper I and III) and is considered to be important for marine fish larvae (Carvalho *et al*., 2004).

There were severe changes in protein quality during the production of the protein cross-linked diet (Paper I) and heat coagulated diet (Paper III). Nearly all water soluble proteins were made insoluble during the cross-linking process, but the concentration of TCA soluble N was not largely affected by this process (Paper I) as earlier suggested (López-Alvarado *et al.,* 1994; Yùfera *et al.,* 2002). However, most of the TCA soluble N was lost by leaching during the following washing stages (see Fig. 1, Paper I). It may be difficult, if not impossible, to produce a protein cross-linked diet with significant amounts of water soluble protein. However, FAA and peptides may be delivered in sufficient amounts if the particles are not exposed to aqueous solution after the cross-linking according to method by Langdon (1989). Due to denaturation, the heat coagulated diet had a reduction in non-TCA soluble N, from 66 % to 30 % with increased inclusion of pepsin hydrolyzed protein (Table 7). According to the results in paper II, the fresh cod fillet contains 16 % non-TCA soluble N while the pepsin hydrolyzed cod fillet contains 26 % non-TCA soluble N. This suggests that fish fillet contains heat sensitive native proteins, while the pepsin hydrolyzed cod fillet consists of a larger fraction of heat stable, partly digested, soluble protein and large peptides. The separation of the non-TCA soluble fraction and inclusion into heat treated diets will both reduce the loss of soluble N during production and reduce the leaching rate compared to the use of FAA and small peptides as shown by Kvåle *et al*. (2006). The inclusion of heat stable, soluble proteins such as casein, may also be a good alternative for diets that are exposed to heat. Casein appears to be highly digestible, according to several *in vitro* digestibility studies (Lan and Pan 1993; Lazo *et al*., 1998; Gopalkrishnan and Prakash 2000; Paper II) and has been used in

several compound diets with positive results (Cahu and Zambonino Infante 1995; Robin and Vincent 2003; Carvalho *et al.*, 2004). *Artemia* cysts heat treated for 5 min at 60 °C, 80 °C and 96 °C had an approximate decrease in soluble protein of 30 %, 61 % and 74 % (of tot. soluble protein) respectively (Garcia-Ortega *et al.*, 2000b). This indicates that although heating of the ingredient mixture might be necessary during production (Garcia-Ortega *et al.*, 2000a; Paper III), the temperature used should be kept as low as possible for the respective diet.

Table 7: Concentration of non – TCA soluble N in heat coagulated diets with 0, 15, 30 and 45 % of the protein being pepsin hydrolyzed cod fillet (Paper III). The concentration of non-TCA soluble N is based on the total concentration of soluble N minus the concentration of TCA soluble N (Paper III).

	0	**15**	**30**	**45**
Non - TCA soluble N	(% of tot N)	(% of tot N)	(% of tot N)	(% of tot N)
Feed ingredients	21	22	23	24
Produced diets	7	9	17	17

The concentration of FAA and peptides does not seem to be affected by heat treatment (Paper III) and it is not presumed that other production processes will severely modify FAA and peptides. For diets exposed to aqueous solution during the production process (Polk *et al.*, 1994; Kelly *et al.*, 2000; Önal and Langdon 2000; Yùfera *et al.*, 2005), leaching may occur during production as shown for the protein cross-linked diet (Yùfera *et al.*, 2002). The commercial diet Minipro^{TM} had a high content of TCA soluble N (32 ± 1.9 %, Paper II). As described in section 1.6, most chitin particles are cross-linked in an aqueous solution were severe leaching may occur. The cross-linking of chitosan or alginate particles by “falling” through a fog of either alkaline solution or calcium chloride (Høgøy, 2005) seems to be a good method to prevent production losses of soluble nutrients. Unfortunately, the leaching rate during feeding of this diet is not known.

Both cross-linking and heat treatment led to a decrease in *in vitro* digestibility of protein compared to the digestibility of the raw ingredients (Table 8 and 9). The *in vitro* digestibility of the mixed ingredients for the heat coagulated diet is calculated from the *in vitro* digestibility of the respective ingredients (Table 9). Since the digestibility of the ingredient mixture of the cross-linked diet was equal to the sum of the digestibility of the respective ingredients (Paper II), it is presumed that the calculation of the raw ingredients digestibility for the heat coagulated diet is reliable.

Table 8: Protein *In vitro* digestibility of raw ingredient mixture, crushed protein cross-linked particles and whole protein cross-linked particles at different time intervals. The protein digestibility is evaluated as the concentration of TCA soluble N.

	0 h (%)	**1 h** (%)	**12 h** (%)	**Relative dif. 1 h and 12 h** (%)
Raw ingredients	8.3 ± 0.6	54.6 ± 2.1	70.6 ± 1.4	23
Crushed particles	1.5 ± 0.9	30.4 ± 0.8	54.3 ± 1.0	44
Whole particles	1.0 ± 0.4	29.4 ± 1.2	53.0 ± 2.3	
Relative dif. raw ingredients/whole particles		46	25	

Table 9: Change in protein *in vitro* digestibility in the raw ingredients (calculated), the produced diet, and the leached heat coagulated diet after a pancreatic digestion for 12 h.

Concentration of hydrolyzed protein	**0** (%)	**15** (%)	**30** (%)	**45** (%)	**Ref**
Sum of digested ingredients	80	81	82	82	Paper II
Heat coagulated diet	65	64	65	65	Paper III
Leached heat coagulated diet	62	60	62	60	Paper III

The reduced protein digestibility of heat treated diets is according to previous *in vitro* and *in vivo* studies (Garcia-Ortega *et al*., 2000ab; Garcia-Ortega *et al*., 2001; Bustos *et al*., 2003). Larvae of African catfish (*Clarias gariepinus*) had a reduced growth rate using *Artemia* cysts exposed to more than 40 °C and *in vitro* digestion of a microbound diet containing heat treated *Artemia* cysts had a significant lower digestibility than a microbound diet containing untreated cysts (Garcia-Ortega *et al*., 2001). Diets produced without high temperatures (Table

10), and with a drying temperature below 40 °C (Garcia-Ortega *et al*., 2001) are therefore recommended.

Table 10: Experimental diets produced without the addition of heat, which may be able to deliver soluble proteins without denaturation or loss by leaching during the production process.

Experimental diets	**Ref**
Microextrusion marumerization (MEM)	Barrows and Lellis, 2006
Particle-assisted rotational agglomeration (PARA)	Barrows and Lellis, 2006
Zein bound particles	Önal and Langdon, 2005a
Carboxymethyll-cellulose bound particles	Pousao-Ferreira *et al*., 1999
Alginate bound particels (not bound in an aqoues solution)	Guthrie *et al*., 2000

Due to crushing and sieving, the protein heat coagulated diet has a uniform structure throughout the particle, while the protein cross-linked diet has a protein membrane that differs from the inner structure (Jones, 1980). *In vitro* digestion of whole cross-linked particles and cross-linked particles that were thoroughly crushed showed no significant difference at either 1 h or 12 h (Table 8) (Paper II and unpublished results). This indicates that it is the cross-linking process that reduces the protein digestibility (Paper II) and not the production of an indigestible outer membrane that can not be penetrated by proteolytic enzymes. In addition, there was a more rapid digestibility of the raw ingredients than of the produced cross-linked diet (Table 8). The time for complete digestion of different feed and feed ingredients, may be essential due to the short retention period of feed in marine larvae and should be considered.

The cross-linked protein walled diet and the protein heat-coagulated diet (Paper I and III) use protein as a binder and it will therefore be possible with a complete enzymatic degradation of the feed particles (Paper II and III). However, the commercial Maripro diet (Paper II) use chitin as a binder (Høgøy, 2005) and the binder will therefore not be digested in the pancreatic *in vitro* digestibility system. Although the binder was indigestible, there was a 24 % better protein digestibility compared to protein cross-linked capsules (Paper II). *In vitro* digestibility

of diets containing non-protein binders is in accordance with previous studies showing a good protein digestibility in systems not containing enzymes to digest the respective binders (Garcia-Ortega *et al*., 2000b; Garcia-Ortega *et al*., 2001). Although binders that are presumed to be indigestible can be used successfully (Partridge and Southgate 1999; Garcia-Ortega *et al*., 2001; Yùfera *et al*., 2005; Barrows and Lellis 2006), the use of indigestibility binders should be more thoroughly investigated.

There was no change in the AA profile due to the production process of the cross-linked diet (Paper I), except for a total loss of taurine due to leaching (see chapter 4.5.3). Although the production process led to a decrease in digestibility for both the heat coagulated and the cross-linked diet, the digested solutions had the same AA profile as the produced diet (Paper III and results not shown). This indicates that both diets may be used to deliver the wanted AA profile to fish larvae.

4.3 Feed ingredients

The water soluble N fraction left in the heat treated marine meals consists mostly of TCA (10%) soluble N (Table 11) which according to Greenberg and Shipe (1979) consists of nitrogenous compounds below 380 D. The fish meal investigated in paper II (Table 11) is made from fresh frozen fish fillet (Coalfish) and had similar concentration of soluble N fraction as the heat coagulated diet (0 % hydrolyzed protein; Table 11), indicating a similar change in protein quality during the fish meal production as in production of the heat coagulated diet (Table 11). With similar N solubility in the fish meal and the heat coagulated diet (Table 11) and a lower digestibility of the heat coagulated diet compared to the fish meal (Paper II and III), there may be no benefit using fresh fish fillet instead of fish meal as long as the diet is exposed to heat. Considering the high leaching rate of FAA and peptides, the use

of fish fillet in a compound diet not exposed to heat (Table 10) should be considered due to the relative high concentration of soluble large molecular weight N compared to fish meal (Paper II).

Table 11: The concentration of water soluble and TCA soluble N in several ingredients and larval diets.

Sample	**Soluble fraction** (% of tot N)	**TCA soluble fraction** (% of tot N)	**Ref**
Rotifers	56.9	-	Srivastava *et al*., 2006
Artemia (nauplii)	67	30	Paper II
Calanus (copepod stage)	54	-	Paper II
cod fillet	34	18	Paper II
Pepsin digested cod fillet	65	39	Paper II
Pankreatic hydrolyzed (12 h)		79.9 ± 9.1	Paper II
Pepsin + pankreatic (24 h + 12 h)		86 ± 2.7	Paper II
Squid meal	11	15	Paper II
Fish meal	17	15	Paper II
Roe meal	11	12	Paper II
Stick water	100	63 ± 0.6	Paper II
Casein	0	5 ± 0.0	Paper II
Na^+-caseinate	100	6 ± 0.6	Paper II
Protein encapsulated diet	2.9 ± 0.9	3.4 ± 0.5	Paper II
Minipro		32 ± 1.9	Paper III
Heat coagulated diet (0 % hydr.)	17.9 ± 0.9	10.6 ± 0.6	Paper III
Heat coagulated diet (15 % hydr.)	21.7 ± 4	13 ± 1	Paper III
Heat coagulated diet (30 % hydr.)	36.7 ± 0.8	20.3 ± 0.7	Paper III
Heat coagulated diet (45 % hydr.)	38 ± 1	21.6 ± 0.9	Paper III

Stick water is the water soluble nitrogenous fraction left during fish meal production and consists mostly of low molecular weight N (Table 11) (Paper II). The nutritional quality of stick water for marine fish larvae has not been thoroughly investigated, but the high content of TCA soluble N suggests that it may be a promising feed ingredient for larval diets. However, the *in vitro* digestibility indicated a very poor protein digestibility of the non TCA soluble N fraction (Paper II). If a diet containing stick water is exposed to severe leaching, there will be

a rapid loss of the low molecular weight N (Kvåle *et al.*, 2006) and the diet may be left with the stick water fraction of low digestibility.

As suggested by Garcia-Ortega *et al.* (2001) and Carvalho *et al.* (2004) the results in paper II and III indicate that the soluble protein fraction has a higher digestibility than the insoluble protein fraction. There was no difference in the digestibility of the soluble and non-soluble casein (12 h) as presumed according to an *in vitro* digestibility study with protease extract from the midgut gland of mature grass shrimp (*P. monodon*), showing a three fold increase in digestibility (Lan and Pan 1993). However, after 1 hour digestion, the soluble casein had a 24 % higher protein digestibility than the insoluble casein (Paper II), indicating a more rapid digestion according to results by Lan and Pan (1993).

4.4 Hydrolysis of feed ingredients

It is well accepted that addition of hydrolyzed protein will improve the performance of marine fish larvae (Zambonino Infante *et al.*, 1997; Cahu *et al.*, 1999; Carvalho *et al.*, 2004). However, a wide variety of hydrolyzed proteins have been investigated, with large qualitative differences dependent on the enzymatic hydrolyzation method and the feed ingredients hydrolyzed (Table 12). Previous studies have aimed to find an optimal level for inclusion of hydrolyzed protein (Zambonino Infante *et al.*, 1997; Kvåle *et al.*, 2002; Kvåle *et al.*, 2007b), but the initial concentration of low molecular protein compounds in the other feed ingredients or the change in quality during the production process has been given little attention. Using fresh fish fillet in a compound diet not exposed to heat, the concentration of soluble N would be similar to the concentration of soluble N in the heat coagulated diet containing 30 % pepsin hydrolyzed protein (paper III), although there would be significant qualitative differences in the soluble fraction. The need for extra inclusion of hydrolyzed protein is probably lower in a

diet based on fresh fillet than if the diet is based on fish meal. It may therefore be misleading to recommend inclusion levels of hydrolyzed protein, if not an additional evaluation of the other feed ingredients and the feed process is performed.

Table 12: Concentration of soluble N (Phosphate buffer pH (8) and TCA soluble N in cod fillet exposed to different hydrolysation methods.

Hydrolyzation method	**Soluble N**	**TCA soluble N**
	% of total N	% of total N
Untreated cod fillet[1,2]	34 (n=1)	18 ± 2.4
Pepsin hydrolyzed (24 h)[1,2]	65 ± 3	40 ± 4.2
Pancreatic hydrolyzation (12 h)[2]		79 ± 9.1
Pepsin + pancreatic (24 h + 12)[2]		86 ± 2.7

[1]Paper III
[2]Paper II

The pepsin hydrolyzed cod fillet (Paper II and III) was not separated into the respective soluble and insoluble fractions; instead the complete hydrolyzed product was incorporated into the respective diets investigated in paper III. When hydrolyzing the main protein source of the diet instead of adding increasing amounts of a different hydrolyzed protein source, the diet will keep the same AA profile only differing in solubility of the N fraction. This is in accordance with Dabrowski *et al.* (2003), that argued that the same source of protein had to be fed in both intact and hydrolyzed forms to investigate if inclusion of protein hydrolysis's leads to enhanced growth in larval fish. However, the non soluble fraction of the hydrolyzed protein may have a lower digestibility than the insoluble fraction of the non hydrolyzed protein (paper II). There were only small differences in AA profile of the leached and not leached diet containing 30 % hydrolyzed protein (paper III), indicating that there are no severe differences in AA profile between the soluble and insoluble fraction of the hydrolysed cod fillet (Paper II and III). The pancreatic digestion of the heat coagulated (paper III) and cross-linked diet (results not shown) led only to small differences in AA profile between the digested TCA soluble fraction and the not digested diet (Paper III). The inclusion of only the

TCA soluble fraction of pancreatic hydrolyzed feed ingredients (Paper II) may therefore be an appropriate method and still have a similar AA profile as argued by Dabrowski *et al*., (2003). Due to the indication that the insoluble fraction of hydrolyzed ingredients has a lower digestibility (Table 8 and 9), it may be recommendable to separate the insoluble fraction prior to inclusion of hydrolyzed ingredients to formulated diets.

4.5 Leaching from formulated feeds

4.5.1 Leaching of proteins, Peptides and FAA

The leaching of the heat coagulated diets (Paper III) confirms previous studies showing a high loss of both soluble protein and low molecular weight nitrogenous compounds when compound diets are exposed to aqueous solution (López-Alvarado *et al*., 1994; Kvåle *et al*., 2006). In addition, there was an increased loss of low molecular weight nitrogenous compounds compared to those of larger molecular size (Paper III; Kvåle *et al*., 2006). Interestingly, there was no significant difference in the retained concentration of FAA and peptides between the diets with increased concentration of hydrolyzed protein after exposure to leaching for 6 min (Paper III). The data supports the suggestions that loss of soluble N may mask the effect of increased inclusion of hydrolyzed protein in feeding trials with marine fish larvae (Kvåle *et al*., 2002, 2007b). However, it should be emphasized that 6 min is a long exposure to leaching and most particles will most likely be ingested within this period using proper feeding routines.

4.5.2 Other micronutrients

The amount of soluble vitamins and minerals lost during leaching from larval diets has been given little attention, but may be severe (Marchetti *et al*., 1999; Langdon 2003; Yùfera *et al*., 2003). The nearly complete loss of zinc and thiamin due to water exposure of the cross-linked

diet (Paper I) confirms that the loss of micronutrients may be comparable to that of FAA (López-Alvarado *et al*., 1994; Kvåle *et al*., 2006), although the cross-linked diet was exposed to water for approximately10 min. The minerals of feed and feed ingredients are naturally found in the ash fraction. By washing pink Perch meat for surimi production there was a decrease in ash from 5 % (dw) to 1 % (Karthikeyan *et al*., 2006), indicating that most ash compounds in fish fillet are water soluble. Dependent on concentration of hydrolyzed protein, the weight loss by leaching from the heat coagulated diets was 5 - 10 % higher (dw) than the calculated weight loss of soluble N (Paper III). The weight loss is similar to the concentration of ash found in the four investigated diets (Kvåle unpublished) and indicates a complete loss of ash and thereby minerals during the leaching trial. In comparison to most EAA, that can be delivered as part of an insoluble complex protein, concentrations of bound soluble micronutrients present in conventional marine feed ingredients seems to be small (Paper I).

4.5.3 Loss of Taurine

Taurine is an organic acid that is abundant in the tissues of many animals. It exists only in free form and not as part of a complex protein. Taurine is described as a non-essential AA, but there are several indications that taurine is essential at early larvae stages. Japanese flounder (*Paralichtys olivaceus*) (Chen *et al.,* 2004, Chen *et al.,* 2005, Kim *et al.,* 2003, Kim *et al.,* 2005, Matsunari *et al.,* 2003, Chen *et al.,* 2002, Takeuchi *et al.,* 2001) and Sea bream (*Pagrus major*) (Chen *et al.,* 2004) fed taurine enriched rotifers showed increased growth and according to studies by Kim *et al*. (2005) juvenile Japanese flounder required at least 15 mg g^{-1} taurine in the diet. Both the protein encapsulated diet and the heat coagulated diet had a 100 % loss of taurine after exposure to water (Paper I and III). The initial concentration of taurine in the raw ingredients of the protein cross-linked diet (Table 13) was approximately 6 times lower than in copepods and 4.5 times lower than *Artemia* (Table 13; Van der Meeren

et al., 2003). The concentration of taurine in the heat coagulated diet was 3.9 times lower than in copepods and 2.8 times lower than in *Artemia* (Table 13; Van der Meeren *et al.,* 2003). However, the concentration was 7.5 - 22 times higher than in rotifers (Table 13; Van der Meeren, 2003; Srivastava *et al.,* 2006). This indicates that most ingredients used for larval diets are insufficient in taurine and extra addition should be considered. In addition, there will most likely be a severe loss of taurine from all conventional diets (Paper I and III).

Table 13: Concentration of taurine in ingredients and live feed for marine fish larvae.

Feed and feed ingredients	**Taurine** mg g^{-1} (dw)	**Ref.**
Rotifers	0.21 – 0.62	Van der Meeren, 2003; Srivastava *et al.*, 2006
Artemia	13	Van der Meeren, 2003
Copepods	18	Van der Meeren, 2003
Heat coagulated diet	4.7	Paper III
Raw ingredients protein encapsulated diet	2.9	Paper I
Protein cross-linked diet	ND	Paper I
Fish fillet	0.4 -3.2	Zhao et al., 1998

4.6 Use of LSB to prevent leaching

Lipid particles containing soluble micronutrients, drugs or other chemical compounds have shown promising for delivering of highly leachable compounds to marine organisms (Buchal and Langdon 1998; Önal and Langdon 2000; 2004b; 2004a; Önal and Langdon 2005a; Clack 2006; Templee 2006). Briefly, the production method involves melting of a lipid solution and mixing core materials with the molten lipid. The molten lipid solution is then sprayed into a cold chamber where the lipid particles solidify. The core material can either be crystals or an aqueous solution/slurry (Figure 7). All nutrients, either soluble or insoluble, may be possible to incorporate into LSB. The only restriction for delivery of soluble compounds with LSB is presumed to be compounds not resistant to the temperature necessary to melt the lipid

solution. For the LSB investigated in paper IV, the melting and spraying temperature was approximately 60 °C, but if producing LSB containing higher concentration of phospholipids than used in paper IV, a higher temperature may be necessary. Although solely feeding of LSB to fish larvae has been accomplished (Önal and Langdon, 2004a), the most promising use of LSB is incorporated into complex particles (see chapter 1.6).

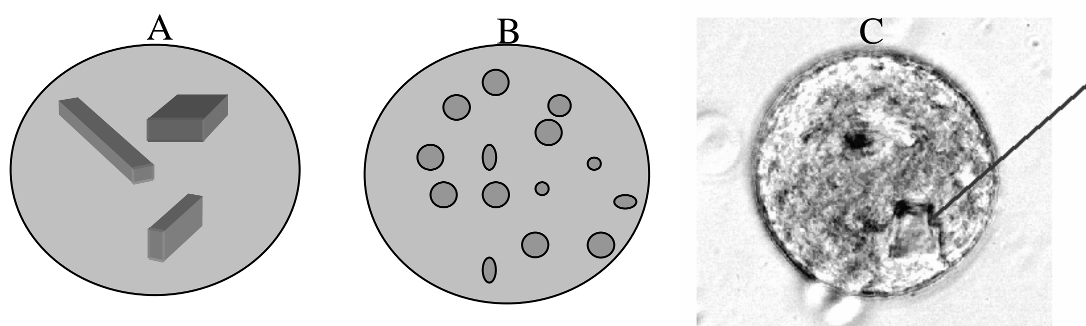


Figure 7: A: LSB containing crystal compounds. B: LSB containing aqueous slurry of soluble compounds. C: LSB made of menhaden stearine and 40 % soy lecithin containing crystal glycine (Paper IV). The picture is taken after 10 min exposure to leaching and the retained glycine crystal is marked with a red arrow.

4.6.1 Lipid quality of LSB

Previous studies of LSB have been performed with LSB made of triacylglycerides and waxes (Buchal and Langdon 1998; Langdon and Buchal 1998; Önal and Langdon 2004ab; 2005b; Önal and Langdon 2005a). To be of practical use, the LSB have to be solid at room temperature and may therefore not contain large concentrations of unsaturated fatty acids. However, LSB made with menhaden stearine have shown to be digestible in fish larvae (Önal and Langdon 2004b) and contains 10.6 % EPA (of tot. FA) and 9.1 % DHA (of tot. FA) (results not shown). In addition to be in-dispersible in water (Paper IV), LSB made of menhaden stearine has a relative low melting point thus making them impractical to use if not incorporated into a complex particle (Önal and Langdon, 2005ab). In addition to the benefit of producing water dispersible lipid particles (Paper IV), the use of phospholipids are beneficial

due to the high melting point even with a high content of unsaturated fatty acids. LSB containing 75 % krill phospholipids has successfully been made (Nordgreen and Langdon, unpublished) and the acetone precipitated krill phospholipids had an EPA content of 27 % (of tot FA) and a DHA content of 12 % (of tot. FA) (Nordgreen and Hamre, unpublished). Although a significant higher content of EPA and DHA than in menhaden stearine, the krill phospholipids were much harder at room temperature than the menhaden stearine.

The LSB used to enrich *Artemia* contained 22.7 % wax, 16 % fish oil and 55 % lecithin. They dispersed nicely and were easier to handle than the LSB containing menhaden stearine. This was probably due to the higher melting point of paraffin wax and increased lecithin concentration. Although the LSB were more heat stable, it was still important that the beads were kept cold during storage so the LSB did not get sticky, causing the particles to clump before they were fully dispersed in the water. Beads that would withstand a higher mechanical stress and higher temperatures during storage would be of great importance for large scale production of LSB. Increasing the melting point without jeopardizing the fatty acid profile is an unsolved problem which needs further investigation. It is questionable if a non-digestible wax should be chosen instead of highly saturated fat, such as palmitic acid (16:0). The use of an indigestible wax may be beneficial since only the more unsaturated fatty acids in the phospholipids or fish oil would be digested. Possible negative aspects of the use of waxes would be reduced digestion and/or increased gut evacuation.

4.6.2 Leaching from LSB

The leaching rate of the investigated LSB were severe (80 %; Paper IV) compared to previous data (Önal and Langdon 2004ab; Buchal and Langdon 1998; Clack 2006). As discussed in section 4.6.2,this is probably due to clumping of the LSB in previous studies. However, there

were a large increase in leaching when LSB were incorporated into complex particles (Önal and Langdon, 2005). This increase in leaching rate, is most likely because incorporated LSB will be in contact with water on all sides equivalent to the dispersed LSB (paper IV). The LSB incorporated into a complex particle (Önal and Langdon, 2005a) had the same loss within 20 min as the loss from the LSB in this study (Paper IV). This indicates that the LSB containing soy lecithin (Paper IV) did not have a higher leaching rate than LSB produced with menhaden stearine (Önal and Langdon, 2004b). There was a similar retention of the OTC after exposure to leaching for 20 min as for glycine, and interestingly there was a good retention of OTC after the first minutes of burst release (Paper IV). Although the loss of soluble compounds is presumed to be too high for delivering of nutrients needed in large quantities, such as FAA and peptides, the LSB may have a retention efficiency sufficient to deliver micronutrients. LSB containing similar levels of marine phospholipids as soy lecithin (Paper IV) have shown similar retention efficiency as investigated in this work (Langdon, unpublished). LSB produced with marine phospholipids may be incorporated into the complex particles in larger quantities due to the improved fatty acid quality.

4.6.3 Leaching in ethanol

When producing complex particles containing LSB, the feed ingredients and the LSB are dispersed in a solution of 90% ethanol and spray dried (Önal and Langdon, 2005a). FAA are sparingly soluble in ethanol and there will be insignificant losses from the LSB during the production process (Clack, 2006). As previously discussed, the use of complex particles may be a more appropriate tool to deliver specific AA and micronutrients instead of crude FAA. Unfortunately, many of the micronutrients are highly soluble in ethanol and may be lost during the production (Table 14). OTC is successfully incorporated into LSB (Paper IV), but OTC is highly soluble in ethanol and LSB made of 100 % menhaden stearine had a 90 % loss

after 10 min leaching in ethanol (Nordgreen, unpublished). This indicates that the use of the complex particles (Önal and Langdon, 2005a) for delivering ethanol soluble nutrients should be considered carefully.

Table 14: Solubility of selected micronutrients in water and ethanol.

Water soluble compounds	**Water**	**Ethanol**
FAA in general	Highly soluble	Low solubility
L-Proline	Highly soluble	Highly soluble
OTC	Highly soluble	1 gram per 35 ml
Taurine	Highly soluble	Insoluble
Thiamine	Highly soluble	Highly soluble
Riboflavin	110 mg l^{-1}	45 mg l^{-1}
Ascorbic acid	Highly soluble	Highly soluble
Iodide	Highly soluble	Highly soluble
Iron (III) chloride	Highly soluble	Highly soluble
Sodium sulfate	Highly soluble	Insoluble

4.6.4 Live feed enrichment with LSB

The recommended optimum particle size for *Artemia* is 6.8 µm and 27.5 µm (Gelabert Fernandez, 2001) and the smallest size class of *Artemia* (1-1.99 mm) had the highest ingestion rate of particles measuring 10 µm (Gelabert Fernandez 2003). This is significantly larger than the reported particle size in standard lipid emulsions (Han *et al*., 2005; Leger *et al*., 1987). Han *et al*. (2005) reported that 50 % of the volume-weighed diameter of emulsion droplets (ICES; 30 % DHA and 50 % DHA) was bellow a particle size of 1.07 ± 0.03 and 0.11 ± 0.03 µm dependent on blending procedures and lipid quality. A Selco product was reported to have an emulsion particle size of approximately 2 µm (Leger *et al*., 1987). The LSB can be produced within the size ranges suggested to be optimal for *Artemia* at all stages (Gelabert Fernandez, 2001) and were efficiently ingested by *Artemia* (Paper IV). The seven fold increase in riboflavin concentration with one hour enrichment (Paper IV), suggests that

dispersible LSB may be an effective tool for enrichment of water soluble nutrients. However, riboflavin has a low solubility (Fig 8), and Önal and Langdon (2004ab) showed a several fold increase in leaching rate with glycine compared to riboflavin. This suggest a less efficient enrichment of more soluble compounds as glycine (Paper IV).

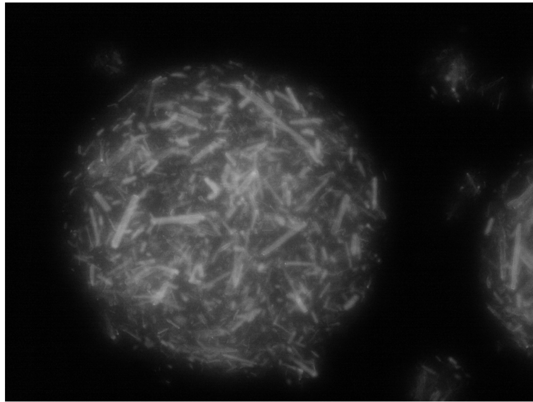


Figure 8: Left: LSB prepared with paraffin wax, soy lecithin and cod liver oil (227 g kg^{-1}, 545 g kg^{-1} and 164 g kg^{-1}) and 64 g kg^{-1} riboflavin (Table 3, LSB type 1). The picture was taken using an epifluorescent microscope (Leica DM1000, Leica inc) fitted with a UV light source (excitation of 450 - 490nm and emission of 515 - 528nm). LSB were dispersed in seawater for one hour and 55 min and riboflavin particles were still visible within the beads (Paper IV).

There were indications that *Artemia* masticated captured beads before ingestion (Figure 9), and the particle size appropriate for enrichment may therefore be larger than the particle size recommended by Gelabert Fernandez (2001) using hard latex particles. Larger LSB, may reduce the leaching rate (Kvåle *et al*., 2006) and the incorporated nutrients may be more rapidly released if the LSB are ruptured upon ingestion. Previous studies have indicated that the intestinal content of *Artemia* constitutes for a insignificant part of the total lipid content of *Artemia* (Smith *et al*., 2002). However, the lipid content of newly hatched *Artemia* (*A. franciscana*) is 145 mg g^{-1} dw (Evjemo *et al.,* 2001) while the lipid content of enriched *Artemia* is reported to be 220-250 mg g^{-1} dw (Evjemo *et al.,* 2001; Vander Meeren, 2003). The present enrichment study (Paper IV) suggests that the intestinal lipid content would represent from 18 to 30 % of the total lipid content of the *Artemia* (dw).

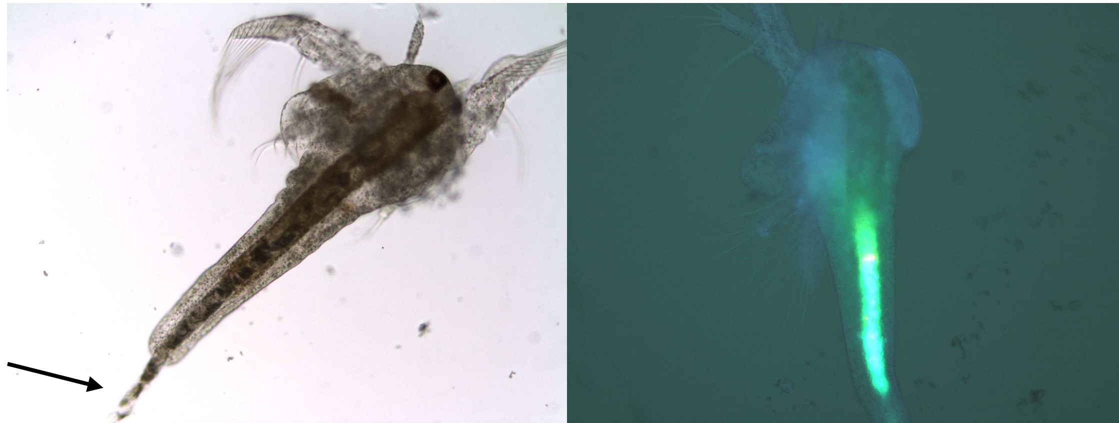


Figure 9. Left*: Artemia* enriched with LSB containing riboflavin after one hour enrichment; a string of faeces can be seen (Leica, DM 1000). Right: Digestive tract of *Artemia* fed on riboflavin-containing LSB, filled with fluorescent riboflavin, shows that *Artemia* ingested LSB. Whole LSB are not visible, indicating that they were broken down. The picture was taken using an epifluorescent microscope (Leica, DM1000, Leica inc) fitted with a UV light source (excitation of 450 - 490nm, emission of 515 - 528nm) (Paper IV).

The lipid class composition in *Artemia* does not seem to be influenced by enriching with polar lipids (Rainuzzo *et al*., 1994; Harel *et al*., 1999), and it may therefore be questionable to use marine phospholipids in enrichment products, due to the high price of these products. On the other hand, the use of enrichment products containing marine phospholipids have shown significantly increase the incorporation of DHA (McEvoy *et al*., 1996; Harel *et al*., 1999). *Artemia* enriched with herring roe phospholipids in tuna orbital oil and Super Selco had DHA concentrations of 19.4 ± 1.1 % and 7.4 ± 1.1 % (of tot FA) respectively, although the two enrichment products had similar concentration of DHA (McEvoy *et al*., 1996). In addition, delivering of DHA in PL form gives a significant higher DHA content in the polar fraction of the *Artemia* (Harel *et al*., 1999). The environment for live feed enrichment is optimal for aggressive per-oxidation due to heavy aeration, high temperature and strong illumination. DHA in PL form has shown to be less exposed to peroxidation than DHA in TAG form (King *et al*., 1992b; 1992a; Song *et al*., 1997). During a 10 week oxidation trial, 90 % of the phospholipids bound DHA was retained while 97 % of the TAG bound DHA was lost by oxidation (Song *et al*., 1997).

Although marine phospholipids (from Krill) has a relative high n-3 HUFA (47.9 % of tot FA) (Monroig *et al*., 2006), there is a high concentration of palmitic acid (16:0) (26.5 % of tot FA) compared to Super Selco (2.8 % of tot FA) (Monroig *et al*., 2006). Interestingly there were no significant difference in the HUFA n-3 profile between *Artemia* enriched with LUV detergent liposomes made of krill phospholipids compared to the Super Selco enriched *Artemia*, and only an increase in 16:0 from approximately 9 % to approximately 11.5 % (Monroig *et al*., 2006). This indicates that marine phospholipids may be used for enrichment without a severe increase in the concentration of 16:0.

4.6.5 Co-feeding fish larvae with LSB

LSB are not appropriate as a complete diet due to the high lipid content, but may be a good tool as a supplement to deliver an additional concentration of chosen nutrients or bioactive compounds which are difficult to deliver through live feed or compound diets. LSB have previously been successfully start fed to larvae of Zebra fish (*Danio rerio*) and glowlight tetra (*Hemigrammus erythrozonus*) (Önal and Langdon 2004a). Zebra fish start fed with LSB (menhaden stearine) containing OTC had a concentration of 119 ng OTC per larvae after one single meal lasting for 2 h and a retention of 39 ng OTC per larvae after gut evacuation (Temple and Langdon, unpublished). However, when using LSB containing 100 % triacylglycerol the LSB must be dispersed with an emulsifier prior to feeding (Templee, 2007) and it is questionable if this is a practical method if LSB are to be used in large scale experiments. The dispersible LSB (Paper IV) were successfully start fed to three days old gobies and rockfish larvae (Nordgreen, unpublished). Start feeding gobies at day three after hatching with LSB containing fluorescent riboflavin, 61 ± 15 % (n= 3 tanks) ingested LSB within one hour (Figure 10). The capability of the larvae to digest the LSB was not investigated and needs further investigation, although as seen figure 10, there is release of

riboflavin seen in the hind gut indicating a rupture of the LSB.

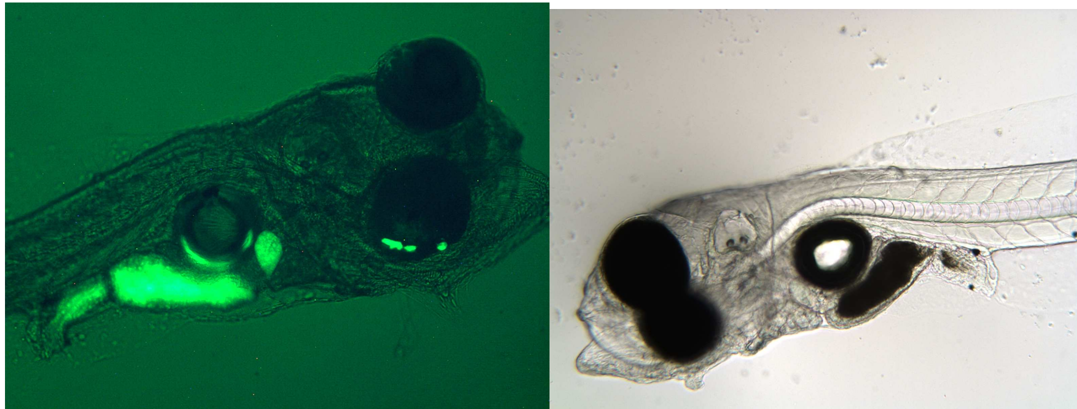


Figure 10: A three days old goby larvae with a length of approximately 2 mm. The goby has been eating LSB containing fluorescent riboflavin. Left: Picture taken with fluorescent microscope. LSB can be seen as green fluorescent light in mid gut and hind gut. The picture was taken using an epifluorescent microscope (Leica DM1000, Leica inc) fitted with a UV light source (excitation of 450 - 490nm and emission of 515 - 528nm). Right: Picture taken with normal light (Leica DM 1000). The LSB can bee seen as black “spots” filling the mid gut of a three days old goby larvae.

5 Conclusions

1. During production of the protein encapsulated diet nearly all water soluble micronutrients were lost (Paper I). It is not recommendable to use this diet for studying the effect of water soluble nutrients without changes in the production protocol. There was a reduction in *in vitro* protein digestibility due to the production process (Paper III) that should be considered and investigated more thoroughly.

2. Leaching of the formulated feeds in these studies led to a 100 % loss of Taurine (Paper I and III). In addition, there is a low concentration of Taurine in fish meal based diets (Paper I and III) compared to copepods and additional supplementation should be considered. If Taurine proves to be an essential AA for pre-metamorphic fish larvae, *Artemia* or Rotifers may be enriched with Taurine by using the dispersible LSB (Paper IV).

3. More than 50 % of the crude protein in live feed and unprocessed fish fillet is water soluble N where half of this fraction is larger peptides and soluble proteins (Paper II). Three different fish meals had a concentration of soluble N ranging from 11 - 17 % where nearly 100 % were FAA and small peptides (Paper II).

4. *In vitro* digestibility indicated that digestibility of the water-soluble N fraction was general higher than that of the insoluble N fraction (Paper II and III).

5. By inclusion of high concentrations of phospholipids into LSB, we were able to produce LSB that dispersed freely and were good vehicles for delivering both lipid and water-soluble compounds to suspension-feeders such as *Artemia.* The concentration of riboflavin was increased 7 fold within 1 hour enrichment.

6 Future perspective

6.1 True leaching rate from formulated feed

This work support the hypothesis that high loss of water soluble nutrients from formulated feed during feeding may significantly effect larval growth and survival. Although there have been done numerous studies on the *in vitro* leaching rate from different formulated feeds, no studies have to our knowledge tested the true leaching rate upon ingestion. There might be significant variations in feeding rate between species. Therefore, a formulated diet fed to one specie under optimal conditions might be eaten within less than a minute, while a slow feeding specie fed in access might use several min to eat most of the feed. It would be of great interest to investigate the real loss of nutrients upon ingestion with diets of known *in vitro* leaching rate. This could be done by incorporating a soluble and a non soluble marker in different compound diets for marine fish larvae. By doing this, we may be able to compare the difference in ratio between the soluble and non soluble marker in the diet and in the fish after feeding. This may show if *in vitro* leaching studies have under or over estimated the leaching rate. We may also be able to compare the difference in feeding rate between species, feeding systems and tank systems.

6.2 Fat soluble derivates

Although microspheres as LSB and liposomes are promising tools delivering highly soluble compounds to marine fish larvae, although they may not be suitable for large scale commercial production. Existing commercial diets, have a high leaching rate. A way to solve this problem may be to substitute water soluble micronutrients with a fat soluble derivate (Table 15). To successful use a fat soluble derivate, the derivate must be converted into its bio

available and bio active form by the larvae. Ascorbyl palmitate (AP) (Merchie *et al*., 1995b; Merchie *et al*., 1996a; Merchie *et al*., 1996b; Merchie *et al*., 1997a; Merchie *et al*., 1997b) and Lipiodol™ (Moren *et al*., 2006a) enriched *Artemia* have successfully been used to increase the supply of their respective water soluble derivate (ascorbic acid and iodine) to fish larvae, although it has been shown that European sea bass fed AP enriched dry diets had a lower assimilation and deposition compared to European sea bass fed a diet with supplemented ascorbic polyphosphate (Merchie *et al*., 1996b). Benfotiamine (Geyer *et al*., 2000) and riboflavin tetra butyrate (Yagi *et al*., 1970) which gave promising results for chicken and rats and the potential for supplementing thiamine and riboflavin to fish larval diets should be investigated. Phospholipids might be an important phosphor source for bone development. For marine fish larvae the use of phosphor salts may lead to leaching and reduction in the delivery.

Table 15: Fat soluble derivates of water soluble vitamins used in feeding trials.

Water soluble vitamin	**Derivate**	**Tested fish**	**Ref**
Iodide	Lipidiol	Halibut	Moren *et al*., 2006a
Vitamin C	ascorbyl palmitate	Turbot	Merchie et al., 1996a; Merchie *et al*., 1996b
Vitamin C	ascorbyl palmitate	Sea bass	Merchie *et al*., 1996b
Vitamin C	ascorbyl palmitate	African catfish	Merchie *et al*., 1995b; Merchie *et al*., 1997b
Vitamin C	ascorbyl palmitate	Rohu carp	Mitra and Mukhopadhyay, 2003
Thiamin	thiamin propyl disulfide	Chicken	Geyer *et al*., 2000
Thiamin	thiamin tetrahydrofurfuryl disulphide	Chicken	Geyer *et al*., 2000
Thiamin	Benfotiamine	Chicken	Loew 1996
Riboflavin	Riboflavin Tetrabutyrate	Rats	Yagi *et al*., 1970

6.3 Further development of LSB

The dispersible LSB containing high concentration soy lecithin proved to be a promising tool for enrichment of water soluble nutrients to *Artemia* (Paper IV) and Rotifers (unpublished results). However, the success of delivering highly soluble micronutrients will most likely be reduced compared to the less soluble riboflavin (Paper IV) and needs further investigation. The enrichment study in paper IV was terminated after on hour, and most of the riboflavin is therefore most likely still in the gut, the capability for the *Artemia* to digest incorporated nutrients are therefore unknown. If LSB are to be used as a vehicle for lipid enrichment, the LSB have to contain marine phospholipids instead of soy lecithin. Stable LSB containing 75 % krill phospholipids have been successfully produced (Nordgreen, unpublished). However a vast amount of work has to be accomplished to verify both technical and nutritional aspects of using LSB in lipid enrichment of live feed.

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Paper I

Evaluation of cross-linked protein capsules for delivering nutrients to marine fish larvae and suspension-feeders

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24 # Abstract

25

26 Cross-linked protein capsules as a vehicle for delivery of nutrients to marine fish larvae and
27 marine suspension-feeders were investigated. The effects of the production process on both
28 qualitative and quantitative changes in protein, lipid and micronutrient concentrations were
29 evaluated. There were no changes in lipid concentration and only minor (but statically
30 significant) differences in crude protein concentrations as a result of the encapsulation
31 process. However, there was nearly a complete loss of water-soluble nitrogen during capsule
32 production - almost 100 % of the water-soluble protein was cross linked and made insoluble
33 and 79 % of the TCA-soluble N was lost. Peptides and free amino acids were lost during the
34 capsule washing stages, but except for a 100 % loss of taurine, small changes in the amino
35 acid profile were observed. There was a more than 90 % loss of water-soluble micronutrients
36 such as thiamine, vitamin C and zinc during capsule production, and only minor increase in
37 concentration of thiamine and zinc in the diet by increasing the levels of these minerals and
38 vitamins were possible. The fat-soluble vitamin E was not affected by the production process
39 and can be delivered at controlled concentrations, but vitamin A had loss ranging from 4-57
40 % with increased inclusion of vitamin A. With the existing production protocol, the results
41 suggest that cross-linked protein capsules are not suitable for the delivery of water-soluble
42 nutrients to fish larvae and marine suspension-feeders.

43

44

45

46

47 # Introduction

48

49 There are many factors that influence survival, growth and deformities when rearing marine
50 fish larvae, but malnutrition is one of the major factors responsible for reduced growth and
51 survival (see reviews by: Rainuzzo *et al*., 1997; Koven, 2003; Kvåle *et al*., in press). To gain
52 more knowledge of the nutritional requirements of different species of marine larvae, we need
53 to carry out numerous basic nutritional dose-response studies were larvae are fed controllable
54 concentrations of the nutrient of interest. Live feed, such as rotifers and *Artemia,* are
55 successfully used for feeding fish larvae and efforts have been undertaken to develop
56 enrichment protocols for live feed to increase growth and survival (Watanabe *et al*.,1983;
57 Nichols *et al*., 1989; Rainuzzo *et al*., 1989; Olsen *et al*., 1993). Although the improved
58 enrichment protocols have increased both growth and survival (Kolkovski *et al*., 2000;
59 Olivotto, 2006; Park *et al*., 2006), the use of live feed for nutritional studies has major
60 drawbacks due to limited possibilities in delivering live feed with different and controllable
61 concentration of nutrients. Although it is possible to increase and, to some degree, control the
62 concentration of essential fatty acids in both *Artemia* (Sorgeloos *et al.*, 2001; McEvoy *et al.*,
63 1996; Narciso *et al.*, 1999; Evjemo & Olsen, 1997a; Takeuchi *et al.*, 1992) and rotifers (Dhert
64 *et al.*, 2001; Castell *et al.*, 2003), there are severe problems with rapid lipid metabolism and,
65 thereby, an uncontrollable change in the nutrients investigated (Olsen 1993; Evjemo *et al*.,
66 1997b). In addition to metabolism of enriched lipid, there are major technical problems with
67 delivering water-soluble nutrients with live feed. The replacement of live feed with
68 formulated feeds has been thoroughly investigated during the last 30 years (see review by
69 Langdon, 2003; Teshima *et al.*, 2000). There has been significant progress, but still
70 formulated diets that give results comparable to live feed for rearing larvae from the first
71 feeding stage have not been developed (Baskerville-Bridges & Kling, 2000). A formulated

72 diet needs to fulfill several technical and nutritional criteria to be used for rearing early fish
73 larvae with success (Langdon 2003). In addition to good growth and survival, a formulated
74 diet used for nutritional studies needs to deliver controlled concentrations of nutrients. It is,
75 therefore, important to develop a flexible particle type for delivery of diets with a wide range
76 of compositions. In addition, it is important to develop a dietary particle that can incorporate
77 and deliver water-soluble nutrients to fish larvae. There are severe problems delivering water-
78 soluble nutrients to marine larvae due to leaching (Lopez Alvarado *et al.*, 1994; Kvåle *et al.*,
79 2006), although improvements have been made (Yúfera *et al.*, 2002; Onal & Langdon,
80 2004ab).
81 Nylon-protein walled capsules (Chang *et al.*, 1966) were first evaluated in nutrition
82 studies with marine organisms by Jones *et al.* (1974). The invention is based on a process
83 whereby an aqueous dietary solution is emulsified in an organic solvent. Small emulsified
84 aqueous droplets, containing dietary nutrients and a wall forming nylon monomer, are
85 exposed to a chemical cross-linking agent producing stable microcapsules that contain the
86 nutrients (Chang *et al.*, 1966; Jones *et al.*, 1974). Nylon-protein capsules proved to be
87 indigestible by aya *(Plecoglossus altivelis)* and red sea bream *(Chrysophrys major*) at an early
88 stage (Kanazawa *et al*., 1982; Teshima *et al*., 1982), but further developments (Jones, 1980;
89 Hayworth, 1983; Langdon, 1989) led to a diet that had improved digestibility and did not
90 contain non-digestible compounds such as nylon-protein. The modified capsules were used in
91 several feeding trials with different larval species, but the protein-encapsulated diet proved to
92 be poorly broken down by sea bass larvae (*Lates calcarifer*) (Chu & Ozkizilcik, 1999) and
93 striped bass larvae (Chu & Ozkizilcik, 1999), although zebrafish (*Danio rerio*) larvae could
94 break down the cross-linked, protein-walled capsules (Onal & Langdon, 2000). By
95 resuspending cross-linked, protein-walled beads in a gelatin solution, beads with a softer and
96 thinner wall were developed (Fernández-Díaz & Yúfera, 1995). This modification led to

97 improved digestibility and growth of gilthead seabream (*Sparus aurata*) fed on encapsulated
98 diets (Yúfera *et al.*, 1995; Yúfera *et al.*, 1996; Fernández-Díaz & Yúfera, 1995). Feeding
99 trials with gilthead sea bream (*Sparus aurata*) showed that the diet could be successfully used
100 to rear larvae from day four after first-feeding (Yúfera *et al.*, 1999). In addition to improved
101 growth and survival, the modified cross-linked, protein-walled capsules were reported to
102 show good retention of free amino acids (FAA) (Yúfera *et al.*, 2002; Kvåle *et al.*, 2006).
103 Considering these promising reports, we evaluated the potential of using cross-linked,
104 protein-walled capsules for delivery of different nutrients to marine fish larvae and
105 suspension-feeders. The effects of the capsule-preparation process on qualitative and
106 quantitative changes in both macro and micronutrients were investigated.
107

108 Materials and methods

109

110 ***Production process***

111 Microencapsulated diets were prepared by interfacial polymerization of the dietary protein
112 according to method described by Yúfera *et al.* (1999; Fig. 1). In the present study 20 g of
113 feed ingredients were used for each batch produced. The dietary ingredients (20 g dw) were
114 dispersed in 180 ml of Tris buffer (Ph 9). Two parts of this solution were emulsified in five
115 parts of a soy lecithin (2 % w/v) solution in cyclohexane (Fluke Chemica, > 99.5 %) with
116 homogenization (IKA, 45-mm ‘ship propeller’ stirrer) for 8 min at 1000 rpm at room
117 temperature (22 ºC) (Fig. 1, step 1). Nine g of the cross-linking agent (1,3,5-
118 benzenetricarboxylic acid chloride (trimesoyl chloride)), dissolved in 20 ml diethyl ether, was
119 then added to the emulsion with continuous stirring and the reaction continued for 8 min (Fig.
120 1, step 2). The microcapsules formed were allowed to settle (Fig. 1, step 3) and the

121 cyclohexane–lecithin solution was removed by decantation (Fig. 1, step 4). After washing
122 with cyclohexane twice (altogether approximately 800 ml) and decanting of remaining debris,
123 the microcapsules were dispersed in an aqueous solution of gelatin (5% w/v; Type B: From
124 Bovine skin, G9385, Sigma) while stirring at low speed for 5 min (Fig. 1, step 5) at 38 ºC.
125 After dispersion in the aqueous solution of gelatine, the capsules were retained on an 80 µm
126 sieve (Retsch, test sieve) and rinsed under pouring tap water (Fig. 1, step 6), and then
127 dispersed in a 38 ppt sodium chloride solution (JOZO® sea salt, Akzo Nobel) for 5 minutes
128 (Fig. 1, step 7) before freezing and freeze drying.
129
130 The concentration of sodium caseinate was kept constant at 390 mg kg^{-1} for the investigated
131 diets.
132
133 **Exp. 1: Change in protein quality and quantity**
134 Three stages of the production process were investigated to determine when changes in
135 protein quality and quantity occurred during the production process. The first step to be
136 analyzed was after mixing the ingredients and adding the Tris buffer at pH 9. The dietary
137 mixture was freeze-dried before further analysis. The next step to be analyzed was after cross-
138 linking the capsule walls and washing the capsules with cyclohexane (Fig. 1, step 4) - instead
139 of adding the capsules to the gelatin solution they were frozen and freeze-dried. The third step
140 to be analyzed was the finished encapsulated diet. The diets' composition is given in Table 1
141 (diet 1) and their micronutrient content is given in Table 3 (diet 3). Three replicate batches
142 were produced for each of the three investigated production steps. The concentrations of
143 crude protein (N x 6.25), soluble N and TCA (10 %) soluble N were investigated at the three
144 described production steps. The amino acid (AA) profile were also analysed at the three
145 different stages during the production process.

146

147 **Exp 2: Change in lipid quality and quantity**

148 Three stages of the production process were investigated (according to Exp.1) to determine
149 when changes in lipid quality and quantity occurred. Total lipid and lipid classes were
150 analysed at each production step. The diets' composition is given in Table 1 (diet 1) and their
151 micronutrient content is given in Table 3 (diet 3).

152

153 Changes in lipid classes and fatty acid composition were investigated to evaluate if an uptake
154 of the soy lecithin used as surfactant and if an eventual uptake of soy lecithin from the
155 cyclohexane solution was influenced by changes in the dietary lipid source (Table 2). The
156 possibility of using marine phospholipids instead of soy lecithin as a surfactant was also
157 investigated (Table 2). The different investigated lipid sources investigated (Table 2)
158 constituted for 17 % (dw) of the raw ingredients. The diets` composition of other macro
159 nutrients are given in table 1 (diet 2).The described diets were produced in triplicates. The
160 concentration of thiobarbituric acid reactive substances (TBARS) is a measure of lipid
161 peroxidation and was analyzed to determine if the cross-linking process led to an oxidation of
162 the lipid and if this oxidation was affected by changing the lipid sources in both the diet and
163 the surfactant.

164

165 **Exp. 3: Retention of micronutrients**

166 To investigate changes in micronutrient content and possible correlations between added and
167 encapsulated micronutrients, a micronutrient mix consisting of two water-soluble minerals,
168 two water-soluble vitamins and two fat-soluble vitamins were chosen (Table 3). Five diets
169 were prepared in duplicate with increasing concentrations of micronutrients (Table 3). Diet
170 number 3 (Table 3) had a micronutrient concentration based on recommendations from NRC

171 (1993) for larger fish and the nutrient content of copepods (Hamre, unpublished). The diets`
172 composition of macronutrients are given in table 1 (diet 1).
173
174 To investigate where during the production process quantitative changes occurred, three
175 stages of the production process were investigated (according to Exp.1). The concentration of
176 zinc, thiamine and vitamin C were investigated at the three described productions steps. The
177 diets' composition is given in Table 1 (diet 1) and their micronutrient content is given in
178 Table 3 (diet 3).
179
180
181 **Analytical methods**
182 Dry matter content of the diets were determined gravimetrically after drying for 24 h at 104
183 ºC. Particle size was evaluated by taking pictures under the microscope of several random
184 capsule samples of each production batch and measuring particle size automatically with
185 Image Pro plus (version 4). A minimum of 400 capsules were measured per capsule batch.
186 Crude protein (N x 6.25) was determined by total combustion using a nitrogen analyzer (Leco
187 FP-528, St. Joseph, MI), Total AA profiles were analyzed according to method described by
188 Cohen *et al*., (1989). The samples were hydrolyzed in 6 N HCl for 22 h at 110 °C. The
189 hydrolyzed solution containing free AA was then analyzed by using the Waters high pressure
190 liquid chromatography (HPLC) analyzer system (Pico Tag) after pre-derivatization with
191 phenyl isothiocyanate (PITC) using norleucine as internal standard. The AA were identified
192 by differences in retention time and detected by UV at 254 nm. Total lipid was determined
193 gravimetrically after extraction with chloroform:methanol 2:1 (Bligh & Dyer, 1959). Total
194 fatty acid composition was analyzed according to Lie & Lambertsen (1991) and individual-
195 fatty acids were identified by known-purified standards. Lipid-class composition was
196 analysed by HPTLC analyser (Iatron Laboratories, Inc., Tokio Japan), using the method

197 described by Jordal *et al*. (2007), modified after Bell *et al*. (1993).. Thiamin was determined
198 by HPLC analysis and fluorescence detection (excitation: 366nm, emission: 435 nm)
199 according to method given by CEN (Comitè Europèen de Normalisation; TC 275 WI
200 002750053 (2002) N134, Foodstuffs -Determination of Vitamin B1). Vitamin C was analyzed
201 by HPLC using electrochemical detection of ascorbic acid according to method by Maeland *et*
202 *al*. (1999). Zinc and iron were analyzed by first wet digesting the capsule samples in a
203 Milestone microwave laboratory system (Milestone, Sorisole, Italy; Julshamn *et al.*, 2000)
204 and then determining the zinc and iron concentrations by flame atomic absorption
205 spectrometry on a Perkin-Elmer 3300 AAS instrument (Norway, CT) (Julshamn *et al.*, 1998;
206 Liaset *et al.*, 2003). Vitamin A was measured as all-trans-retinol (all-trans-ROH) and was
207 analyzed by HPLC with a UV detector (325 nm) according to Moren *et al*. (2002) (modified
208 method of Noll (1996)). Vitamin E (α- tochopherol) was analyzed by normal phase HPLC
209 with fluorescence detection (excitation 289 nm, emission 331 nm) according to Lambertsen
210 and Brækkan (1959) with modifications by Lie *et al*. (1994). Thiobarbituric acid-reactive
211 substances (TBARS) were determined as described by (Hamre *et al.*, 2001). For the analysis
212 of water-soluble nitrogen and TCA (10 %) soluble nitrogen, capsule samples were ground
213 (Retsch MM301) for 30 seconds with a frequency of 30 Hz. Then 0.2 g of capsule samples
214 were weighed into 15 ml Nunc plastic tubes. Seven ml of phosphate-buffered saline (PBS, pH
215 7.4) were added to each sample and agitated well (Vortex) before being shaken for 2 hours on
216 a shaking table at room temperature (22 ºC). The samples were centrifuged for 15 minutes at
217 4000 RPM at 20 ºC, the supernatant was removed and the pellet was re-suspended before
218 being centrifuged again. The tubes with the pellet were frozen and freeze-dried before the
219 pellet was analyzed for crude protein (N x 6.25).
220

221 **Statistical methods**

222 Data are expressed as means ± standard deviations were three or more replicates were
223 measured and means ± ranges are used when duplicates were measured. Crude protein content
224 (N x 6.25), soluble nitrogen and TCA (10 %) soluble nitrogen were analyzed by one-way
225 ANOVA followed by Tukey's HSD multiple comparison test. Changes in Amino acid (AA)
226 profiles due to the production process were analyzed by one-way ANOVA (Sokal and Rohlf,
227 1969) followed by Tukeys's HSD multiple comparison test. Change in lipid content, lipid
228 class composition and fatty acid profile was analysed with one way ANOVA followed by
229 Tukeys's HSD multiple comparison test. The effect of increased inclusion of micronutrients
230 was analyzed by regression analysis. Means were considered significantly different at $P \leq$
231 0.05. All statistical analyses were performed using Statistica 7.1 (Statsoft, Inc, Tulsa, USA).

232

233 Results

234

235 Protein encapsulation proved to be an efficient method to produce diets for delivery of lipid
236 and complex protein. However, large volumes of organic solvents were needed for the
237 production of the protein cross-linked diet, approximately 1.3 l of cyclohexane was needed to
238 produce 15 g of protein capsules (dw). The overall capsule diameter was 114 ± 68µm. The
239 total efficiency of encapsulation (mass of capsules obtained from the total mass of ingredient
240 mixture) was 76 ± 4 % on a dry weight basis. Changes in diet composition did not affect
241 encapsulation efficiency ($P \geq 0.05$). The water content of produced and freeze dried capsules
242 were 1.8 ± 0.4 and was not affected by the investigated diets composition ($P \geq 0.05$).

243

244

245

246 **Exp. 1: Change in protein quality and quantity**

247 The protein cross-linked capsules could deliver a controllable concentration of crude protein
248 (N x 6.25) with relative small differences between included and analyzed concentration in the
249 produced diet. However, significant effects between included and analysed concentration
250 were found (Table 4).

251

252 Although significant ($P \leq 0.05$), the changes in crude protein content during the production
253 process were rather small (Table 4), but the qualitative changes in the protein fraction were
254 large. Nearly all water-soluble protein was cross-linked during the encapsulation process
255 (Table 4). The TCA-soluble nitrogen was not cross-linked (Table 4), but was largely lost due
256 to leaching during the washing stages (Table 4). Except for taurine there was no significant
257 change ($P \geq 0.05$) in the AA profile during the production process (Fig. 2). There was no
258 significant difference ($P \geq 0.05$) in taurine concentration between the raw ingredients and the
259 cross-linked diet, but taurine was not detectable in the finished diet (Fig. 2).

260

261 **Exp 2: Change in lipid quality and quantity**

262 No significant differences ($P \geq 0.05$) in total lipid concentration between the ingredients and
263 the finished diet were found, but there was a significant increase in the concentration of
264 phospholipids (Table 5). There was a significant increase ($P \leq 0.05$) in lipid after the cross-
265 linking process that was reduced during the washing stages (Table 5). The production of
266 protein-encapsulated particles without use of surfactants was not successful. Marine
267 phospholipids were successfully used as a surfactant and the capsules seemed to have a
268 similar quality as capsules made with soy lecithin as the surfactant. There was no significant
269 difference ($p > 0.05$) in encapsulation efficiency among the different diets - the mean
270 encapsulation efficiency for all diets was 77 ± 6 %. In diets which had no inclusion of soy

271 lecithin in the ingredient mixture, there was a 5 fold increase 18:2n-6 when soy lecithin was
272 used as surfactant, compared to use of marine phospholipids as the surfactants (Table 6). This
273 indicates an uptake of surfactant during the production. An interaction between increased
274 amounts of phospholipids added to the diet and increased uptake of surfactant during the
275 production process could not be detected. There were no significant difference ($P \geq 0.05$) in
276 phospholipid concentration in the encapsulated diet that used marine phospholipids as a
277 surfactant and those which used soy lecithin (Table 6).

278 The production process did not lead to any severe peroxidation of lipids (TBARS) and
279 there were no significant difference ($P \geq 0.05$) between the four investigated diets. The
280 TBARS concentration for the four diet combined was 14 ± 2 nmol g^{-1}.

281

282 **Exp. 3: Retention of micronutrients**

283 There was an overall high loss of the water-soluble nutrients except iron during production
284 (Fig. 3). No loss of vitamin E was demonstrated, but there was an increased loss of vitamin A
285 with increased inclusion (Fig. 3). A positive correlation ($P=0.017$) was found between the
286 inclusion of thiamine and loss of thiamine, the loss of thiamine ranged from 92.3 % - 96.6 %
287 (Fig. 3). However, the total concentration of thiamine increased 7.6 times from 0.49 mg kg^{-1}
288 to 3.7 mg kg^{-1} as the amount of added thiamine increased in diets 1 to 5 (Fig.3). There was an
289 overall large loss of zinc during production and a positive correlation ($P=0.0005$) was found
290 between the inclusion of zinc and loss of zinc. As the inclusion of zinc increased from 8 mg
291 kg^{-1} to 120 mg kg^{-1}, the loss increased from 86 % in diet 1 to 95 % in diet 5 (Fig. 3). As seen
292 in Table 7, there was no loss of zinc and thiamine during the first part of production, but a
293 nearly a complete loss during the washing part of the production process. Vitamin C was
294 completely lost during the cross linking process (Table 7) and vitamin C was not detected
295 (detection limit, 1.1 mg kg^{-1}) even at the highest inclusion level. Iron was retained in the

296 capsules during the production process (Table 7) and there was also a good correlation
297 (P<0.00001) between added and analyzed iron (Fig. 3). A good correlation (P<0.00001)
298 between the amount of α-tocopherol in the diet and the concentration added was demonstrated
299 and little or no α-tocopherol was lost during the production process (Fig. 3). Increased
300 amounts of all-trans-ROH added to the diet correlated (P<0.00001) with analyzed amounts in
301 the diet, but as the inclusion of all-trans-ROH increased from 0.625 mg kg^{-1} to 10 mg kg^{-1}, the
302 loss increased from 4 % in diet 1 to 57 % in diet 5 (Fig. 3).
303

304 Discussion

305 The main objective of this study was to characterize and quantify changes of macro and micro
306 nutrients that occur during the production of cross-linked protein-walled capsules.
307

308 ***General production process***

309 For all ingredient mixtures, the capsules seemed visually to have been successfully
310 encapsulated. The overall encapsulation efficiency was approximately 75 % (dw) and was
311 similar to previous results (Yúfera *et al.*, 2002). There was a considerable amount of debris
312 decanted during the cyclohexane washing process (Fig. 1, step 3-4), however there was no
313 loss of dry matter after cross-linking and cyclohexane washing. The mean particle size of the
314 freeze-dried capsules were 114 ± 68µm, however, the capsules were only investigated in dry
315 form and the size was not measured after re-hydration. The sieve used to rinse the capsules,
316 had a mesh size of 80 µm. With a sieve of this size, it is most likely that a significant amount
317 of the capsules were lost during washing. This may explain the 25 % loss of dry matter during
318 the production process, the loss of water-soluble compounds by leaching should however also

319 be considered. The different diet ingredients did not affect the particle size, however, these
320 results may have been affected by loss of the smaller capsules due to sieving.
321

322 ***Change in protein quality during production process***

323 There were changes in protein quality due to chemical cross-linking and leaching
324 during the different washing stages, as shown by a nearly complete loss of both soluble N and
325 TCA-soluble N. The loss of TCA-soluble N was in accordance with Yúfera *et al*. (2002) who
326 reported a 92.5 % loss of lysine during production. All water-soluble protein was rendered
327 insoluble after cross-linking, but the concentration of TCA-soluble N was minimally affected
328 by this process. This indicates that most low molecular weight N was not linked to protein by
329 the polymerization process as earlier suggested as an explanation for the loss of low
330 molecular weight N (Lopez –Alvarado *et al*., 1994; Yúfera *et al*., 2002). However, most of the
331 TCA-soluble N was lost by leaching during the following washing stages.

332 There were no major effects of either cross-linking or the washing process on the AA
333 profile, except for a 100 % loss of taurine at the gelatin dispersion and washing stages.
334 Taurine exists only in free form and not as part of proteins. Taurine will therefore be exposed
335 to severe leaching if not incorporated into a diet with good retention properties. Although
336 taurine is described as a non-essential AA, there are indications that taurine is an essential AA
337 at early fish larvae stages. Japanese flounder (*Paralichtys olivaceus*) (Chen *et al.*, 2004; Chen
338 *et al.*, 2005; Kim *et al.*, 2003; Kim *et al.*, 2005; Matsunari *et al.*, 2003; Chen *et al.*, 2002;
339 Takeuchi *et al.*, 2001) and sea bream (*Pagrus major*) (Chen *et al.*, 2004) fed on taurine-
340 enriched rotifers showed increased growth compared with un-enriched rotifers. The
341 concentration of taurine in the raw ingredients (2.9 mg g^{-1} dw) was approximately 6 times
342 lower than in copepods (18 mg g^{-1} dw, Van der Meeren 2003) and 4.5 times lower than
343 *Artemia* (13 mg g^{-1} dw, Van der Meeren 2003). This study confirms that diets made with

344 marine meal as a main ingredient have a low concentration of taurine (Nordgreen *et al.*,
345 submitted) and additional supplementation of taurine may be considered. According to Kim *et*
346 *al.* (2005), juvenile Japanese flounder required at least 15 mg taurine per g^{-1} capsule. To
347 produce a protein-encapsulated feed containing 15 mg g^{-1} (dw) taurine, approximately 200 mg
348 g^{-1} would have to be included in the capsule ingredients, assuming that losses during
349 production were equal to the loss of lysine reported by Yúfera *et al.* (2002) or similar to the
350 losses of zinc and thiamine in the present.

351 Yúfera *et al.* (1996) reported that the crude protein content in diets made with gelatin
352 was 18.6 % higher than that of diets that were washed in alcohol. These results suggested that
353 there was an uptake of gelatin during the dispersion in the hot gelatin solution. Change in
354 crude protein concentration due to increased loss of protein in the alcohol washed diet is
355 unlikely since protein solubility will be markedly lower in ethanol than in water (Pace, 2004).
356 Gelatine has a high concentration of the AA glycine (27 %) and proline (15%), and an uptake
357 of gelatin may lead to a change in the concentration of the respective AA. The small changes
358 in AA profile during the production process indicates that uptake of gelatin in the capsules are
359 limited.

360 Due to the interfacial cross-linking process, large fractions of the dietary protein has to
361 consist of water-soluble or water-dispersible protein containing free amine groups (Jones,
362 1980). According to Jones (1980) any hydrophilic protein with sufficient reactive amino
363 groups may be used, especially if the proteins have a relatively high concentration of lysine,
364 histidine and arginine that contais two amine groups (Jones, 1980). Protein capsules prepared
365 with nylon-protein (Jones, 1974), purified crab protein (Langdon 1989; Langdon and
366 DeBevoise, 1990), whey protein (Lee and Rosenberg 2000), albumin (El-Mahdy *et al.*, 1998;
367 Yúfera *et al.*, 2000), or caseine (Fernández-Díaz *et al.*, 1994; Ozkizilcik and Chu 1996;
368 Yúfera *et al.*, 1996) have been successfully made. Most investigated protein encapsulated

369 diets, had a casein fraction of 500 g kg^{-1} (dw) (Yùfera *et al.*,1999, 2000, 2002, 2003; Alarcon
370 *et al.*, 1999), however a protein encapsulated diet containing 260 g kg^{-1} casein (dw) was
371 successfully produced and investigated for leaching (Kvåle *et al.*, 2006). Protein encapsulated
372 diets containing less than 250 g kg^{-1} casein (dw) may give unstable capsules (M. Yufera,
373 unpublished observations). In this study the casein fraction constituted for 390 g kg^{-1} (dw) and
374 approximately 55 % of the total crude protein. With such a high proportion of the protein
375 source being the cross-linking protein, it is of major importance that it is has an acceptable
376 AA profile and good digestibility. Water soluble Na caseinate as used in this study has
377 successfully been used to produce cross-linked protein microspheres (Millar, 1991, Quigg *et*
378 *al*., 1992, Heelan & Corrigan 1997, Corrigan & Heelan 2001). However, cross-linked
379 microspheres used as larval feed have mostly been produced with acid precipitated casein
380 (Yùfera *et al*.,1999, 2000, 2002, 2003, Chu & Ozkilcik 1999, Kvåle *et al*., 2006). Acid
381 precipitated casein is insoluble in water, but is solubilized at alkaline pH and therefore
382 alkaline solutions are used when producing cross-linked casein microspheres. It should be
383 considered that the use of Na caseinate instead of casein, may affect technical properties such
384 as leaching rate, but considering the similar loss of FAA during the production process in the
385 present study and in the study of Yùfera *et al*. (2002), it is unlikely that there are large
386 differences.

387 Casein has been used as the main protein source in several experimental diets for fish
388 larvae and juveniles (Carvalho *et al.*, 1997; Carvalho *et al.*, 2004; Fontagne *et al.*, 2006;
389 Ostaszewska *et al.*, 2005; Alam *et al.*, 2005; Wang *et al.*, 2004). However, Carvalho *et al.*
390 (2004) showed that insoluble casein had a negative influence on larval *Cyprinus carpo*
391 performance during the first two weeks of exogenous feeding, compared to soluble sodium
392 casein. *In vitro* digestibility of the protein-encapsulated diet and the ingredient mixtures for
393 the same diet, showed a decrease in protein digestibility from 71 ± 1.4 % to 53 ± 2.3 % due to

394 the production process (Tonheim *et al*., 2007). In vitro digestibility of soluble casein (Na
395 Casein) and in-soluble casein was similar after 12 h (Tonheim *et al.*, 2007), indicating
396 that the decline in *in vitro* digestibility was due to the covalent cross-linking reactions and not
397 a reduction in solubility. It is not likely that large fractions of water-soluble protein can be
398 delivered with the protein encapsulated diet, considering that all the soluble protein in this
399 study was cross-linked. There may be a possibility that soluble protein with low
400 concentrations of free amino groups (if existing) could be delivered without being
401 cross-linked.

402 Previous studies indicates that protein-encapsulated diets have reduced leakage rates
403 compared to other investigated formulated diets (LopezAlvarado *et al.*, 1994; Kvåle, 2004;
404 Yúfera *et al.*, 2002). Lopez Alvarado (1994) reported leaching of 60 % FAA in 2 minutes
405 from a protein-encapsulated diet and Ozkizilcik & Chu (1996) reported a loss of 45 % lysine
406 within 4 minutes of exposure to leaching while Yúfera *et al*. (2002) reported a loss of 17 %
407 FAA in 60 minutes and Kvåle *et al*. (2006) reported a 30 % loss of serine in 60 minutes. The
408 large differences in leaching rates among these reports are probably due different initial
409 concentrations of FAA. The diets investigated by Lopez-Alvarado *et al.* (1994) and Ozkizilcik
410 and Chu (1994) were produced according to Langdon and Debevoise (1990) where the diet
411 was only washed in cyclohexane and freeze-dried without gelatin dispersion and aqueous
412 washing steps (Yúfera *et al*., 1999). The concentration of FAA in the microencapsulated diet
413 was 0.53 % (dw) in the diet investigated by Yúfera *et al*. (2002), while the diet investigated
414 by Lopez Alvarado (1994) had approximately 20 % FAA (dw).

415

416 ***Change in lipid quality***

417 There was a significant increase in crude lipid concentration after cross-linking, but during the
418 washing processes the lipid concentration returned to the initial level. However, the

419 phospholipid content increased from 26 to 33 %. The changes in crude lipid and lipid class
420 composition are not fully understood and need further investigation. The decrease in lipid
421 concentration in the diet from the cyclohexane-washed stage to the finished diet may be due
422 to a loss of lipid during washing or an increase in protein by uptake of gelatin, but as
423 discussed above, there were no change in the AA profile that could explain the change in lipid
424 concentration. It is therefore unlikely that the decrease in lipid concentration were due to
425 uptake of gelatin. The increase in phospholipid concentration was to small to explain the total
426 increase in lipid during this production step.

427 The use of a surfactant, such as soy lecithin, affects the rate of the wall formation
428 (Jones, 1980) and may be used to control particle size (Jones, 1980). The surfactant helps
429 emulsify the aqueous droplets and is supposed to improve contact between the hydrophilic
430 and hydrophobic phases (Jones, 1980). According to Jones (1980), surfactants are not
431 essential in the process; however, in this study, we were not able to produce stable protein
432 capsules without using either marine phospholipids or soy lecithin as surfactants.

433

434 ***Micronutrients***

435

436 Fat-soluble vitamins, such as vitamin E and A, can be delivered in a controlled manner
437 without severe losses during production. There was no loss of vitamin E during production,
438 but there was an increase in relative loss of vitamin A from 4 % to 57 % with increased
439 inclusion amounts. It is not known whether this is due to analytical problems, leaching into
440 the cyclohexane or chemical destruction during cross-linking. However, losses of vitamin A
441 were relatively small compared to those for the water-soluble micronutrients.

442 Both zinc and thiamine were efficiently encapsulated, but high losses occurred during
443 the washing stages. The increased loss (%) of both zinc and thiamine with increased inclusion

444 may be due to better retention of the zinc and thiamine naturally occurring in the capsule
445 ingredients or that increases in concentration lead to increases in leaching rates. Vitamin C
446 (ascorbyl monophosphate) was not detectable after the production process, but in contrast to
447 thiamine and zinc, vitamin C was degraded during the cross-linking process. This may be,
448 because the vitamin C was covalently bond to the protein matrix during the crosslinking
449 process as earlier reported for other incorporated compounds (Willmot et al., 1989,
450 Cummings *et al*., 1991, Quigg *et al*., 1992, Willmott et al., 1992). Willmott et al. (1992)
451 reported that doxorubicin (anti-tumour agent) was covalently bound during encapsulation in
452 casein cross-linked particles. However, *in vitro* trypsin digestion of the cross-linked particles
453 released the covalently bound doxorubicin which still had its anti-tomour activity intact. This
454 may also be the case for vitamin C. In comparison to the complete loss in this study, Yúfera *et*
455 *al*. (2003) reported an incorporation efficiency of 0.8 – 1.2 % for both ascorbyl-
456 monophosphate and ascorbyl-polyphosphate. The reason for the complete loss of vitamin C in
457 this study compared to the study by Yúfera *et al*. (2003) is probably due the higher initial
458 incorporation of vitamin C (30 -70 g kg^{-1}; Yufera et al., 2003) compared to that used in this
459 study (0.1 – 1.6 g kg^{-1}). Although the iron added to the diet was water-soluble, there was no
460 loss during the encapsulation process. This is most likely due to precipitation when iron was
461 added to the Tris buffer (pH 9), as indicated by visual observations. The precipitation of iron
462 may influence the availability of this mineral for marine fish larvae and should be considered.
463

464 ***Conclusion***

465 The main goal of the investigation of the protein-encapsulated diet was to evaluate the change
466 in nutritional quality during the production process and to better understand how the
467 encapsulation process affected the nutrients so that the final concentration of encapsulated
468 nutrients could be predicted. There were no large differences in the concentration of added

469 and analysed crude protein and crude lipid. All water-soluble protein was made insoluble by
470 cross-linking, while nearly all the TCA-soluble N was washed out during the washing stages
471 of the production process. With the existing production protocol, the diet can not deliver
472 soluble N. Nearly all water-soluble micronutrients were lost during the production process.
473 The results suggest that this particle type is unsuitable for use in nutritional studies on the
474 requirements for water soluble nutrients. To use this particle type for requirements studies, it
475 is suggested not to carry through the gelatine dispersion and washing stages. However, the
476 reported decrease in digestibility by not accomplishing these production steps has to be
477 considered carefully.

478

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485

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487

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734 **Legions to figures**

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736 Figure 1: 1) The dietary solution is emulsified in cyclohexane with stirring. 2) The cross-
737 linker (trimesoil) is dissolved in ether and added under constant stirring for 8 minutes 3) The
738 stirrer is stopped and stabile cross-linked capsules settle. 4) The cyclohexane-lecithin solution
739 is decanted, the capsules are washed with cyclohexane twice. 5) The capsules are stirred in a
740 gelatin solution for 5 minutes. 6) The capsules are washed in fresh water. 7) The particles are
741 dispersed in saltwater for 5 minutes. 8) The capsules are rinsed in fresh water before freezing
742 and freeze drying.

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744 Figure 2: Concentration of AA at three different stages in the capsule production process.
745 Concentrations of the different AA are given as % of total AA. Error bars represent standard
746 deviations (n=3).

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748 Figure 3: Concentrations of encapsulated micronutrients in five diets with increasing levels of
749 added micronutrients. Two separate batches of each encapsulated diet were produced. Error
750 bars represent means ± ranges (n=2).

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758 Table 1: Diet "1" was used to investigate changes in nutritional quality and quantity of
759 protein, lipid and micronutrients during the production process (Exp. 1, 2 and 3). Three
760 separate batches of capsules were produced for each of the three production steps
761 investigated. The micronutrient concentrations in the capsule are those of diet "3" given in
762 Table 3. Diet "2" was used to investigate the affect of different types of lipid in the diet (Exp.
763 2) (see table 2 for lipid used).

Diet **Ingredients**	**1** **Concentration** **g kg^{-1}**	**2** **Concentration** **g kg^{-1}**
Sodium caseinate[1]	390	390
Squid meal[2]	120	120
Pepsin hydrolysated cod roe[2]	120	110
Cod roe[2]	120	110
Dextrin[3]	100	10
Soy lecithin[4]	50	(see table 2)
Cod liver oil[5]	100	(see table 2)
Marine phosholipid[6]		(see table 2)

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765 [1] Tine, Oslo, Norway

766 [2] Rieber & Sønn ASA. Bergen, Norway.

767 [3] Grinded Sagogryn, Varenr: 8051, HOFF, Norway

768 [4]Lecithin Granulat, Biosym A/S, Ikast, Denmark

769 [5] Möller's tran naturell, Peter Möller, Oslo, Norway

770 [6]PhosphoNorse, Eximo AS, Tromsø Norway

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776 Table 2: Four different diets (each prepared in triplicate) were produced to investigate the
777 effect of lipid source on the production process. Diets 1 and 2 were produced to investigate if
778 emulsifying phospholipids were taken into the diet during the production process. Diets 3 and
779 4 were produced to investigate if there was increased uptake of surfactant phospholipids when
780 the diet had a higher content of phospholipids. Lipid peroxidation was analyzed for all diets.
781 The included lipid constituted for 17 % (dw) of the different diets. The diets compositions of
782 other nutrients are given in Table 3 and the micronutrient content is given in Table 4 (diet 3).

Diet	Lipid in diet	Lipid in cyklohexane solution
1	50 % fish oil[1] and 50 % marine phospholipid[2]	soy lecithin[3]
2	50 % fish oil[1] and 50 % marine phospholipid[2]	marine phospholipids[2]
3	marine phosholipid[2]	soy lecithin[3]
4	fish oil[1]	soy lecithin[3]

783 [1] Möller's tran naturell, Peter Möller, Oslo, Norway
784 [2]PhosphoNorse, Eximo AS, Tromsø Norway
785 [3]Lecithin Granulat, Biosym A/S, Ikast, Denmark
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797 Table 3: Concentrations of micronutrients added to the five different diets. Two batches were

798 made for each micronutrient concentration.

Diet **Micronutrients**	**1** **mg kg^{-1}**	**2** **mg kg^{-1}**	**3** **mg kg^{-1}**	**4** **mg kg^{-1}**	**5** **mg kg^{-1}**
Thiamine mononitrate[a]	5	10	20	40	80
Vit C[b]	100	200	400	800	1600
Vit E[c]	50	100	200	400	800
Vit A[d]	0.625	1.25	2.50	5	10
Iron[e]	15	30	60	120	240
Zinc[f]	8	15	30	60	120

799 [a] Rovimix B1, Roche

800 [b] Rovimix®, STAY-C® 35, Roche

801 [c] Rovimix E, Roche

802 [d] Rovimix A, Roche

803 [e] Iron (II) Sulfat ($FeSO_4$) * 7 H_20

804 [f] Zinc Sulfat ($ZnSO_4$)* 7H_2O

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818 Table 4: Concentration of encapsulated crude protein (N x 6.25), soluble nitrogen (% of tot N)
819 and TCA (10 %) soluble nitrogen (% of tot N) at three different stages in the production
820 process. The proportion of soluble nitrogen is given as % of total N in the diet. Three separate
821 batches of capsules were produced for each of the three production steps (mean ± SD, n=3).

	Raw ingredients (%)	Cyclohexane washing (%)	Finished diet (%)
Crude protein (%)	$63.1^a \pm 0.5$	$51.4^b \pm 0.6$	$58.8^c \pm 0.2$
% Sol. N of tot. N	$62.2^a \pm 2.1$	$12.6^b \pm 1.2$	$2.9^c \pm 0.9$
% TCA-sol N of tot. N	$15.9^a \pm 0.3$	$12.8^b \pm 0.3$	$3.4^c \pm 0.5$

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838 Table 5: Total lipid (% dw), total polar lipids (% of total lipid) and produced amount of

839 microcapsules (g dw) at three different stages during the encapsulation process. Three

840 separate batches of feed are produced at each of the three production steps (mean ± SD, n=3).

	Rawmaterial	After cross-linking	Finished diet
Lipid (% dw)	$29^{a} \pm 2.2$	$42.1^{b} \pm 1.1$	$29.0^{a} \pm 1.1$
Tot PL % of tot lipid	$26^{a} \pm 0.5$	$31.9^{b} \pm 0.5$	$33.3^{b} \pm 1.1$
Produced amount (g)	$19.5^{a} \pm 0.1$	$19.9^{a} \pm 1.1$	$13.5^{b} \pm 1.5$

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859 Table 6: Concentration of 18:2n-6 given as % of total fatty acids (FA) in diets with different
860 concentrations of marine phospholipids and diets produced with either marine phospholipids
861 or soy lecithin as the surfactants in the cyclohexane solution (mean ± SD, n=3).

Lipid in diet	Lipid in cyclohexane solution	18:2n-6 % of FA	Phospholipid % of tot. lipid
50 % fish oil and 50 % marine phospholipids	soy lecithin	4.2 ± 1.1	20.0 ± 4.3
50 % fish oil and 50 % marine phospholipid	marine phospholipids	0.83 ± 0.07	18.3 ± 3.0
marine phospholipids	soy lecithin	6.8 ± 0.2	35.8 ± 1.4
fish oil	soy lecithin	4.9 ± 0.4	14.8 ± 1.2

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878 Table 7: Concentrations of zinc, thiamine and vitamin C at three different stages during the

879 production process. Three batches of capsules were produced and analyzed for each

880 production step. The concentrations of micronutrients are given in mg kg^{-1} (mean ± SD, n=3).

881 nd = not detected

	Ingredients mg kg^{-1}	**Cyclohexane washing mg kg^{-1}**	**Finished diet mg kg^{-1}**
Zinc	78.9^{a} ± 1.7	66.1^{b} ± 1.8	4.4^{c} ± 1.6
Thiamine	29.4^{a} ± 1.9	21.7^{b} ±0.7	1.3^{c} ±0.3
Vitamin C	415 ± 23	nd	nd

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886 **Figure 1**

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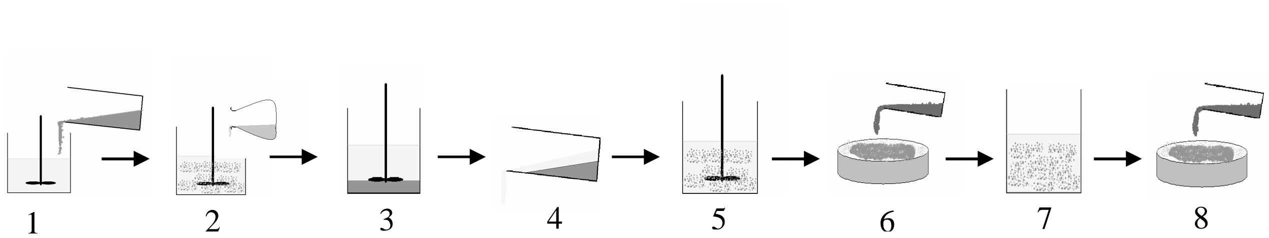

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906 **Figure 2**

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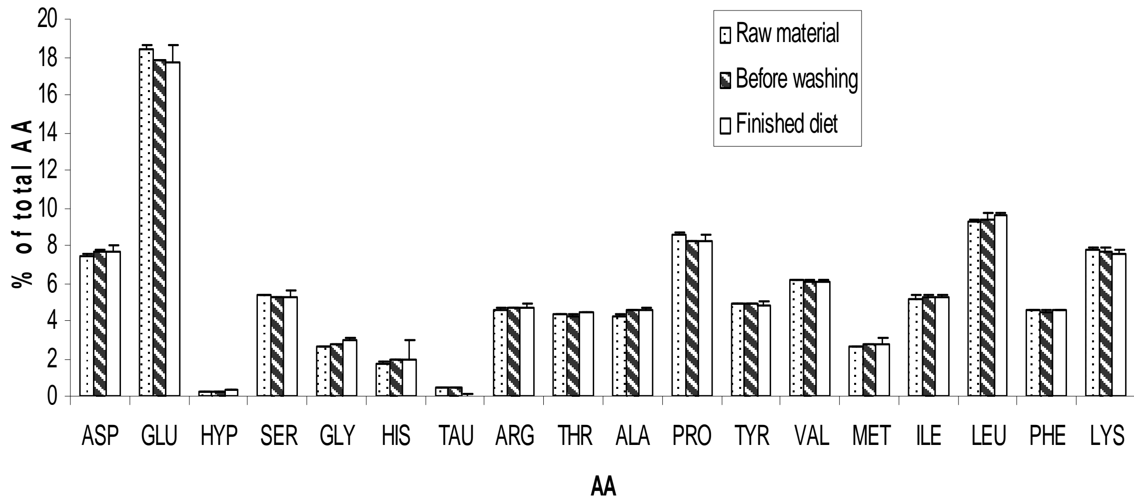

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924 **Figure 3**

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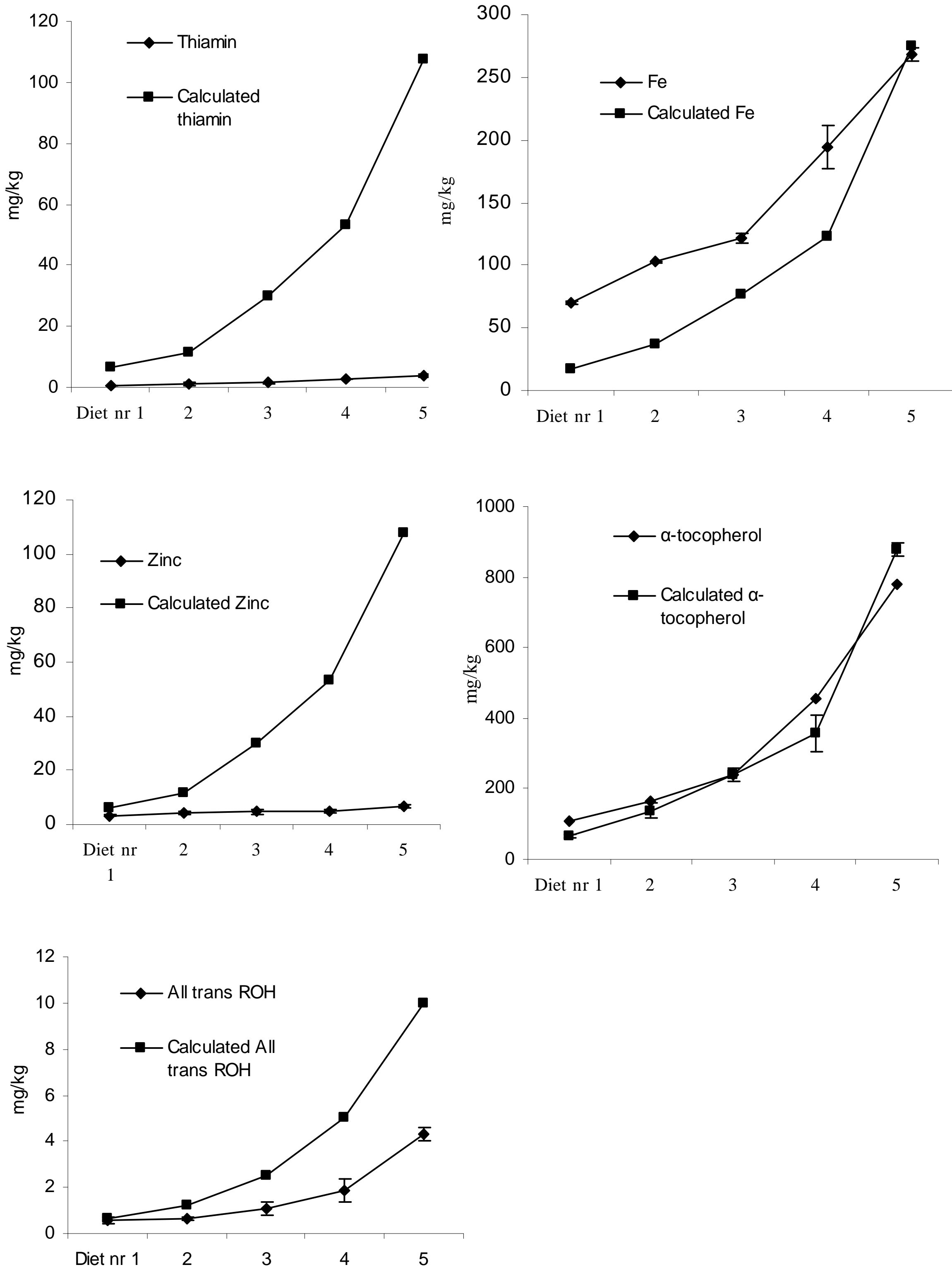

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Paper II

Errata

Paper II

“Trypsin-digested cod fillet” should be changed to “pepsin-digested cod fillet” throughout paper II

Table 2:

”Trypsin- digested cod fillet”, should be changed to “pepsin- digested cod fillet” as shown bellow.

Sample	Water-soluble fraction (%)	Insoluble fraction (%)	S.D.	n (analytical sample)
Artemia (nauplii)	67	33	2.8	4
Calanus (copepod stage)	54	46	2.2	4
Squid meal	11	89	2.6	4
Fish meal (Rieber, Norway)	17	83	1.6	4
Roe meal	11	89	1.1	3
Pepsin-digested cod fillet	65	35	3.0	1
Fresh frozen cod fillet	34	66		
Stick-water	100			
Casein		100		
Na+-caseinate	100			
Whey protein	100			

The errata is in bold.

“Hydrolysed cod fillet” refers to “**pepsin**-digested cod fillet” (Page 430; Table 3)

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In vitro digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients

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Abstract

In vitro methods have previously been utilised for the rapid and reliable evaluation of protein digestibility in fish. In this study we used *in vitro* methods to compare the digestibility of various live and artificial larval feeds and feed ingredients. Given previous suggestions that water-soluble dietary proteins are efficiently digested and utilised by stomachless fish larvae, we also analysed the content of water-soluble nitrogen in the feeds and feed ingredients and then measured the specific *in vitro* digestibility (simulated midgut conditions) of the water-soluble and insoluble fractions. The soluble nitrogen fractions were generally more digestible than the insoluble nitrogen fractions ($P<0.05$). A soluble reference protein (Na^{+}-caseinate) was digested faster than the similar but insoluble reference protein (casein) although their final digestibility was the same (94%). Frozen live feeds (*Artemia fransiscana* and *Calanus finmarchicus*) contained high fractions of soluble nitrogen (54–67%) and also had high digestibility *in vitro* (84 and 87%, respectively). The *in vitro* digestibility of two formulated larval feeds tested was lower (53 and 70%) than the frozen live feeds. The digestibility of the ingredients of the protein-encapsulated feeds particles was reduced as a result of the production process (from 71 to 53%, respectively). Three meals of marine origin (fish meal, squid meal and fish roe meal) all had low contents of water-soluble nitrogen (11–17%) but showed different degrees of digestibility (77, 77 and 49%, respectively). The results also demonstrated that while pre-hydrolysis of a feed ingredient (fresh frozen cod fillet) almost doubled the water-soluble nitrogen fraction (from 34 to 65%) the positive effect of pre-hydrolysis on *in vitro* digestibility was much lower (from 80 to 86%). This demonstrates the complexity of assessment of bioavailability of dietary protein sources in larval fish; a number of factors such as leaching rates from feed particles, digestibility, digestion rates and absorption rates all need to be taken into account.
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Keywords: Protein; Digestibility; Fish larvae; Soluble protein; Compound diets

1. Introduction

Most marine fish species that are either established or candidates for aquaculture start exogenous feeding at an early stage in ontogeny, and long before the digestive system has fully matured (Vu, 1983; Blaxter, 1988; Luizi et al., 1999). The lack of a functional acid-secreting stomach in particular may negatively affect

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protein digestion (e.g. Jany, 1974; Rønnestad et al., 2003; Tonheim et al., 2005). In a functional gastric stomach, proteins are exposed to proteolytic active pepsin under denaturing acid conditions (pH 2 to 5), which may accelerate the proteolysis of ingested dietary proteins. In stomachless larvae dietary protein digestion has to rely solely on the intestinal digestion which is performed by pancreatic proteases and mucosa-associated proteases. It has also been shown that dietary protein can be taken up by pinocytosis in larvae and be digested intracellularly in the enterocyte by lysosomal proteolysis (Watanabe, 1981; Govoni et al., 1986), although the quantitative significance of this route of absorption remains to be determined (Rønnestad and Conceição, 2005).

Fish larvae have high dietary amino acid (AA) requirements due to their high growth potential (e.g. Houde, 1989; Conceição et al., 1997; Otterlei et al., 1999) and extensive combustion of AA in their energy metabolism (Rønnestad and Naas, 1993; Finn et al., 1995, 2002). The digestibility of dietary proteins is therefore crucial to their utilisation efficiency and an understanding of the specific digestibility of feed ingredients is important for the formulation of optimal diets for larvae in aquaculture. However, little is known about the digestibility of various protein sources used in compound diets for first-feeding marine larval fish. The digestibility of different sources of protein in adult fish and other animal models is of only limited relevance to larvae, because of differences in the digestive physiology of larval and juvenile/adult stages.

Current best practice for first-feeding fish larvae involves the extensive use of live feeds such as rotifers, *Artemia* and copepods (e.g. Kolkovski, 2001). Experimental studies have shown that it is possible to first-feed some stomachless marine fish larvae exclusively on compound diets (sea bass, *Dicentrarchus labrax*, Cahu et al., 1998; Japanese eel, *Anguilla japonica*, Pedersen et al., 2003; gilthead sea bream, *Sparus aurata*, Robin and Vincent, 2003. However, both sea bass and gilthead sea bream larvae grew more poorly than larvae fed live feeds (Cahu et al., 1998; Robin and Vincent, 2003). Growth on compound diets was also poor in the Japanese eels (Pedersen et al., 2003), but this species had not previously been successfully first-fed in an artificial system. A sufficient supply of dietary amino acids is a prerequisite for high growth rates. Compound feeds usually have high nutrient density, high relative protein content and are based on ingredients that have good amino acid balance as far as sustaining growth in juvenile fish is concerned. It is therefore unlikely that inferior growth in fish larvae fed compound diets is the result of low quantitative protein content or a poor amino acid profile, but is more likely to be a result of other qualitative differences and specific larval dietary requirements.

Carvalho et al. (2004), who studied freshwater common carp, *Cyprinus carpio*, larvae, found that replacing insoluble casein with soluble Na^+-caseinate in a starter diet improved both growth and survival. The protein sources used in their study are very similar except for their water solubility. For this reason, and because common live feeds, unlike formulated feeds, contain a high proportion of water-soluble protein (Fyhn et al., 1993; Hamre et al., 2002; Helland et al., 2003; Carvalho et al., 2003), Carvalho et al. (2004) suggested that protein solubility is important as a determinant of digestibility in larvae.

FAA and small peptides are usually water soluble and are efficiently absorbed from the intestine without previous digestion, and can thus be regarded as pre-digested dietary protein. Although pre-hydrolysis will enhance digestibility, substantial supplementation of compound diets with pre-hydrolysed protein has been reported as having a negative impact on the growth and survival of some marine fish larvae (*S. aurata*, Kolkovski and Tandler, 2000; *D. labrax*, Cahu et al., 1999). On the other hand, Day et al. (1997) found that substituting 100% of fish meal with pre-hydrolysed fish protein in a compound weaning diet for Dover sole (*Solea solea*) resulted in improved survival.

The specific digestibility of soluble versus insoluble proteins has not been studied in fish larvae. The positive effects of Na^+-caseinate as reported by Carvalho et al. (2004) may be due to soluble proteins being more exposed to intestinal proteases or being more efficiently taken up by pinocytosis. High feed intake and gut evacuation rates have been reported in fish larvae, (Govoni et al., 1986), and these may be important factors in poor protein digestion and utilisation in larvae (Govoni et al., 1986, Tonheim et al., 2005). An important prerequisite of successful compound starter diets for fish larvae may thus be sufficient quantities of highly digestible dietary proteins.

The true digestibility of different protein sources in larvae can only be measured *in vivo*. However, methods for *in vitro* digestion assessment using commercial available proteases have been used for rapid, easy and reproducible evaluation of the digestibility of feeds and feedstuffs (Hsu et al., 1977; Saterlee et al., 1979; Lazo et al., 1998). Chong et al. (2002) compared dry matter and protein digestibility in discus fish (*Symphysodon aequifasciata*) assessed by three different methods: the *in vitro* protocols of Hsu et al. (1977), Saterlee et al.

(1979), Lazo et al. (1998); *in vitro* digestion using gut extract from the discus fish; and *in vivo* digestibility assessed in feeding trials with fish itself. They found that relative digestibility, as measured by simple methods involving only a few proteases in a single reaction step, correlated well with digestibility measured *in vivo*. *In vitro* digestibility can thus be a useful first approach to selecting candidate protein sources for follow-up studies in larvae.

In order to improve our understanding of bioavailability of larval feeds, we investigated the solubility and *in vitro* digestibility of the water-soluble and water-insoluble fractions of live feeds and selected protein sources that are widely used or have a potential for use in larval compound diets.

2. Materials and methods

2.1. Feed and feed ingredients

A total of 13 feeds and feed ingredients was evaluated (Table 1). The live feeds, squid meal, fish roe meal, fish meal, fresh frozen cod fillet and pepsin-hydrolysed cod fillet were homogenised in phosphate buffer (pH 8.0) at 4 °C. The insoluble fraction of each homogenate was separated from the soluble fraction by centrifugation. Pellets were resuspended in phosphate buffer and centrifuged twice in order to wash the pellet.

Two compound larval diets were included in the study: one commercial microbound diet (Minipro™) and one protein-encapsulated micro-diet. Minipro™ was based on fish meal, fish protein hydrolysate, squid meal and herring stick-water as the main ingredients and the feed particles were prepared by using a carbohydrate binder and a spray technique. The protein-encapsulated diet was based on Na^+-caseinate, 39%; squid meal, 12%; cod roe, 12%; pepsin hydrolysed cod roe, 12%; dextrin, 10%; soya lecithin, 5%; cod liver oil, 10%. The microcapsules were prepared according to Yufera et al. (1999), with some modifications. The dissolved feed ingredients (water phase) were immersed in a non-polar solvent before the addition of an agent (1,3,5-benzene-tricarboxylic acid chloride), which induced cross-linking between the dietary protein molecules at the interface between the two phases.

2.2. In vitro measurements

Several different methods and procedures have previously been used to measure protein digestibility *in vitro*. In this study, digestibility was assessed as a percentage of nitrogen in the soluble fraction after treatment of the protein source with proteases and subsequent precipitation of undigested protein by trichloroacetic acid (TCA).

Crude protein content ($N \times 6.25$) was determined in all separated fractions and in the compound feeds by an FP-528 nitrogen analyser (Leco, MI, USA). The previously separated insoluble fractions, dry compound feeds and insoluble casein were resuspended in 1 ml of phosphate buffer (pH 8.0) in Eppendorf tubes in amounts corresponding to 20 mg of crude protein. The previously separated soluble fractions, soluble Na^+-caseinate, whey protein and stick-water were added to Eppendorf tubes in amounts corresponding to 20 mg of crude protein and diluted with phosphate buffer (pH 8.0)

Table 1
Feeds and feed ingredients evaluated

Feeds and feed ingredients	Origin
[1]*Artemia franciscana* (nauplii)	Decapsulated and hatched at IMR*, Austevoll, Norway
Calanus finmarchicus (copepod)	Captured and frozen at sea, Calanus AS, Tromsø, Norway
**Minipro™	Produced by Maripro AS, Austevoll, Norway
**Protein-encapsulated feed	Produced by NIFES according to Yufera et al., 1999
Micronised squid meal	Obtained from Rieber, Bergen, Norway
Micronised fish meal	Obtained from Rieber, Bergen, Norway
Micronised fish roe meal	Obtained from Rieber, Bergen, Norway
[2]Pepsin-digested cod fillet	Wild captured, hydrolysed at NIFES, Bergen, Norway
[3]Fresh frozen cod fillet	Wild captured
Stick-water	Water-soluble fraction from boiled herring, produced by Maripro AS, Austevoll, Norway
Casein	Tine AS, Oslo, Norway
Na^+-caseinate	Tine AS, Oslo, Norway
Whey protein	Tine AS, Oslo, Norway

*Institute of Marine Research;** Formulated diets; [1]Decapsulated, hatched and enriched for 20 h according to standard protocols at IMR before being rinsed with water, frozen and stored at −20 °C; [2]Ground fillets (136 g) were acidified by addition of 6 M of HCl (46 ml, pH 4.2). Hydrolysis was initiated by adding a mixture of water (18 ml), glycerol (9.5 ml) and pepsin (10.2 g) and hydrolysis allowed to continue at room temperature over night. The hydrolysate was thereafter neutralised to pH 7.6 by adding 6 M NaOH (55 ml). [3]A fresh fillet, frozen and stored at −20 °C.

to a final volume of 1 ml. A mixture (final volume 100 μl) of trypsin (type IX, *bovine* pancreas), chymotrypsin (type II, *bovine* pancreas) and bacterial protease (type XIV, *Streptomyces griseus*), all obtained from Sigma-Aldrich, MO, USA, was added to each tube to final concentrations of 73, 145 and 64 mg ml^{-1}, respectively. Digestion was performed at room temperature (22 °C). Proteins were precipitated and the *in vitro* digestion terminated by adding 250 μl of 40% TCA. For each component analysed, four parallel tubes were terminated and sampled 0, 1 and 12 h after addition of the proteases. The tubes were centrifuged and the crude protein content in the supernatant (350 μl), which contained proteolysis products, FAA and small peptides, was analysed and regarded as representing digested protein. Crude protein in the precipitate, containing intact protein and large peptides, was regarded as representing undigested protein. Casein was introduced as a reference protein every time *in vitro* digestion was performed, as a control of reproducibility.

Minor errors (<3%) in the calculation of supernatant volume due to changes in suspended material according to the degree of proteolysis were corrected for.

3. Results and discussion

Digestibility was assessed by elementary nitrogen analysis of the TCA solubles after treatment with crystalline proteases in an alkaline buffer. Compared to the results of Garcia-Ortega et al. (2000), who separated digested nitrous compounds from undigested nitrous compounds by filtration, the present analyses of the soluble fraction represent an improvement as most of the samples contained more nitrogen in the soluble than the insoluble fraction. In future studies, further improvements could be made by determining the increase in free amino acids by amino acid analysis, or by detecting free amino groups, as demonstrated by Lindner et al. (1995).

3.1. Contents of water-soluble nitrogen

The distribution of crude protein into water-soluble and insoluble fractions varied among the feeds and feedstuffs (Table 2). The live feeds, *Artemia* and *Calanus*, contained high fractions of soluble nitrogen; 67 and 54% of total N respectively. This was in accordance with high levels of water-soluble nitrogen in *Artemia* and rotifers (54 and 61%, respectively), as previously reported by Carvalho et al. (2003). Fresh frozen cod fillet contained 35% of water-soluble nitrogen. Pepsin hydrolysis almost doubled the water-soluble nitrogen fraction in cod fillet to 65%. The squid, fish and roe meals contained lower levels of 11, 17 and 11% respectively.

Table 2
Distribution of crude protein ($N\times6.25$) into water-soluble and water-insoluble fractions

Sample	Water-soluble fraction (%)	Insoluble fraction (%)	S.D.	*n* (analytical)
Artemia (nauplii)	67	33	2.8	4
Calanus (copepod stage)	54	46	2.2	4
Squid meal	11	89	2.6	4
Fish meal (Rieber, Norway)	17	83	1.6	4
Roe meal	11	89	1.1	4
Trypsin-digested cod filet	65	35	3.0	3
Fresh frozen cod fillet	34	66		1
Stick-water	100			–
Casein		100		–
Na^{+}-caseinate	100			–
Whey protein	100			–

3.2. In vitro digestibility

The *in vitro* digestibility after 12 h, assessed as percentage of analysed nitrogen in the TCA-soluble fraction, showed that the water-soluble fractions from the live feeds, the hydrolysed cod fillet and the meals were digested to 90% or above (Table 3). The water-soluble fraction of the fresh frozen cod fillet, together with whey protein and stick-water, both of which consists entirely of water-soluble proteins, were digested to some lesser extent, 75±6.4%, 68±1.9 and 71±0.7, respectively.

The insoluble fractions of the live feeds, hydrolysed (pepsin-digested) cod fillet, fish and squid meals were all digested to above 70% (71–76%), while the insoluble fraction of the fish roe meal was digested to a lesser extent (43±1%). The insoluble fraction of the fresh frozen cod fillet had the highest digestibility of the insoluble fractions (82±2.5%). These results suggest that, in a pancreatic digestive system, the water-soluble protein fractions of live feeds and feed ingredients are more digestible than the insoluble protein fractions ($P<0.05$), when all insoluble fractions are compared with all soluble fractions, All water-soluble fractions contained a high initial proportion of TCA-soluble nitrogen (36–93%). This fraction was, according to the definition used in this study, already digested before the proteases were added.

The initial levels of TCA-soluble nitrogen in the *Calanus* soluble fraction consisted of 93% of total

Table 3
In vitro digestibility of some feeds and feed ingredients used in starter diets for fish larvae in Aquaculture

	Digestibility of water-soluble fractions (%)				Digestibility of insoluble fractions (%)				[a]Total digestibility (%)			
	0 h	1 h	12 h	*n* (analytic)	0 h	1 h	12 h	*n* (analytic)	0 h	1 h	12 h	*n* (analytic)
Artemia	37±1.9	66±6.7	91±7.0	4	17±0.2	46±0.4	71±3.6	2	30	59	84	–
Calanus	93±3.8	88±8.9	96±6.1	4	9±0.2	45±1.0	72±0.8	2	54	68	87	–
Cod fillet	36±2.5	55±7.4	75±6.4	4	8±0.1	55±1.3	82±2.5	2	18	55	80	–
Hydrolysed cod fillet	55±2.7	80±4.8	92±2.7	4	10±1.4	42±1.9	76 (*n*=1)	2	39	67	86	–
Fish meal	56±7.0	99±4.8	103±2.4	4	6±0.9	46±0.8	71±0.2	2	15	55	77	–
Squid meal	79±7.5	85±4.3	90±7.1	4	7±1.4	42±0.5	75±2.3	2	15	47	77	–
Fish roe meal	57±5.4	71±5.6	91±0.9	4	6±0.4	21±0.8	43±1.0	2	12	27	49	–
Casein									5±0.0	61±4.0	94±3.0	2
Na^+-caseinate									6±0,6	82±2.4	94±1.7	6
Whey protein									14±1.2	24±3.9	68±1.9	4
Stick-water									63±0.6	73±0.0	71±0.7	4
Minipro™									32±1.9	47±2.0	70±3.0	2
Protein-encapsulated diet									1±0.4	29±1.2	53±2.3	4
Mixed ingredients for protein-encapsulated diet									8±0.6	54±2.1	71±1.4	4

[a] Total digestibility was calculated as the weighed digestibility based on the digestibility of the water-soluble and water-insoluble fractions. Hence, errors and analytical parallels are not presented.

nitrogen. This strongly suggests that autolysis had taken place in this sample, a possibility that cannot be rejected, as the *Calanus* sample was obtained frozen from a commercial supplier and its history of treatment from capture to freezing was not known in detail. The *Artemia* sample, on the other hand, was rapidly frozen and kept frozen at −20 °C until analysis, and during extraction of water-soluble nitrogen the temperature was kept at 4 °C in order to avoid autolysis. In spite of this *Artemia* also contained a high initial level of TCA solubles (37.8%), far above the levels of free amino acids (FAA) that have been reported for *Artemia* in the literature (Hamre et al., 2002; Helland et al., 2003). Carvalho et al. (2003) found that 89% of the *Artemia* nitrogen was in macromolecules larger than 500 Da. As peptides larger than this may be soluble in TCA, these values may represent the true level of FAA and small peptides in *Artemia*. Alternatively, they may include some hydrolysis products of autolysis in spite of the rapid freezing and low temperature during extraction. However, if autolysis during the extraction contributed to the high initial content of water-soluble nitrogen in the *Artemia*, the same contribution from autolysis to the total proteolysis in the gut after ingestion by fish larvae would be expected. The high initial levels of TCA-soluble components in the live feed samples thus suggest high protein availability in larvae. On the other hand, there is a possibility that the freezing and thawing of the *Artemia* sample in the present study contributed to some extent to protein degradation and the increased solubility and digestibility of the *Artemia*, beyond that of live *Artemia* ingested by fish larvae.

The measured digestibility of the soluble Na^+-caseinate after 12 h did not differ ($P>0.05$) from that of insoluble casein. After 1 h, however, the digestibility of soluble Na^+-caseinate was significantly higher ($P<0.05$), suggesting a lower initial digestion rate of insoluble casein. One possible reason for the difference observed after 1 h is the poor hydroscopic properties of the casein, which formed large hydrophobic aggregations in the reaction tubes, which would have reduced the effective contact surface between the casein molecules and the proteases. Carvalho et al. (2004) reported significantly improved survival and some improved growth in first-feeding carp larvae on compound diets when insoluble casein was replaced by Na^+-caseinate as the only protein source. Since feed ingestion rate was not monitored in their experiment, the possible positive effect of the solubility of Na^+-caseinate cannot be separated from possible differences in palatability between diets. However, no evidence for such a different effect on palatability has been presented and due to the high molecular weight of casein, differences in leaching between the two diets were unlikely to occur. The measured difference in initial digestibility between insoluble casein and Na^+-caseinate in the present study supports

the notion of improved digestibility as an explanation for the improved performance of the soluble Na^+-caseinate in the early feeding larvae in the study of Carvalho et al. Visual observations made during this study showed that all suspended insoluble fractions were more hydrophilic than the insoluble casein and were therefore better dispersed in the buffer in the reaction tubes. Even so, the average initial digestibility (at 0 h) of insoluble casein exceeded that of all the other insoluble fractions (Table 3). This suggests that the insoluble fractions of the fish-, squid- and fish roe-derived ingredients are even less available than the insoluble casein that produced poor growth and survival in carp larvae (Carvalho et al., 2004). This further supports the idea that low dietary protein availability could be a limiting factor for growth and survival in first-feeding larvae fed compound diets based on such protein sources. Feeding experiments with compound diets for marine larvae (sea bass, *D. labrax*) have shown that casein may support growth and survival in the early feeding stage (sea bass, Cahu and Zambonino Infante, 1995; sea bream, *S. aurata*, Robin and Vincent, 2003) when intestinal proteolysis is considered to be poor. On the other hand, diets with casein as the only protein source did not to support growth in sea bass larvae over time just as well as fish-based or mixed protein sources (Cahu and Zambonino Infante, 1995), suggesting that casein as the only protein source did not fully meet the amino acid requirements of the larvae of this species. However, in these experiments insoluble casein or a mix of insoluble casein and soluble casein and pre-hydrolysed casein were used. To the best of our knowledge, marine larvae have yet to be fed with compound diets based on Na^+-caseinate as the only protein source, as in the study of Carvalho et al., 2004 on freshwater carp larvae.

The total digestibility of the live feeds and the complex ingredients was not analysed *per se*, but was calculated from the specific digestibility of the water-soluble and insoluble fractions (Table 3). Theoretically, this value can differ from the digestibility of the unseparated feed ingredients due to protease inhibition from other ingredient constituents (Alarcón et al., 1999). The *in vitro* digestibility of the live feeds (84 and 87%) was greater than that of the compound diets (Minipro™, 70±3%, protein encapsulated diet, 53±2.3%). Of the feed ingredients, cod fillet was more digestible *in vitro* than the squid, fish and fish roe meals. Fish roe meal distinguishes itself as the least digestible protein source in this study, at 48%. All the other feeds, feedstuffs and reference proteins were digested to between 68 and 95%.

3.3. Pre-hydrolysis of dietary protein

The two live feeds tested contained high levels of TCA-soluble nitrogen which, as pointed out above, seems to be readily available for utilisation by the fish larvae. The feed ingredients tested contained various levels of TCA-soluble nitrogen, although these levels may be increased by hydrolysis before incorporation into feed particles, as shown for cod fillet (Table 3). Pre-hydrolysed protein sources are frequently added to compound larval diets (Cahu et al., 1999; Hamre et al., 2001; Kvåle et al., 2002) in order to increase the availability of dietary AA. However, this strategy may fail if nutrient leaching is not prevented due to the increased solubility and lowered molecular weight of the hydrolysis products and thus the potential leaching from feed particles before ingested by the larvae. Rapid rates of loss (>90% in 2 min) have been reported for free amino acids supplementing compound diets (López-Alvarado et al., 1994; Kvåle et al., 2006). Free amino acids should be regarded as extremes with regards to leaching rates; however, extensive leaching of nitrous compounds from microdiets based on intact fish protein sources (Opstad and Hamre, 2003) demonstrates that leaching will occur also for water-soluble macromolecules. Leaching rates of peptides should be regarded as intermediate and a function of molecular weight and to a certain extent, of the amino acid composition too. Our cod fillet results show that pre-hydrolysis with pepsin almost doubles the content of water-soluble nitrogen, while total digestibility increased only from 55 to 67% at 1 h and from 79 to 86% at 12 h. In a feeding trial, pre-hydrolysis of a protein source such as cod fillet might therefore reduce rather than improve the total availability of the protein if a significant proportion of the water-soluble cod fillet or pre-hydrolysed cod fillet is lost by leaching to the water before being ingested. Nutrient leaching is time-dependent and nutrient loss may therefore also depend on tank design, feeding protocols and the feeding activity of the species concerned. Supplementing pre-hydrolysed protein sources in compound diets may thus be advantageous in species that ingest formulated feeds rapidly and that can be kept in small systems, while this strategy may fail and even have negative effects in species whose feeding behaviour is more sedate and thus eat more slowly, and that also require larger tank systems, such as larvae of Atlantic halibut (Kvåle et al., 2006). Negative absorptive and metabolic effects of pre-hydrolysed protein sources in compound larval diets have been observed and discussed by several authors (e.g. Cahu et al., 1999; Kolkovski and Tandler, 2000; Dabrowski et al., 2003). Unfortunately, the leaching

rates of the micro-particulate diets in these experiments were not reported, making it difficult to distinguish between the possible negative effect of ingested pre-hydrolysed protein on absorption and metabolism (flooding) and the negative effects of reduced total dietary and digestible protein ingested due to substantial leaching from the feed. In our opinion this makes it very difficult to evaluate the effects of such inclusions in feeding trials. At the same time, some leaching of nutrients are positive due to their roles as attractants (Kolkovski et al., 1997; Koven et al., 2001).

3.4. The effect of feed preparation on dietary protein digestibility and availability

The *in vitro* digestibility of the mixed ingredients of the protein-encapsulated particles was equal to the sum of the analysed digestibility of the individual protein sources, suggesting that there were no significant protease inhibitor effects of any of the ingredients of this diet formulation (Alarcón et al., 1999). The *in vitro* digestibility of the finished protein-encapsulated particles, however, was significantly ($P<0.05$) reduced in comparison with that of the mixed ingredients. After 1 h, the digestibilities of the particulated feed and the mixed ingredients were 29% and 54% respectively, but the relative difference decreased thereafter, eventually ending at 53% and 71% after 12 h (Table 3). A lowering of the digestibility as a result of the diet formulation process could be expected, due to the cross-binding of proteins which probably yields fewer degradable reaction products.

The final digestibility at 12 h was higher for the other formulated feed tested (Minipro™, 70±3%) than for the protein-encapsulated particles (53±2.3%). In comparison with the protein-encapsulated particles, Minipro™ contained substantially higher initial levels of TCA solubles (31.8±2.0% vs 1±0.4%) and was thus more similar to the composition of live feeds. However, in contrast to live feeds, in which the nutrient content is protected by biomembranes and homeostatic regulation, small soluble components supplementing compound feeds may largely be lost by leaching in water (López-Alvarado et al., 1994, Kvåle et al., 2006). The availability of the Minipro™ TCA soluble protein fraction to larvae will thus depend on the rate and extent to which it leaks from the feed particles after it has been added to the water. The finished protein-encapsulated feed leaches its water-soluble added ingredients at a low rate (Yufera et al., 2002). However, a recent study (A. Nordgreen, NIFES, Bergen, Norway, pers. comm.) indicates that repeated washing of microcapsulate feeds in water during the production process seems to leave little of leachable components. Any leakage during production may alter the composition of the microcapsules in favour of the water-insoluble fractions. This may thus also have direct implications for the total protein digestibility of this feed. The other micro-diet was presumedly more similar to the list of ingredients with regards to biochemical composition when the *in vitro* digestibility was performed. Thus, it is difficult to compare and evaluate these specific micro-diet preparation methods in terms of their impact on protein digestibility, on the basis of the present study.

A potential strategy for improving dietary protein availability in compound feeds would be to prevent leaching by efficient encapsulation of feed particles that are supplemented with high levels of FAA, peptides and soluble protein, in order to mimic live feeds. Another potential strategy would be to design feeds that contain intact protein sources which would resist leaching due to their large molecular size, but that are still highly digestible. Na+-caseinate appears to be a candidate in the latter category, due to its high digestibility, although it might be necessary to supplement it with other amino acid sources in order to satisfy the larval demands for amino acids. However, our results confirm that the digestibility of intact proteins that are highly digestible *per se* may be significantly reduced during the feed preparation process. Thus, some effort still needs to be put into developing micro-feed preparation techniques that combine particle stability and nutrient leaching on one side with adequate digestibility on the other.

3.5. Correlation between in vitro and in vivo digestibility

Various approaches have been tried in order to develop reliable and cost-efficient methods for the evaluation of protein digestibility. Compared to *in vivo* measurements, *in vitro* assays are easier to perform and are much more cost efficient; however, their relevance can only be confirmed by *in vivo* experiments. *In vitro* studies may be based on intestinal extract from a species of particular interest (e.g. Jany, 1974; Nankervis and Southgate, 2006) or on commercial available crystalline proteases (e.g. Hsu et al., 1977; Saterlee et al., 1979). In this study, our aim was to evaluate the digestibility of some feeds and potential protein sources in stomachless fish larvae in general. Commercial enzymes were thus used as this was expected to improve the reproducibility of data as compared to using larval intestinal extracts from one species of fish. This approach was also supported by the findings of Chong et al. (2002), who

showed that *in vitro* protein digestibility rates, as evaluated by three different protocols, correlated well among themselves and also with *in vivo* digestibility in juvenile discus fish. The protocol used in the present experiment was a combination of two of the protocols (Hsu et al., 1977; Saterlee et al., 1979) tested by Chong et al. (2002), with the exception that Trizma-base (pH 8.0) was replaced by the nitrogen-free phosphate buffer (pH 8.0) since our protocol was based on nitrogen analysis. Although Chong et al. found some differences in absolute digestibility between protocols, the feed ingredient digestibility ranking made by all protocols agreed well with the *in vivo* digestibility ranking. They used juvenile discus fish that had developed functional gastric digestion and thus differed from the typical marine fish larvae. However, the *in vitro* digestion protocol consists of a single chamber reaction at alkaline pH, which is more similar to the type of digestion that takes place in marine larvae. The results of Chong et al. suggest that *in vitro* protocols based on commercial crystalline enzymes can be used with success to predict the relative digestibility of different feedstuffs *in vivo*. However, in the end, the true digestion of a diet has to be measured in the target larvae for the studies. Such *in vivo* digestibility studies are not easily performed in small larvae, due to technical difficulties concerning the quantification of ingestion and faeces collection. An assessment of the digestibility of dietary protein can be performed in fish larvae by controlled tube-feeding of a radioisotope-labelled protein followed by compartmental analysis of the distribution of the tracer in a hot-chase approach (Rønnestad et al., 2001). Using this method, Tonheim et al. (2004, 2005) measured absorption rates and the utilisation efficiency of a salmon serum protein in Atlantic halibut larvae. However, this method requires labelling techniques that do not alter the properties of the protein (Rojas-García and Rønnestad, 2003; Tonheim et al., 2004) and so far only model proteins have been tested. If this problem can be adequately dealt with, tube-feeding experiments may be included in future studies to assess the correlation between the *in vitro* digestibility of a particular dietary protein and the digestibility of the identical but labelled protein in larvae. A range of other tracer methodologies for the assessment of digestibility is currently available (Conceição et al., 2006 but due to their individual constraints it is advisable that long-term effects should also be assessed in subsequent validation growth trial-type experiments. However, *in vitro* digestibility experiments can be a very useful tool for screening larval feeds and feed ingredients and reducing the number of dietary treatments to be tested in growth-trial studies.

4. Conclusions

1. The live feeds and the cod fillet contained high fractions of water-soluble protein.
2. The digestibility of the water-soluble protein was generally higher than that of the insoluble protein fraction.
3. The digestion rates of soluble Na^{+}-caseinate were higher than those of insoluble casein, although they reached the same final value for digestibility after 12 h.
4. Pepsin hydrolysis almost doubled the water-soluble nitrogen fraction in cod fillet, but produced only a small increase in total digestibility.
5. The final assessment of protein sources for diets for marine fish larvae should include evaluation of digestibility and bioavailability from prepared particles that have been immersed in water for a realistic period of time.

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Paper III

1 # Effect of heat treatment and leaching on the protein quality of fish larval
2 # feed with increased concentration of hydrolyzed protein

3

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19 Keywords: Formulated diet, fish larvae, Protein hydrolysate, *In vitro* digestibility, protein
20 quality

21

Abstract

Four heat coagulated diets with increasing concentrations of pepsin hydrolyzed protein used for early weaning of cod and halibut larvae were investigated concerning change in protein quality during feed production and exposure to leaching. Water-soluble N, TCA (10 %) soluble N and amino acid profiles were determined in the finished diets and in the diets after being submerged in water and allowed to leach for 6 minutes. *In vitro* digestibility of the diets were measured and related to the increasing inclusion of hydrolyzed protein and N leakage. A large fraction of the soluble N in the feed ingredients was made insoluble by heat denaturation during production of the diets, but the concentration of peptides and FAA was not influenced by the production process. Most of the water-soluble protein and all peptides and free amino acids (FAA) were lost after 6 minutes exposure to leaching by hydration in phosphate buffer (pH 8). Increased inclusion of hydrolyzed protein in the diets significantly increased the loss of crude protein (% of crude protein), however there was no significant difference in relative loss (%) of soluble N. All taurine and 30 % of histidine was lost during leaching, however, no other major changes in AA profile due to leaching were found. There was no significant difference in *in vitro* digestibility between the diets exposed to leaching. However, the leached diets showed significantly reduced digestibility *in vitro* as compared to diets that had not been exposed to leaching.

46 # Introduction

47

48 Marine fish larvae have a great growth potential (Houde, 1989; Kamler *et al.*, 1992;
49 Conceicao *et al.*, 1997) and even in cold water species such as the Atlantic cod (*Gadus*
50 *morhua*) specific growth rates exceeding 25 % day^{-1} have been reported (Otterlei *et al.*, 1999).
51 The larval growth is lean and thus mainly the result of protein deposition (Houlihan *et al.*,
52 1995). Accordingly, both high quality and quantity of dietary protein is required. At an early
53 stage the larvae lack a functional stomach (Govoni *et al.*, 1986; Pittman *et al.*, 1990; Segner *et*
54 *al.*, 1994) and may have low digestive enzyme activity (Cousin *et al.*, 1987; Kvåle *et al*,
55 2007). The larval ability to digest complex protein has thus been questioned (Tonheim *et al.*,
56 2004). Tube feeding of larval Atlantic Halibut (*Hippoglossus hippoglossus*) showed a higher
57 absorption efficiency of hydrolyzed protein as compared to intact soluble protein (Tonheim *et*
58 *al.*, 2005). Stomach-less fish larvae fed formulated diets have suppressed growth and survival
59 compared to larvae fed live feed (Howell *et al*., 1998; Kolkovski, 2001; Cahu and Zambonino
60 Infante, 2001). The high concentration of water-soluble nitrogen (N) (copepods 54 ± 2 %,
61 rotifers 56.9 ± 0.8 % and *Artemia* 54 ±0.4 % of total N, respectively, Tonheim *et al*., 2007;
62 Srivastava *et al.*, 2006; Carvalho *et al.*, 2003) and high concentration of low molecular
63 weight N (Carvalho *et al.*, 2003; Tonheim *et al*., 2007) in live feed compared to formulated
64 diets, is a suggested explanation for the reduced growth and survival reported from trials with
65 stomach less fish larvae fed on formulated feeds (Carvalho *et al*., 2004). Carvalho *et al.*,
66 (2003) showed that nearly 90 % of the soluble N in rotifers and *Artemia* was less than 500 D
67 in size.

68 To mimic the N pool of live feed and get improved growth and survival, free amino
69 acids (FAA) or hydrolyzed protein has been added to formulate feeds for early stage larvae
70 (Carvalho *et al.*, 1997; Cahu *et al.*, 1999; Hamre *et al*., 2001; Kvåle *et al.*, 2002; Carvalho *et*

71 *al.*, 2004). Although the results are not always consistent, low inclusion levels of hydrolyzed
72 protein in the diets have shown to improve survival and growth (Zambonino-Infante *et al.*,
73 1997; Cahu *et al.*, 1999; Carvalho *et al.*, 2004) while higher inclusion levels have shown to
74 have negative effects on larval performance (*Sparus aurata* L., Kolkovski and Tandler, 2000;
75 *Cyprinus carpio*, Carvalho *et al.*, 1997; Carvalho *et al.*, 2004; *Dicentrarchus labrax*, Cahu *et*
76 *al.*, 1999; *Hippoglossus hippoglossus* Kvåle *et al*., 2007). Supplementation of 40 % pepsin
77 hydrolyzed protein to a heat coagulated diet (Hamre *et al.*, 2001) improved survival rates in
78 cod (*Gadus morhua*) larvae, compared to lower levels of supplementation (Kvåle *et al*, 2007).
79 On the other hand, Atlantic Halibut (*Hippoglossus hippoglossus*) juveniles fed the identical
80 experimental diets showd a declining survival rate as the inclusion level of hydrolyzed protein
81 increased (Kvåle *et al*., 2007). It is suggested that the reduced survival in halibut fed increased
82 levels of hydrolyzed protein is caused by their typical slow feeding behavior (Stoss *et al.*,
83 2004, Kvåle *et al*., 2007). The feed will therefore stay in the water for a longer time before
84 eaten compared to when fed to species with a more aggressive feeding behavior, and the loss
85 due to leaching of water-soluble nitrogenous compounds will be correspondingly higher and
86 proportional to the inclusion level of hydrolyzed protein. This will consequently result in
87 lowered levels of both total protein and water-soluble N in the eaten feed particles, which will
88 in turn affect the amount of digestible protein and amino acids available for the larvae.
89 Loss of low molecular weight nutrients such as FAA and peptides are severe in almost
90 all tested formulated larvae diets supplemented with such (Lopez alvarado *et al.*, 1994; Kvåle
91 *et al.*, 2006). The loss of FAA was as high as 90 % within less than 2 minutes in diets tested
92 by Carvalho *et al*. (2004). Experimental diets have been developed, which to some extent can
93 reduce the loss of low molecular weight nutrients during feeding (Villamar and Langdon,
94 1993; Lopez Alvarado *et al.*, 1994; Yufera *et al.*, 2002; Onal and Langdon, 2005), however,
95 nearly all studies conducted to investigate the nutritional effect of hydrolyzed protein on

96 different species fish larvae have been carried through with micro bound or grinded diets that
97 all have in common a rapid leaching of soluble compounds (see review by Langdon 2003).
98 In addition to high loss during feeding, water-soluble protein is susceptible to loss or
99 modification during production, such as denatuartion (de Wet, 1983; Boye *et al.*, 1997,
100 Mohammed *et al.*, 2000,), crosslinking (Jones *et al.*, 1974; Langdon, 1989; Yufera *et al.*,
101 1996), Maillard reactions (Plakas *et al.*, 1985, Deng *et al.*, 2005) and leaching (Nordgreen,
102 unpublished). The feed production process can therefore significantly change the protein
103 quality and reduce the digestibility of diet and thereby mask any biological effects of
104 increased supplementations of hydrolyzed protein. When investigating the effect of such
105 supplementations to formulated diets, it is thus of great importance to investigate the effect of
106 production process and leaching on the remaining protein quality.
107 The present study was designed to investigate the changes in protein quality due to
108 production process and leaching in 4 diets with increasing levels of hydrolyzed protein. A
109 heat coagulated diet were used (Hamre *et al.*, 2001), that had previously been used for
110 weaning of cod and Atlantic halibut larvae in several studies (Hamre *et al.*, 2001;Kvåle *et al.*,
111 2002; Hamre *et al.*, 2002; Hamre *et al.*, 2003; Moren *et al.*, 2004; Hamre *et al.*, 2005). The
112 diet had also been used in several studies to investigate possible effects of hydrolyzed protein
113 on the performance of Atlantic halibut larvae and Atlantic Cod larvae (Kvåle *et al.*, 2002;
114 Kvåle *et al.*, 2007). To understand the changes in protein quality and bioavailability during
115 production and leaching of the diets, we investigated changes in crude protein, water-soluble
116 N, TCA-soluble N (FAA and peptides), amino acid profile and *in vitro* digestibility.
117
118

119 ## Materials and methods

120

121 ### Feed and feed ingredients

122 Four heat coagulated diets based on fresh cod fillet and increasing concentrations of pepsin
123 hydrolyzed cod fillet as the major protein sources were produced at National Institute of
124 Nutrition and Seafood Research (NIFES), Bergen, Norway, as described by Hamre *et al.*,
125 (2001). The different inclusion levels of pepsin hydrolyzed cod fillet represented 0 %, 15 %,
126 30 % and 45 % of diet total protein content (table 1). The feed ingredients were blended in a
127 high speed cutter to a thick dough and finger thick strings of the feed blend were fed onto a
128 conveyor belt. The feed was first heated using electromagnetic energy and then dried in a
129 warm air tunnel dryer over night (with immobile conveyor; 60 ºC). Thereafter pellets were
130 crushed and sieved. The size fraction sieved through a 600 µm sieve and collected on a 300
131 µm sieve was studied.

132 The pepsin hydrolyzation was carried out according to the method described by Kvåle
133 *et al*. (2002). Fresh cod fillet was minced and the pH was lowered to 4.2 by addition of 6 M
134 HCl. A solution consisting of pepsin (7.5 g per kg mince; Sigma-Aldrich Corp., St. Louis,
135 Mo, USA), glycerol (93 ml per kg mince; Merck, Darmstadt, Germany) and distilled water
136 (173 ml per kg mince) was mixed into the minced fish solution and incubated at room
137 temperature for 24 hours under continuous shaking. Thereafter the pH was raised to 7.2 by
138 addition of 5 M NaOH. The hydrolyzed solution was frozen (-30 ºC) until use. The enzymes
139 were inactivated by heat during the feed production.

140 Particle size was evaluated by taking pictures under the microscope (Olympus BX 51)
141 at 40 times magnification. The width and length of minimum 90 feed particles of each
142 produced diet were measured with Olumpus DP-soft.

143

144 **Change in protein quality due to the production process**

145 Crude protein (N x 6.25), water-soluble N and trichloroacetic acid (TCA) soluble N weres
146 measured in all raw materials and the concentrations of the different N fractions in the raw
147 material blends were calculated on results from Tonheim *et al*. (2007). Crude protein, water-
148 soluble N and TCA soluble N was also analyzed in the four diets. For analysis of water-
149 soluble N, the diets were crushed and 44-49 mg was weighed into 1.8 ml Eppendorf tubes. A
150 volume of 1ml of 80 mM phosphate buffer (pH 8) was added and the tubes where shaken
151 vigorously on a shaking table for 5 hours. The tubes were then centrifuged at 8000 RPM
152 (Eppendorf Centrifuge 5415 C) for 9 minutes. The supernatant was removed and the pellet
153 was washed and centrifuged twice in phosphate buffer. The pellet was transferred to
154 evaporating tinfoil cups (5 ml, Elemental Microanalysis Limited) and residues in the tube
155 were rinsed into the tinfoil cups. The tinfoil cups were dried at 65 ºC overnight before
156 analyzed for crude protein (N x 6.25) by total combustion. TCA soluble N was analyzed by
157 weighing 30 mg of each of the 4 diets (n=4) into 1.8 ml Eppendorf tubes. A volume of 1.1 ml
158 of phosphate buffer was added and the tubes were shaken vigorously before 250µl of TCA
159 (40 %) was added to each sample. The samples were incubated at 4 ºC until the next day
160 before centrifuged at 8000 RPM (Eppendorf Centrifuge 5415 C) for 9 minutes. Finally 350 µl
161 of supernatant was added to tin capsules (Smooth wall, Round base; Elemental Microanalysis
162 Limited) and analyzed for total N by total combustion.

163

164 **Leaching study**

165 To investigate the difference in leaching between the four diets, 5 parallel samples with 40-50
166 mg of each diet were suspended in tubes containing1.5 ml of phosphate buffer for 6 minutes.
167 The tubes were rotated slowly during the 6 minutes to keep the feed in suspension. The
168 contents of the tubes were then filtered onto a 0.65 µm N free Durapore membrane filter (25

169 mm). The filters were wrapped in tin foil cones (Elemental Microanalysis Limited) and
170 analyzed for crude protein (N x 6.25) by total combustion. The loss of N was calculated by
171 dividing the N content in the leached samples with the analyzed N content in the pre-leached
172 samples. To calculate the remaining concentration of TCA soluble N after leaching the diet
173 was lyophilised before 30 mg of the each diet (N=4) were weighed into 1.8 ml Eppendorf
174 tubes, precipitated with TCA and analysed as described above.

175

176 ***In vitro* digestibility**

177 The protocol for *in vitro* digestion used in the present study was based on the methods
178 described by Hsu *et al*. (1977) and Saterlee *et al*. (1979) and was carried through according to
179 modified method described Tonheim *et al.* (2007). Diet samples equivalent to 20 mg of crude
180 protein was suspended in 1 ml of phosphate buffer in 1.8 ml Eppendorf tubes. A mixture of
181 trypsin (type IX, bovine pancreas) chymotrypsin (type II, bovine pancreas) and bacterial
182 protease (type XIV, *strepomyces griseus*), all obtained from Sigma-Aldrich, MO, USA were
183 added to each tube to final concentrations of 73, 145 and 64 μg ml^{-1} phosphate buffer,
184 respectively. Digestion was performed at room temperature (22 ºC). The samples were placed
185 on a shaking table at low speed during the digestion period. Undigested protein was
186 precipitated and the *in vitro* digestion terminated by adding 250 µl of TCA (40 %). For each
187 analyzed diet and treatment four parallel tubes were analyzed at 0 h, ½ h, 1 h and 12 h,
188 respectively. All tubes were centrifuged and the crude protein content in the supernatant (350
189 µl), containing proteolysis products, i.e. FAA and small peptides, was analysed and regarded
190 as representing digested protein. Each sample of supernatant (350 µl) was added to a tin
191 capsule (Smooth wall, Round base; Elemental Microanalysis Limited) and analyzed for total

192 N by total combustion. Crude protein (N x 6.25) in the precipitate, containing intact protein
193 and large peptides, was regarded as representing undigested protein.
194 To remove water-soluble N from the four diets prior to *in vitro* digestibility, 1 gram of
195 each diet was suspended in 10 ml phosphate buffer and shaken for 2 minutes. The tubes were
196 centrifuged at 3220 g for 30 sec. and the supernatant was removed. This was repeated once;
197 the feed was in solution for approximately 6 minutes to equal the leaching study. The leached
198 diets were frozen and freeze dried and a sub sample was analyzed for N content before the *in*
199 *vitro* digestibility studies.
200
201 **Change in amino acid (AA) profile due to leaching and *in vitro* digestibility**
202 Change in AA profile due to leaching and *in vitro* digestibility was analyzed with the diet
203 containing 30 % hydrolyzed protein (Table 2). The diet was leached according to above
204 described method. The pre-leached and leached diets were analyzed in triplicates. To
205 investigate the AA profile of the digested diet, the pre-leached and leached diet was *in vitro*
206 digested in triplicates for 12 hours according to described method. Samples of the
207 supernatants were analyzed for AA content.
208
209 **Analytical methods**
210 N was determined by total combustion using a nitrogen analyzer (Leco FP-528, St. Joseph,
211 MI). Crude protein was calculated as N x 6.25. Total AA profile was analyzed according to
212 the method described by Cohen *et al*. (1989). The samples were hydrolyzed in 6 N HCl for 22
213 h at 110 °C. The hydrolyzed solution containing free AA was then analyzed using the Waters
214 HPLC analyzer system (Pico Tag) after pre-derivatization with phenyl isothiocyanate (PITC)
215 using norleucine as internal standard. The AA were detected by UV at 254 nm and identified

216 by retention time. Tryptophane is degraded during the acidic hydrolysis and is therefore not
217 analyzed.
218

219 **Statistical methods**

220 The data are expressed as means ± standard deviations. To investigate the effect of pepsin
221 hydrolyzes on change in crude protein content (N x 6.25), water-soluble N and TCA soluble
222 N the data were analyzed by a T-test. The effects of the production process on water-soluble
223 protein content and TCA soluble content in the different diets were analyzed by regression
224 analysis along with one-way analysis of variance (ANOVA; Sokal and Rohlf, 1969) followed
225 by Tukey's HSD multiple comparison test. Change in AA profile due to production process,
226 leaching and in vitro digestibility was analyzed by one-way analysis of variance (ANOVA;
227 Sokal and Rohlf, 1969) followed by Tukeys's multiple comparison test. The effect of
228 increased inclusion of hydrolyzed protein on particle size was analyzed by regression analysis
229 along with one way ANOVA. Means with P values less than 0.05 were considered
230 significantly different. All statistical analyses were performed using Statistica 7.1 (Statsoft,
231 Inc, Tulsa, USA).
232

233 ## Results

234 Pepsin hydrolyzes of the cod fillet led to a 91 % increase in water-soluble N (Table 2) and a
235 122 % increase in TCA soluble N (Table 2).

236 Due to the crushing and sieving process most of the particles were rod shaped (Figure
237 1) and it was therefore necessary to measure both width and length of the particles. The
238 particles were nearly twice as long as they were wide and had a mean length 593 ± 184 µm
239 and a mean width of 320 ± 81 µm. There was no significant correlation (R^2=0.006, P=0.35)
240 between increased hydrolyzed protein and particle size and no significant difference
241 (ANOVA, $P>0.05$) in particle size between any of the diets.

242

243 **Change in total N**

244 There was a negative correlation (R^2= 0.91, $P<0.00001$ Beta= - 0.96) between concentration
245 of hydrolyzed protein and crude protein (N x 6.25) in the four diets (Figure 2) with a 5 %
246 reduction in crude protein from the diet containing 0 % hydrolyzed protein to diet with 45 %
247 hydrolyzed protein (Figure 2).

248 Leaching was positively correlated (R^2=0.96, P=0.000, Beta= 0.982) with inclusion of
249 hydrolyzed protein. The loss of crude protein ranged from 14.9 ± 0.5 % to 30.3 ± 0.4 % of
250 total crude protein from the diet containing 0 % hydrolyzed protein to the diet containing 45
251 % hydrolyzed protein (Figure 2).

252

253 **Change in water-soluble N**

254 The calculated increase in water-soluble N in the raw ingredients with an increase of
255 hydrolyzed protein from 0 % to 45 % hydrolyzed protein was 38 % (Figure 3). Increased
256 inclusion of hydrolyzed protein was positively correlated (R^2=0.89, $P<0.00001$, Beta= 0.943)
257 with concentration of water-soluble N in the diet (Figure 3), but there was a significant

258 decrease (R^2=0.73, P<0.00001, Beta= -0.86) in relative loss (%) of water-soluble N with
259 inclusion of hydrolyzed protein during feed production (Figure 3). The loss of water-soluble
260 N due to heat denaturation ranged from 46,8 ± 2.7 % to 17.4 ± 2.4 % of water-soluble N from
261 the diet containing 0 % hydrolyzed protein to the diet containing 45 % hydrolyzed protein
262 (Figure 3).

263 Total loss of water-soluble N due to leaching was positively correlated with
264 concentration of hydrolyzed protein (Figure 3), and the remaining concentration of water-
265 soluble N ranged from 3.0 ± 0.5 % water-soluble N for the diet containing 0 % hydrolyzed
266 protein to 11.0 ± 0.5 % water-soluble N for the diet containing 30 % hydrolyzed protein
267 (Figure 3). There was no correlation between concentration of hydrolyzed protein and relative
268 loss of water-soluble N by leaching and the relative loss of water-soluble N ranged from a
269 minimum of 70 % to a maximum of 83 % of total soluble N in the respective diets.

270

271 **Change in TCA-soluble N**

272 The raw ingredients had a 77 % increase in TCA soluble protein from the diet containing 0 %
273 hydrolyzed protein to the diet containing 45 % hydrolyzed protein (Figure 4). The
274 concentration of TCA soluble N was not affected by the feed production (Figure 4) and the
275 produced diets had a significant increase (R^2=0.9 P<0.0001, Beta= 0.949) in TCA soluble N
276 from 10.6 ± 0.6 % of total N to 21,6 ± 0.9 % of total N (Figure 4).

277 There was a nearly complete loss of TCA soluble N due to 6 minutes leaching and no
278 significant difference (P>0.05) in concentration of retained TCA soluble N between the diets
279 (Figure 4).

280

281

282

283 ### *In vitro* digestibility

284 There was a significant increase (ANOVA, P<0.05) in digestibility with time for both the
285 leached and not leached diets (Figure 5). The not leached diets all had a significantly higher
286 (ANOVA, P<0.05) digestibility at all investigated time intervals than the leached diets (Figure
287 5). Increased digestibility correlated with increased concentration of hydrolyzed protein for
288 time 0, ½ h and 1 hour for the not leached diets and there was no significant difference in
289 digestibility between the four diets at 12 hours in vitro digestibility (Figure 5). For the leached
290 diets, no significant differences (ANOVA, P≥0.05) in digestibility between the diets at any of
291 the investigated time intervals were found (Figure 5).
292

293 ### Change in AA profile

294 There were significant changes in the AA profile due to leaching for 6 minutes, but the only
295 severe change was a 100 % loss of taurine and a 30 % loss of histidine (Figure 6). No
296 significant (P≥0.05) differences in AA profile were found comparing the pre-leached diet with
297 the digested pre-leached diet and the leached diet with the digested leached diet (Figure 6).
298 The significant difference in the AA profile between the *in vitro* digested pre-leached and
299 leached diet was a consequence of the changes occurring during the leaching process (Figure
300 6).
301

302 ## Discussion

303 These experiments were performed to evaluate changes in protein quality as a consequence of
304 production procedures and leaching in a heat coagulated diet (Hamre *et al.*, 2001) with
305 increasing inclusion levels of pre hydrolyzed protein. The results clearly indicated that both
306 the production process and leaching can dramatically change the protein quality and could
307 possible mask biological effects of including a high concentration of hydrolyzed protein.

308 There were only minor differences in soluble N and no difference in TCA soluble N between
309 the four diets after exposure to water for 6 minutes. This shows the difficulties in
310 accomplishing feeding studies to find the optimal concentration of hydrolyzed protein for
311 different species of marine fish larvae and could explain the varying results in feeding trials
312 with formulated diets containing different concentrations of hydrolyzed protein, as suggested
313 by Kvåle *et al*., (2006).
314

315 **Quality of the hydrolyzed protein**

316 Fresh cod fillet has a relative high initial concentration of soluble N compared to
317 commonly used feed ingredients such as heat processed fish meal (Tonheim *et al*., 2007).
318 Pepsin hydrolyzes of cod fillet lead to a doubling in concentration of both water-soluble N,
319 from 34 (n=1) to 65 ± 3 %, and TCA soluble N, from 18 ± 2.4 to 40 ± 4.2 % of total N.
320 Tonheim *et al*. (2007) showed that 12 hours pancreatic hydrolyzes of cod fillet gave a product
321 with 79 ± 9.1 % TCA soluble N while a pepsin + pancreatic (24 h + 12 h) gave a product with
322 86 ± 2.7 % TCA soluble N. This shows that different degree of hydrolyzes leads to large
323 changes in product quality which might affect fish larvae during feeding trials. Due to
324 possible large differences in protein quality between hydrolyzed protein sources, it is of
325 importance to describe the quality of hydrolyzed protein fractions and the total protein of diets
326 used in feeding trials. Due to the high initial concentration of soluble N in cod fillet,
327 inclusion of 45 % hydrolyzed protein led only to a 37 % increase in water-soluble N in the
328 raw ingredient mixture in this diet, compared the diet containing 0 % hydrolyzed protein.
329 However, there was a 104 % increase in TCA soluble N.

330 Inclusion of pure peptides and FAA mixtures could be used instead of hydrolyzed
331 marine ingredients (Dabrowski *et al*., 2003; Zhang *et al*., 2006). Although there might be a
332 better control with the concentration and quality of the soluble N fraction by using pure

333 peptides and FAA mixtures, the use of hydrolyzed marine products (random peptide
334 mixtures), such as fish fillet used in this study, is a cheap and effective way of producing a
335 product with a good AA profile. When hydrolyzing the main protein source of the diet instead
336 of adding increasing amounts of a different hydrolyzed protein source the diet will keep the
337 same AA profile only differing in solubility of the N fraction. This is in accordance with
338 Dabrowski *et al*. (2003) that argued that the same source of protein had to be fed in both
339 intact and hydrolyzed forms to investigate if inclusion of protein hydrolysates leads to
340 enhanced growth in larval fish.
341
342 **Binding and particle quality**
343 A protein bound heat coagulated diet is bound together by thermal denaturizing of the
344 protein. A diet with increased hydrolyzed protein will contain less protein which is able to
345 bind and contribute to matrix formation and might therefore result in decreased stability of the
346 feed particles. The binding properties of the 4 diets was not investigated, however feeding
347 trials with heat coagulated diets containing similar concentration of hydrolyzed protein have
348 been accomplished without detecting problems with the particle stability (Kvåle pers med,
349 NIFES, Bergen, Norway). Crushing and sieving led to rod shaped particles with shorter
350 distance to the center of the particles and larger surface area compared to round particles with
351 the same volume. Increase of surface to volume ratio can lead to an increased leaching rate
352 (Lee and Rosenberg 2000, 2001; Kvåle *et al*., 2006). However, rod shaped particles can make
353 it possible for marine fish larvae to swallow larger particles that might compensate for the
354 increased leaching due to the increased surface area.
355
356
357

358 **Effect of production process on protein quality**

359 As expected there was a reduction in soluble N due to thermal denaturizing, but the
360 concentration of TCA soluble N did not seem to be affected by heat treatment. However the
361 concentration of TCA soluble N in the raw ingredients was calculated from the pooled
362 ingredients (Tonheim *et al*., 2007) so no statistics could be run. Increasing inclusion levels of
363 hydrolyzed protein increased the total content of water soluble N from the ingredients and a
364 relative higher percentage of this water soluble N remained water soluble after feed
365 formulation as compared to feeds with lover inclusion levels of hydrolyzed protein. The most
366 likely reason for this is that the hydrolyzed protein to a lesser extent participate in matrix
367 binding due to the reduced molecular size, and thus remains unbound and still water soluble
368 after particle formation. The decrease in soluble N was reduced from 45 ± 4 % in the diet
369 containing 0 % hydrolysate to 17 ± 2 % in the diet containing 45 % hydrolysate. The use of
370 protein as the binder can be desirable in order to reduce the concentration of binders with low
371 or non nutritional qualities or to reduce the concentration of carbohydrates for species with a
372 low tolerance such as halibut (Hjertnes, 1993; Hamre *et al.*, 2003). However, different
373 processes that cause protein matrix formation such as heat or cross binding (Jones, 1980),
374 might also negatively affect the digestibility. For diets exposed to heat during feed
375 production, it is necessary to include soluble N in form of FAA, peptides and thermally stable
376 proteins such as casein, to avoid reductions in the concentration of soluble N due to
377 denaturation,

378

379 **Effect of leaching on protein composition of submerged feed particles**

380 This study confirms the high loss of water-soluble N fractions when formulated diets
381 with a small particle size are submerged in water (Lopez Alvarado *et al.*, 1994; Kvåle *et al.*,
382 2006). However, the present study showed an increased rate of leaching compared to studies

383 on heat coagulated diets produced after the same protocol (Kvåle *et al*., 2006). Kvåle *et al*.
384 (2006) investigated the leaching with the use of radioactive marked nitrogenous compounds
385 that were included in a very small concentration compared to the concentration of soluble N
386 investigated in this study. The different analytical method and the low initial concentration of
387 the investigated compound may explain the difference in leaching rate.

388 The loss of soluble N from diets with high concentrations of hydrolyzed protein will
389 not only cause a shift in quality of the remaining protein, but also to a high extent affect
390 protein quantity. The soluble N lost from the diet containing 45 % hydrolyzed protein
391 accounted for 30 % of the total protein fraction. As earlier hypothesized by Kvåle *et al*.
392 (2006) among others, it might be the reduction in dietary crude protein levels that led to the
393 reduced survival of Atlantic halibut juvenile fed increased concentration of hydrolyzed
394 protein (Kvåle *et al*. 2007).

395 Dependent on the hydrolyzation method there may be a significant change in the AA
396 profile between the soluble and insoluble fraction. For diets containing a large concentration
397 of soluble N, this may lead to a change in the AA profile of the ingested diet, compared to the
398 diet as formulated, due to leaching. For the investigated diet containing 30 % hydrolyzed
399 protein there were no major changes in AA profile due to leaching except a 32 % loss of
400 Histidine and a 100 % loss of Taurine.

401 Histidine is an essential AA and a high loss may lead to malnutrition, but even with a
402 32 % loss the diet contained a similar concentration of histidine (% of tot. AA) as rotifers
403 (Srivastava *et al*., 2006). Unlike histidine, taurine, which actually is no amino acid at all, but
404 an imido acid and a metabolite in the sulfur amino acid metabolism, is not incorporated in
405 protein synthesis. Taurine can thus only be ingested in the free form and not as part of dietary
406 protein. Taurine will therefore be lost rapidly from most formulated diet as reported for a
407 protein encapsulated diet that also had a 100 % loss after exposure to water (Nordgreen *et al*.,

408 submitted). Taurine is described as a non essential nutrient, however, there are several
409 indications that taurine is essential during early development of fish. Enrichment of live feed
410 with taurine leads to an increased growth of Japanese flounder (*Paralichtys olivaceus*) (Chen
411 *et al.*, 2004; Chen *et al.*, 2005; Kim *et al.*, 2003; Kim *et al.*, 200;, Matsunari *et al.*, 2003; Chen
412 *et al.*, 2002; Takeuchi *et al.*, 2001) and Sea bream (*Pagrus major*) (Chen *et al.*, 2004). In
413 addition to a complete loss by exposure to leaching, the initial concentration of taurine (4.7
414 mg g^{-1} dw) was one fourth of that in copepods (18 mg g^{-1} dw, Van der Meeren 2003) and 2.8
415 times lower than in *Artemia* (13 mg g^{-1} dw, Van der Meeren *et al.*, 2003). However, the
416 concentration was 7.5 - 22 times higher than in rotifers (0.21 – 0.62 mg g^{-1} dw , Van der
417 Meeren, 2003; Srivastava *et al.*, 2006). Kim et al. (2005) found that juvenile Japanese
418 flounder required at least 15 mg g^{-1} taurine in the diet and confirms this study that formulated
419 diets based on meals needs extra addition of taurine to reach this level (Kim et al., 2005;
420 Nordgreen Unpublished). However, the taurine requirement for Japanese flounder established
421 by Kim *et al*. (2005) might be overestimated due to leaching.
422

423 **Effect of heat treatment on digestibility**

424 Heat processing during feed production can decrease both the in vivo and in vitro
425 protein digestibility of marine protein sources significantly (Yanes *et al*., 1970; Opstvedt *et*
426 *al*., 1980; Lan *et al*., 1993; Garcia Ortega, *et al*., 2000). The in vitro digestibility of the raw
427 ingredient mixture of the feeds investigated in this study was unfortunately not measured. On
428 the other hand, the individual digestibility of the protein sources *in vitro* was reported by
429 Tonheim *et al*. (2007), using the identical batch of enzymes and identical protocol as was
430 used in this study. From this it was indicated that there is a decrease in digestibility from
431 approximately 80 % for the pooled ingredients (Tonheim *et al*., 2007) to 65 % for the finished
432 diets. This may indicate a reduction in protein digestibility due to the production process.

433

434 **Effect of leaching on digestibility**

435 As shown in this study, pepsin hydrolyzes of fish fillet led to a doubling in both TCA and
436 water soluble N, however 35 % of the hydrolyzed product was still insoluble protein. The
437 complete hydrolyzed product with its remaining non soluble N was included into the
438 formulated diet as done in previous studies (Kvåle *et al*.,2002, 2007). It is likely that the N
439 fraction of the raw material that will be solubilized by enzymatic hydrolyzes is the same
440 fraction that first would be digested by the marine fish larvae. A high inclusion of hydrolyzed
441 protein with a following leaching during feeding may therefore lead to an ingested diet with
442 not only a reduced concentration of total protein, but also having lower quality on the
443 remaining protein as compared to a formulated diet using the same protein source non-
444 hydrolyzed. This could explain the lower digestibility in the leached diets compared to the
445 diets that not have been exposed to leaching. When high inclusion of hydrolyzed protein is
446 aimed, separation of the non soluble fraction before inclusion into a formulated diet should be
447 considered. Several studies have indicated improved digestibility (Tonheim *et al*., 2007) and
448 growth with the use of water-soluble proteins compared to insoluble proteins (Carvalho *et al*.,
449 2004). The high concentration of FAA in live feed (Frolov et al., 1991; Hamre et al., 2002;
450 Carvalho et al., 2003; Helland et al., 2003; Meeren 2003; Srivastava et al.,2006) has been
451 suggested to be one of the main reasons for increased growth of larvae fed these feeds,
452 compared to larvae fed formulated diets (reviewed by Rønnestad et al., 1999, 2003).
453 However, later studies have also suggested that larger soluble nitrogenous compounds in the
454 live feed organisms could be as beneficial as FAA for increasing growth. More than 85 % of
455 the soluble N in *Artemia* and Rotifers were larger than 500 D (Carvalho *et al*., 2003). There
456 was a lower loss of water soluble N than TCA soluble N in the present study. This is in
457 accordance with Kvåle *et al*. (2006) who showed that increased size of the soluble N fraction

458 led to a significant reduction in leaching rate. It should therefore be considered to use soluble
459 N of a larger size rather than FAA and small peptides, in order to reduce the leaching rate
460 from formulated diets.

461 For the diets that had not been exposed to leaching, there was a decreasing difference
462 in digestibility between the diets with increase in time of digestibility. The difference in
463 digestibility at ½ and 1 h is most likely a logic consequence of the initial difference in TCA
464 soluble N between the diets. At 12 hours there were no significant differences between the
465 diets, indicating that increased concentration of hydrolyzed protein did no lead to an increased
466 digestibility. The diets tested in the present study performed similar to the commercial
467 available Minipro™ after both 1 and 12 h but better with regards to digestibility in vitro than
468 a protein encapsulated diet that was digested to only 50 % after 12 h (Tonheim *et al*., 2007).

469 The diets exposed to leaching prior to digestibility had a significant lower digestibility
470 at all time intervals compared to the diets that were not leached. At ½ h and 1 h digestibility
471 the lower digestibility is most likely most of all a consequence of the loss of TCA soluble N.
472 After 12 h, on the other hand, the difference in digestibility might be due to difference in
473 protein quality. A similar digestion of the diets exposed to leaching at all time intervals
474 confirms the suggestions by Kvåle *et al*. (2006) that leaching can change the quality of the
475 ingested diets. Using *in vitro* digestibility methods, it is unfortunately not possible to evaluate,
476 the biological effects of reduction in crude protein due to leaching. Which was suggested by
477 Kvåle *et al*. (2006) to be a possible reason to the observed reduction in survival of halibut
478 juvenile fed diets with increased inclusion levels of hydrolyzed protein.

479 The protocol for *in vitro* digestion used in the present study was based on the methods
480 described by Hsu *et al*. (1977) and Saterlee *et al*. (1979) and has been thoroughly discussed
481 by Chong *et al*. (2002) and Tonheim *et al*., (2007). Chong *et al*., (2002) found a good
482 correlation in relative digestibility between *in vitro* and *in vivo* measurements using juvenile

483 discus fish (*Symphysodon aequifasciata*) although there were some differences in absolute
484 digestibility. Thus, if aiming to investigate the true digestion of a feed or feed ingredients, *in*
485 *vivo* measurements in the target species should be performed. As discussed by several authors
486 it is difficult to estimate digestibility of different protein sources in small fish larvae. Tube
487 feeding of radio-labeled protein to marine fish larvae (Rønnestad *et al*., 2001;Tonheim *et al*.,
488 2004. 2005) has proved to be a good method to evaluate the protein digestibility in such small
489 animals, however the method can only be used tube feeding soluble model protein (Tonheim
490 et al., 2004) and partly insoluble dietary proteins can not be investigated by this method.
491

492 **Conclusion**

493 This study clearly indicates that feed production and leaching can have significant
494 effects on protein quality and that optimal level of inclusion of hydrolyzed protein in
495 formulated diets for marine fish larvae may be difficult to determine. The optimum levels
496 may have to be set for every individual diet and protein source. The severe changes in protein
497 quality during feed production and leaching indicates the importance of considering both the
498 protein sources used and the type of diet produced when effects of hydrolyzed proteins are
499 investigated. Production process, leaching rate, and type and quality of the hydrolyzed
500 product used, have to be considered.
501

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732 **Figure legends**

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734 Figure 1: Sample of a diet containing 15 % hydrolyzed protein. The picture is taken with
735 differential interference contrast (DIC) (Olympus BX 51).

736

737 Figure 2: Grey column is concentration of crude protein (N x 6.25) in for 4 diets with
738 increasing inclusion of pepsin hydrolyzed protein ranging from 0 % to 45 % of the total
739 protein (n=3). The white spotted columns are loss of crude protein (N x 6.25) in the same
740 diets after 6 minutes leaching (n=5). The black spotted columns are loss (%) of total weight
741 after 6 minutes leaching (n=4). The weight loss from the diet containing 0 % and 45 %
742 hydrolyzed protein has n=1 and statistic analysis is therefore not performed. Otherwise error
743 bars represent standard deviations. Significant differences between the diets are shown by
744 different letters (ANOVA, followed by Tukey HSD, $p < 0.05$).

745

746 Figure 3: Concentration of water-soluble N in raw ingredients (calculated), produced
747 diet (n=4) and diets leached for 6 minutes (n=5) for 4 diets with inclusion of pepsin
748 hydrolyzed protein ranging from 0 % to 45 % of the total protein. Error bars represent
749 standard deviations. Significant differences between the diets are shown by different letters
750 (ANOVA, followed by Tukey HSD, $p < 0.05$).

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757 Figure 4: Concentration of TCA soluble N in diets with increasing inclusion of pepsin
758 hydrolyzed protein ranging from 0 % to 45 % of the total protein. The concentration of TCA
759 soluble N is investigated in the raw ingredients (calculated) the produced diets (n=4)
760 and in the diets after 6 minutes leaching (n=4). Error bars represent standard deviation.
761 Significant differences between the diets are shown by different letters (ANOVA, followed by
762 Tukey HSD, $p < 0.05$).
763

764 Figure 5: *In vitro* digestibility of protein in four diets with increasing concentration of
765 hydrolyzed protein ranging from 0 % to 45 % of the total protein. In vitro digestibility was
766 investigated for both not leached and leached (6 minutes) diets. The digestibility was
767 investigated at 4 time intervals from 0 h to 12 h (n=4). Error bars represent standard deviation.
768 Significant differences between the diets at each time interval are shown by different letters
769 (ANOVA, followed by Tukey HSD, $p < 0.05$).
770

771 Figure 6: AA profile of the heat coagulated diet containing 30 % hydrolyzed protein before
772 and after exposure to leaching for 6 minutes and the AA profile of the in vitro digested TCA
773 precipitated supernatant of the not leached and leached diet. Error bars represent standard
774 deviations (n=3).
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784 Table 1: Composition of the formulated diets investigated in this study (g kg^{-1} dry weight).

Diets	0	15	30	45
Ingredients (d.w)	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
Suprex wheat[a]	35	35	35	35
Squid mantle[b]	81	81	81	81
Cod fillet[c]	725	604	483	362
Pepsin hydrolysert cod fillet[d]	0	121	242	362
Fish oil[e]	90	90	90	90
Lecithin[f]	30	30	30	30
Vitamin mixture[g]	10	10	10	10
Minerals[h]	30	30	30	30

785 [a]Suprex wheat[a]

786 [b] Fresh frozen squid mantle

787 [c]Fresh cod.

788 [d]See description in materials and methods

789 [e]Epax A/S, Lysaker, Norway

790 [f]Soy lecithin, Norsk Medisinaldepot, Bergen, Norway.

791 [g]As recommended by NRC (1993) except for alfa-tocopheryl acetate supplemented at

792 200 mg kg^{-1} and vitamin C at 400 mg $^{kg-1}$.

793 [h]As recommended by NRC (1993).

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796

797 Table 2: Analysis of the different N fractions of minced cod fillet before and after pepsin

798 hydrolyzes (mean ± SD, n=3).

	Water-soluble N % of tot N	TCA soluble N % of tot N
Cod fillet	34 (n=1)	18 ± 2.4
Pepsin hydrolyzed cod fillet	65 ± 3	40 ± 4.2

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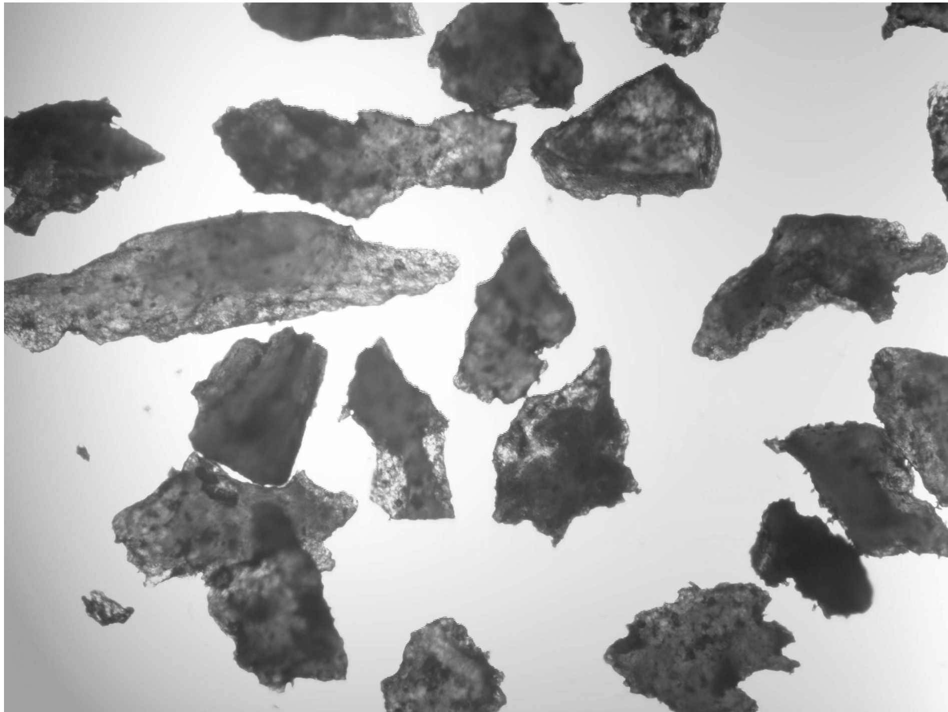

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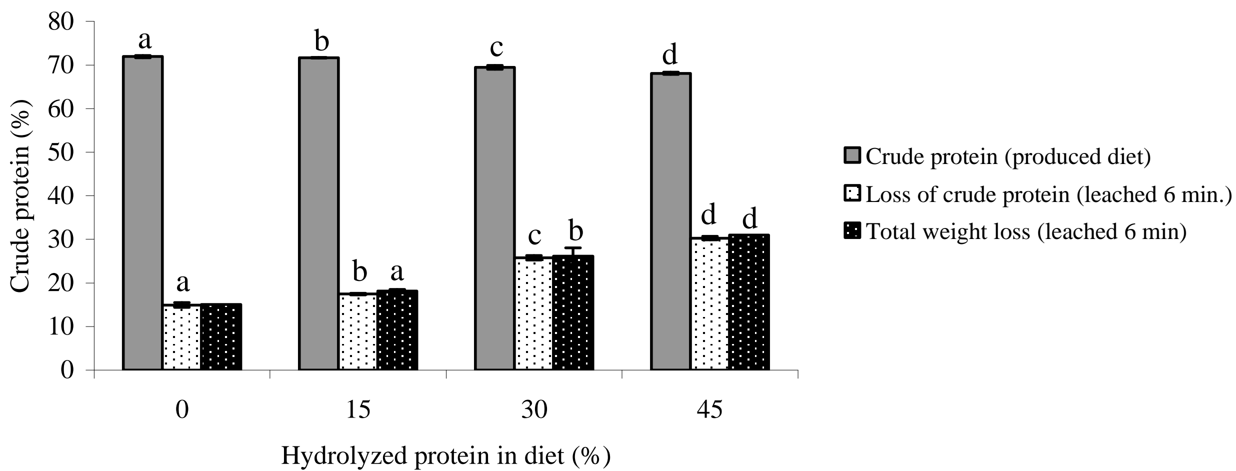

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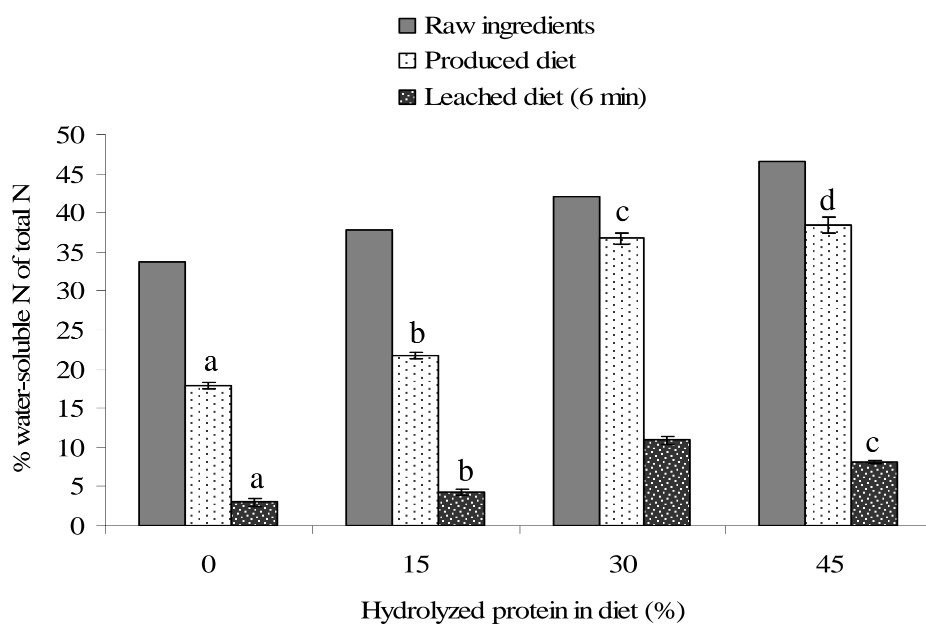

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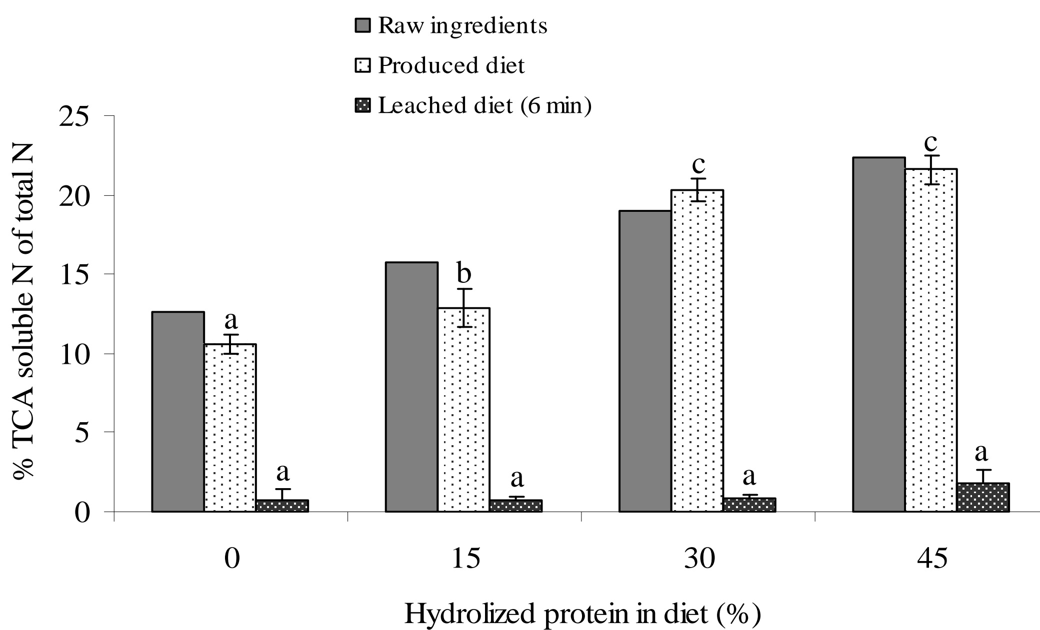

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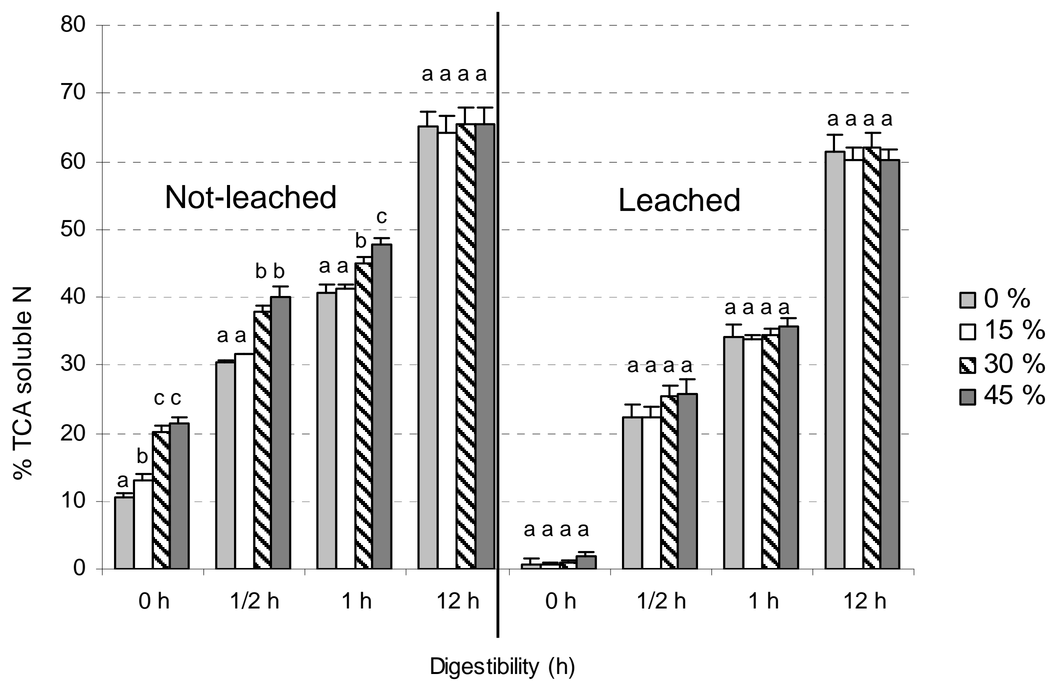

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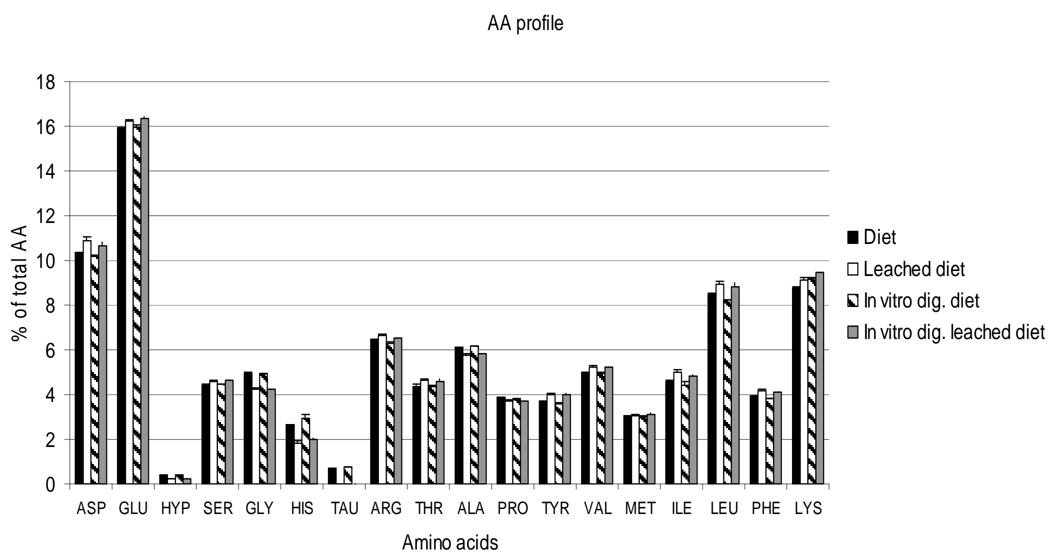

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906 Figure 6:

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Paper IV

1

2 # Development of lipid microbeads for delivery of lipid and water-

3 # soluble materials to *Artemia*.

4

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22 *Keywords:* lipid beads, phospholipid, amino acid, nutrient, glycine, riboflavin,

23 oxytetracycline, *Artemia*

24

25 # Abstract

26

27 Lipid spray beads (LSB) containing high concentrations of phospholipids were produced in
28 order to improve their dispersion in both fresh and saltwater. The beads were developed to
29 deliver both fat-soluble and water-soluble micronutrients to *Artemia* and other suspension-
30 feeders. LSB were prepared by spraying molted lipid into a chamber that was cooled with
31 liquid nitrogen in order to solidify the lipid beads. Addition of soy lecithin to LSB did not
32 affect retention of glycine when the beads were suspended in distilled water. There was an
33 initial loss of 80 % incorporated glycine after LSB were suspended in water for 20 min.
34 *Artemia* readily ingested riboflavin-containing LSB and their full guts were evident within 30
35 min of feeding. The riboflavin content of *Artemia* could be increased from 55 ± 0.6 mg kg^{-1}
36 (dw) to 329 ± 62 mg kg^{-1} (dw) after 1 h enrichment. LSB prepared with phospholipids are
37 promising vehicles for enrichment of suspension-feeding organisms used as feed for larval
38 marine fish and crustaceans as well as other suspension feeders.

39

40 # Introduction

41

42 Compared with natural zooplankton prey, such as copepods, *Artemia* are deficient in several
43 water-soluble micronutrients that may be necessary for high growth and survival of marine
44 fish larvae (Vander Meeren, 2003; Moren et al., 2006). Enrichment of *Artemia* with water-
45 soluble micronutrients is commonly achieved by either direct addition of micronutrients to the
46 culture water or by adding micronutrients to lipid emulsions fed to *Artemia* cultures. The
47 current enrichment methods are not optimal due to low uptake efficiencies and a failure to
48 increase micronutrient concentrations to desired levels (Hamre, unpublished results).

49 Enrichment of *Artemia* with liposomes has been successful (Tonheim et al., 2000; Monroig et
50 al., 2003, 2006), but production of large quantities of liposomes is both expensive and
51 technically difficult.

52 Incorporation of nutrients in a lipid matrix can be accomplished by a method referred
53 to as spray chilling. Molten lipid, containing the active ingredients, is sprayed into a cooled
54 environment where the lipid beads harden. The lipid beads consist of active ingredients
55 randomly distributed in the lipid matrix with some possibly protruding through the surface of
56 the beads. This is in contrast to truly encapsulated material where ingredients are surrounded
57 with an intact wall (see review by Gouin, 2004)

58 Spray-chilling is a commonly used technology in pharmacy and food technology
59 where it has been used for sustained release (Akiyama et al.,1993), taste masking (Yajima et
60 al., 1999), improving stability (Schwendeman et al., 1998) and encapsulation of vitamins and
61 minerals (Gibbs et al., 1999). The development and use of LSB to deliver water-soluble
62 nutrients to marine fish larvae has been successfully accomplished using triacylglyceride
63 (TAG) LSB that were themselves incorporated in a zein-bound feed particle (Onal and
64 Langdon, 2005ab). Encapsulation of water-soluble nutrients in LSB significantly decreased
65 leaching (Onal and Langdon, 2005a).

66 The method to produce LSB by spray-chilling requires that the lipid is solid at low
67 (<20 ^{o}C) temperatures; therefore, it is not possible to deliver significant proportions of
68 unsaturated fatty acids as TAG because they typically have low melting points. In contrast,
69 phospholipids, such as soy lecithin, often have high proportions of unsaturated fatty acids
70 (58% linoleic acid and 8% linolenic acid; Wettstein et al., 2000), but melting temperatures are
71 elevated compared to those of free fatty acids or triacylglycerols due to the presence of the
72 phosphorus group. This characteristic of phospholipids makes them good candidates for
73 making LSB that are solid at room temperature, but may have a high content of unsaturated

74 fatty acids, such as EPA and DHA. In addition, several studies have shown that marine fish
75 larvae benefit from inclusion of phospholipids as a dietary lipid source (Kanazawa et al.,
76 1983ab; Cahu et al., 2003).

77 The goal of this study was to evaluate the effects of additions of soy lecithin, as a
78 phospholipid source, on the characteristics of LSB designed to deliver micronutrients to
79 enrich *Artemia*, a common live feed for rearing marine fish larvae.

80

81 Materials and methods

82

83 ***Production of LSB with different concentrations of soy lecithin***

84 Menhaden stearine was used as the major lipid source in experiments to determine the effects
85 of additions of soy lecithin on the properties of LSB. Menhaden stearine is a by-product of
86 menhaden oil refining and has the consistency of peanut butter and a melting point of 43 ºC.
87 LSB were prepared using a modification of the method described by Önal and Langdon
88 (2000, 2004a). Briefly, this method involved mixing core materials with molten lipid and then
89 spraying the mixture into a chilled chamber to harden the lipid to form beads. Menhaden
90 stearine was melted in a water bath at 60 ºC and soy lecithin was dissolved in the melted
91 menhaden stearine with sonication. Glycine has a low molecular weight (75.05 D) and is
92 highly water-soluble and was, therefore, chosen as the core material to investigate if additions
93 of soy lecithin affected LSB properties (Önal and Langdon, 2004a). LSB were prepared
94 containing glycine as either dry micro-ground crystalline particles or as a saturated solution
95 containing a slurry of suspended particles.

96

97 ***Lipid spray beads with a core of dry particulate glycine***

98 Beads were prepared containing 16 % w/w dry glycine particles. Six diets containing different
99 concentrations of soy lecithin were produced (Table 1). Each batch of LSB was prepared in
100 triplicate with a mixture of 2.88 g glycine and 15.12 g lipid. The lipid/glycine mixture was
101 sonicated to obtain complete dispersion of particles and the mixture was then sprayed
102 immediately to avoid particle separation.

103

104 ***Lipid spray beads prepared with an aqueous slurry of glycine***

105 The volume ratio of aqueous glycine slurry to lipid was chosen based on the ratio that was
106 shown to result in the best retention and delivery efficiencies for glycine incorporated in
107 menhaden stearine LSB (Clack, 2006). Each batch of LSB, consisting of 4 g glycine, 7.2 g of
108 distilled water and 13.8 g of lipid (Table 2), was prepared in triplicate with different
109 proportions of soy lecithin. The glycine slurry was prepared and sonicated before it was
110 mixed with the molten lipid and again sonicated. The mixture was sprayed immediately to
111 avoid separation of the emulsion. The concentration of glycine (ww) is the same in the LSB
112 containing particulate glycine and aqueous slurry of glycine. However, it should be
113 emphasized that there is a difference in glycine: lipid ratio between the two respective types
114 of LSB (Table 1 and 2) due to the water content of the LSB containing an aqueous slurry.

115

116 ***Replacement of menhaden stearine with paraffin wax***

117 A series of preliminary experiments showed that LSB containing paraffin wax were less
118 sticky at room temperature than LSB produced with menhaden stearine, and were also more
119 stable in salt water. Therefore, LSB prepared with paraffin wax were chosen for further
120 investigation of their use for *Artemia* enrichment. Cod liver oil was added to the paraffin to
121 improve LSB nutritional quality and to potentially improve their digestion by *Artemia*.

122 To investigate the properties of LSB prepared with paraffin wax, beads were prepared with
123 cores of ground oxytetracycline (OTC >95 % HPLC 05875, Sigma). OTC was used instead of
124 glycine as an alternative example of a water-soluble material. OTC has earlier been
125 successfully incorporated in LSB (Buchal and Langdon, 1998; Langdon and Buchal, 1998).
126 Based on preliminary experiments, LSB consisting of 545 g kg^{-1} soy lecithin, 227 g kg^{-1}
127 paraffin wax, 164 g kg^{-1} cod liver oil and 64 g kg^{-1} OTC were chosen for further investigation
128 (Table 3). The lipid/OTC mixture was sonicated to obtain complete dispersion of particles and
129 the mixture was then sprayed immediately to avoid particle separation
130
131 To investigate the use of LSB to deliver water-soluble nutrients to *Artemia*, paraffin wax LSB
132 were also prepared containing finely ground (McCrone micronizing mill; McCrone,
133 Westmont, IL) riboflavin ($\geq$98%, R-4500, Sigma) as the core material (Table 3). Riboflavin
134 is a fluorescent water-soluble vitamin and has proved to be a good compound to visually
135 investigate digestion and release of core materials from LSB ingested by fish and bivalve
136 larvae (Langdon et al., 2000; Önal and Langdon, 2004b; Önal and Langdon, 2005b).
137

138 ***Encapsulation efficiency (EE)***

139 EE represents the percentage core material to lipid (% w/w) in LSB after production (Buchal
140 and Langdon, 1998). The concentration of core material (glycine; % w/w) was analyzed
141 according to methods described by Önal and Langdon (2004b). First, duplicate samples of 0,1
142 g of each LSB production batch were weighed into 20 ml glass tubes, 10 ml of chloroform
143 were added and the tubes were shaken until the LSB were completely dissolved. Ten ml of
144 distilled water containing 1.5 mg ml^{-1} SDS were added and the tubes were placed on a rotary
145 mixer for 30 min. The tubes were then centrifuged and the water fraction was removed and

146 collected. This extraction was repeated three times. The collected supernatants from each
147 extraction were pooled and analyzed for glycine by the ninhydrin method, as described in the
148 analytical methods section (below).

149

150 ***Quantifying core material on the surface of LSB***

151 When producing LSB, some of the core material may be situated partly or wholly on the
152 surface of the beads. An attempt was made to measure this surface-associated, non-
153 incorporated glycine by carrying out a rapid rinse of the beads and measuring glycine
154 concentrations in the filtrate. However, glycine in the filtrate also likely contained some
155 glycine leached from the beads; therefore, measurements of surface-located core material
156 were likely to be overestimates of true values. Two replicate 0.1 g samples of each LSB
157 production batch were weighed onto filters (25 mm, 0.45 µm, Metrical membrane filter; Pall
158 Life Science). LSB on the filters were rinsed under vacuum with 10 ml of water. The rinse
159 water was collected and glycine concentrations determined using the ninhydrin method (see
160 below).

161

162 ***Dispersion of LSB***

163 Dispersion in both seawater and distilled water were investigated for all produced LSB (Table
164 1, 2 and 3). LSB were taken directly from a -80 °C freezer and added to cold (1 ºC) seawater
165 or distilled water in 20 ml screw-capped test-tubes. The suspensions were hand-shaken and
166 allowed to settle for 30 seconds before being visually examined under a microscope. All the
167 tubes were kept in an ice bath during the investigation. The temperature of the suspensions
168 with dispersed LSB was increased to room temperature (22 ºC) to investigate the stability of
169 LSB suspensions.

170

171 ***Retention efficiency.***

172 Retention efficiency is the percentage of initial incorporated core material lost after
173 suspension of LSB in water (Önal and Langdon, 2004b). Leaching experiments were carried
174 out based on a modified method described by Önal and Langdon (2004b). Preliminary studies
175 indicated that clumping of LSB significantly reduced leakage rates. To reduce clumping,
176 leakage experiments were carried out in an aqueous solution of 0.1 % sodium dodecyl sulfate
177 (SDS; Sigma). To investigate the effect of SDS on leaching, LSB containing 0 %
178 phospholipid (not dispersible without SDS) and 40 % phospholipid (dispersible without SDS)
179 were suspended in distilled water with and without 0.1% SDS for 10 min and leakage rates
180 compared. To avoid initial clumping and facilitate dispersion, it was important to disperse the
181 LSB in chilled water.
182
183 To investigate leaching, 60 mg of LSB were added to pre-cooled glass tubes and 10 ml of
184 cold (1 ºC) 0.1 % SDS (No. L-4509 Sigma) solution added. It was necessary to use chilled
185 SDS solution to maintain hardened, non-sticky beads to ensure their dispersal. The tubes were
186 stirred vigorously (Vortex-Genie, Fisher Scientific) for a few seconds to disperse the LSB and
187 then placed on a rotary mixer (20 RPM; tissue culture rotator: 099A RD4512; Glas-Col, Terre
188 Haute, IN) for 20 min at room temperature (22 ºC). There was a temperature increase during
189 the 20 min leaching experiment from 1 to 22 ºC, but this temperature increase was similar for
190 all samples. The contents of the tubes were then filtered onto 0.45 µm filters (Metrical
191 membrane filter, Pall Life Sciences). The filtered supernatant was collected and glycine
192 concentrations determined using the ninhydrin method (see below).
193
194 LSB prepared with paraffin wax and soy lecithin with a payload of OTC (Table 3) were
195 leached in distilled water (without SDS) at room temperature (22 °C) for various periods of

196 time. Thirty mg of LSB were weighed into glass tubes and 10 ml of distilled water were
197 added and the tubes were then placed on a rotary mixer (20 RPM) for 2, 10, 30 and 60 min.
198 The contents of the tubes were then filtered onto 0.45 µm filters (Metrical membrane filter,
199 Pall Life Sciences). The filtered supernatant was collected and OTC concentrations
200 determined using a spectrophotometer (Beckman DU 530; absorbance 358 nm).
201

202 ***Enrichment of Artemia***

203 Decapsulated *Artemia* cysts (*Artemia salina*; Salt Creek Inc) were hydrated in fresh water for
204 1 h before being hatched at room temperature (22 Cº) under continuous aeration and light in
205 10-micron filtered seawater. Three days after hatching, unfed *Artemia* were washed onto a
206 250 µm sieve and rinsed with clean seawater. Nine 2-l conical, aerated bottles were filled with
207 1.5 l seawater and 363 ± 13 *Artemia* l^{-1} were added. The *Artemia* were acclimated for
208 approximately 1 h before enrichment. In one treatment (n=3), *Artemia* were fed
209 133 mg L^{-1} LSB that were partially prepared with paraffin wax and contained 6.0 % w/w
210 riboflavin (Table 3). In the second treatment (n=3), *Artemia* were fed 133 mg L^{-1} empty LSB
211 (Table 3) and pre-dissolved riboflavin at the same concentration as that delivered with
212 additions of riboflavin-containing LSB (8 mg L^{-1}). The third treatment (n=3) consisted of
213 unfed *Artemia*. During a 1 h period of enrichment, samples of riboflavin LSB-fed *Artemia*
214 were examined using an epifluorescent microscope (Leica DM1000, Leica inc) fitted with a
215 UV light source and compared with samples from the two control treatments.
216

217 After enrichment, the *Artemia* were washed thoroughly with filtered seawater on a 250 µm
218 mesh sieve. A small sub-sample of washed *Artemia* fed on riboflavin-containing LSB was
219 examined under the microscope to ensure that no LSB were attached to their surfaces.

220 *Artemia* from all treatments were then freeze-dried for riboflavin analysis. No attempt was
221 made to purge the guts of the LSB-fed *Artemia*.
222

223 ***Analytical methods***

224 Glycine concentrations were analyzed based on the method described by Doi et al. (1981).
225 Each one ml sample was acidified by addition of 10 µl of 1 % acetic acid and one ml
226 ninhydrin reagent (2 % solution, Sigma) was added. The samples were placed on a heating
227 block at 100 °C for 10 min, and then transferred to an ice bath before 5 ml ethanol (95 %)
228 were added to stabilize the color. Absorbance was determined spectrophotometrically at 570
229 nm (Beckman DU 530) and converted to glycine concentration using regression equations
230 derived from standard curves. The samples were analyzed in UV-grade
231 polymethylmethacrylate cuvettes (VWR).

232 OTC concentration was determined spectrophotometrically (DU 530; Beckman
233 Coulter, Fullerton, CA) by measuring absorbance at 358 nm. The samples were analyzed in
234 quartz cuvettes (VWR). The absorbance was converted to OTC concentration using
235 regression equations derived from standard curves.

236 Riboflavin concentrations were determined by high-performance liquid
237 chromatography (HPLC) with fluorometric detection according to a method described by
238 Brønstad et al., (2002). Freeze-dried *Artemia* (0.25-0.30 g) were added to 45 ml 0,1 M HCl.
239 The samples were autoclaved at 121 ºC for 30 min, cooled to room temperature and the pH
240 adjusted to 4,0 with 2,5M sodium acetate. Sodium acetate trihydrate was added at a
241 concentration of 100 mg per g sample and the samples were incubated at 37 ºC overnight. The
242 samples were then diluted to 50 ml and the solution was filtered before HPLC analysis (HP
243 1100, Agilent; exitation: 468 nm and emission: 520 nm). The concentration of riboflavin was
244 calibrated with the use of external standards.

245 ***Statistical analysis***

246 Biological and analytical data are expressed as means ± 1 standard deviation. Regression
247 analysis together with one-way analysis of variance (ANOVA; Sokal and Rohlf, 1969) and
248 Tukey's HSD post-hoc multiple comparison test were used to investigate the effect of
249 concentration of soy lecithin on encapsulation efficiency, the proportion of non-incorporated
250 glycine and leaching rate. The effect of LSB enrichment on the concentration of riboflavin in
251 *Artemia* between the groups was analyzed by Student t-Test with a significance level (P
252 value) of 0.05. All statistical analyses were performed using Statistica 7.1 (Statsoft, Inc,
253 Tulsa, USA).
254

255 Results

256

257 ***LSB containing a core of dry particulate glycine***

258 There was an increase in the viscosity of the melted lipid-core mixture with increasing
259 additions of soy lecithin, but all bead types could be sprayed. Small amounts of glycine were
260 lost during the production process (Figure 1) with encapsulation efficiencies ranging from 95
261 ± 3 to 102 ± 2% for the different bead types. There was a significant difference in
262 incorporated concentrations of glycine (ANOVA, P=0.029) between the LSB containing 1 %
263 and 20 % soy lecithin (Figure 1).

264 There was a positive correlation (r^2=0.89, P<0.00001) between the amount of soy
265 lecithin added to the beads and loss of glycine when LSB were rinsed with distilled water.
266 The overall rinse loss of glycine ranged from only 0.1 ± 0.03 % for LSB containing 0 % soy
267 lecithin to 3.2 ± 0.6 % for LSB containing 40 % soy lecithin (Figure 2).

268 Loss of glycine after 20 min of leaching (Figure 3) ranged from 65 ± 7 to 85 ± 5 %.
269 There was no correlation ($P>0.05$) between concentration of soy lecithin and glycine loss, but
270 there was a significant difference ($P=0.041$) in loss of glycine between LSB containing 1 %
271 and 10 % lecithin (Figure 3).
272
273 ***Lipid spray beads prepared with an aqueous slurry of glycine***
274 It was impossible to prepare LSB with an aqueous core with additions of more than 5 %
275 lecithin because with higher lecithin concentrations LSB did not harden in the chilled
276 chamber and they clumped on the chamber walls. There was no significant correlation
277 ($P>0.05$) between soy lecithin concentration and encapsulation efficiency of glycine and no
278 significant differences in glycine encapsulation efficiencies among LSB types (ANOVA,
279 $P>0.05$) (Table 4). The combined encapsulation efficiency of glycine for all slurry LSB types
280 was 105 ± 5 %. There was no significant correlation ($P>0.05$) between soy lecithin
281 concentration and encapsulation efficiencies for water and no significant (ANOVA, $P>0.05$)
282 differences among the LSB types prepared with slurry of glycine (Table 4). The combined
283 encapsulation efficiency of water was 101 ± 3 %.
284 The loss of glycine due to rinsing ranged from 23 ± 4% to 27 ± 2% of the incorporated
285 glycine (Table 4), but there was no significant ($P>0.05$) correlation between soy lecithin
286 concentration and loss. The leaching experiment showed no significant ($P>0.05$) correlation
287 between soy lecithin concentration and loss of glycine due to leaching and no significant
288 (ANOVA, $P> 0.05$) differences in leaching among the bead types (Table 4). The overall loss
289 in 20 min was 98 ± 5 % of incorporated glycine for all the slurry bead types combined.
290
291
292

293 ***Dispersion of LSB.***

294 Addition of 0.1 % SDS to suspensions of LSB containing 0% lecithin and crystal glycine
295 (Table 1) increased the leakage of glycine from 20 % to 64 % after 10 min (Figure 4). The
296 leakage of glycine from LSB containing 40 % soy lecithin and particulate glycine (Table 1)
297 was not significantly (ANOVA, $P \geq 0.05$) affected by addition of SDS to the incubation water
298 (Figure 4). LSB containing 0 % lecithin (Table 2) could not be fully dispersed in either
299 distilled water or 0.1 % SDS solution.

300 LSB containing an aqueous slurry of glycine and 0 %, 1% or 5 % soy lecithin, did not
301 disperse in either salt or freshwater, but clumped immediately. In contrast, LSB containing
302 dry particulate glycine and 40 % soy lecithin dispersed in both fresh and salt water while LSB
303 prepared with less than 40 % lecithin did not disperse in either fresh or sea water (Figure 5).
304 LSB containing paraffin wax and 54.5 % lecithin (Table 3) dispersed well in both fresh and
305 saltwater.

306

307 ***Replacement of menhaden stearine with paraffin wax***

308 Small amounts of OTC were lost during production of LSB containing paraffin wax and the
309 encapsulation efficiency was 95 ± 2 %. Large amounts of OTC were lost within the first min
310 of suspension of the LSB in water (Figure 6), but after 10 min a 76 ± 5 % loss stabilized and
311 the remaining OTC (about 20 %) was retained for 60 min (Figure 6). Riboflavin-containing
312 LSB (Table 3) still had particulate riboflavin visible in the lipid matrix after dispersion in
313 saltwater for more then 1 h (Figure 7). LSB containing paraffin wax was more stable than
314 those with mennhaden stearine, since no signs of disintegration of the beads were detected
315 when dispersed in either fresh or saltwater for 1 h. LSB prepared with 40 % lecithin and
316 menhaden stearine started to disintegrate within this time span.

317 ***Artemia enrichment***

318 *Artemia* ingested riboflavin-containing LSB and their guts became full after 30 min of feeding
319 (Figure 8). *Artemia* appeared to filter and mechanically breakdown the LSB because the
320 digestive tract was filled with material with no whole beads visible (Figure 8). All the beads
321 fed to *Artemia* were eaten within 1 h and no LSB were observed to be attached to the outside
322 surface of washed *Artemia*. There was more than a six-fold increase in riboflavin
323 concentration from 47.9 ± 2.1 to 329 ± 62 mg kg^{-1} (dw) in *Artemia* after 1 h enrichment with
324 riboflavin-containing LSB (Figure 9). There were no differences in riboflavin concentration
325 between the unfed control and the control fed a combination of empty LSB and riboflavin
326 dissolved in the water (Figure 9).
327

328 Discussion

329 Onal and Langdon (2005b) showed that LSB made of triacylglycerol were hydrophobic and
330 that the LSB clumped and did not disperse in aqueous solution. By inclusion of high
331 concentrations of phospholipids in the LSB, we were able to produce LSB that dispersed
332 freely and were vehicles for delivering both lipid and water-soluble compounds to
333 suspension-feeders, such as *Artemia.* Addition of phospholipids can also increase the
334 concentration of unsaturated fatty acids in LSB without causing a decrease in melting point.
335 There were no methodical difficulties producing LSB containing high concentrations
336 of lecithin and particulate glycine or OTC. We did not succeed in melting pure lecithin and it
337 was, therefore, necessary to dissolve the lecithin in either menhaden stearine or a mixture of
338 wax and fish oil. Due to the high melting point of soy lecithin and other phospholipid sources,
339 it is unlikely that it will be possible to produce LSB consisting of 100 % phospholipid by
340 using a spray technique. There was also a problem with cold-hardening LSB before they

341 impacted the walls of the spray chamber for LSB made up of lecithin concentrations of > 5%
342 w/w and containing an aqueous core. This problem might be solved by increasing the height
343 of the spraying tower or by reducing the concentration of water in the beads.

344 All LSB types had high incorporation efficiencies in accordance with earlier studies
345 (Önal and Langdon 2004b, 2005b). Incorporation efficiencies may be increased further when
346 lipid beads contain an emulsifier (Vilivalam and Adeyeye, 1994, Önal and Langdon, 2004b).
347 Not all the core material associated with the beads is necessarily fully incorporated. The
348 results of this study showed that rinsing losses were only 1 % for LSB containing particulate
349 glycine but as high as 25 % for LSB with aqueous cores.

350 Önal and Langdon (2004b) reported that LSB prepared with 5 % of the surfactant
351 sorbitan monoaplmitate or sorbitan sesquioleate showed improved dispersion but higher
352 leaching rates than LSB prepared with triacylglycerol and no surfactant. To compare leaching
353 among LSB with different dispersion properties, it was necessary to use 0.1 % SDS to make
354 the LSB types more equally dispersible. Dispersion of LSB containing 100 % menhaden
355 stearine with 0.1 % SDS led to a three-fold increase in loss of glycine while there was no
356 significant effect of SDS on leakage from more dispersible (less hydrophobic) LSB
357 containing 40 % lecithin. Leaching rates in this study were much higher for all investigated
358 LSB than those reported in earlier studies (Önal and Langdon, 2004ab; Buchal and Langdon,
359 1998; Clack, 2006) and this difference could have been due to the use of a dispersants in the
360 leaching experiments.

361 Inclusion of soy lecithin did not affect leaching rates significantly for any of the
362 investigated LSB types when suspended in 0.1% SDS, but there was an indication that LSB
363 containing particulate glycine and low concentrations of soy lecithin showed reduced losses
364 of glycine. This might have been due to clumping during the leaching trials since LSB
365 prepared with low concentrations of lecithin did not completely disperse in 0.1 % SDS.

366 Earlier studies have shown that LSB containing an aqueous core of both glycine and
367 OTC had lower leakage rates compared to LSB with particulate cores (Önal and Langdon,
368 2004a, Buchal and Langdon, 1998); however, in this study we found that LSB with an
369 aqueous glycine core had a nearly complete loss of glycine after 20 min suspension. It is not
370 clear if this higher loss was due to increased dispersion by the use of 0.1 % SDS or due to
371 other factors.

372 Leaching of OTC from LSB prepared with paraffin wax followed a classic burst
373 release profile, with a rapid loss of 80 % incorporated glycine during the first min followed by
374 a slower rate of leaching over a period of an h. Leakage rates of OTC from LBS prepared with
375 paraffin wax were similar to losses from LSB containing particulate glycine. Loss of OTC
376 was higher than previously reported by Buchal and Langdon (1998), but the leaching profile
377 was similar to that described by Onal and Langdon (2005a). Retention efficiencies for
378 riboflavin-containing LSB were not investigated, but riboflavin has a low aqueous solubility
379 and high concentrations of particulate riboflavin were observed in LSB after dispersion in
380 water for 1 h (Fig. 7).

381 Dispersion in water (without SDS) of LSB containing particulate glycine
382 occurred only with LSB containing 40 % soy lecithin. LSB containing 22.7 % wax, 16 % fish
383 oil and 55 % lecithin dispersed well in water and were more stable than LSB containing
384 menhaden stearine. This was probably due to the higher melting point of paraffin wax and
385 high phospholipid concentration.

386 Beads that can withstand mechanical stress and ambient temperatures during handling
387 and storage would be useful for large-scale production and storage of LSB. Increasing the
388 melting point of the lipid mix of LSB without decreasing the proportion of unsaturated fatty
389 acids by the addition of indigestible waxes needs further investigation. Use of waxes could
390 reduce digestion and increased gut evacuation rates, for example.

391 *Artemia* ingested LSB and the digestive tract was completely filled within 30 min.
392 This was in accordance with previous reports where *Artemia* were fed latex particles
393 (Gelabert, 2003). Microscopic observations indicated that there were no whole beads present
394 in the digestive tract. This may indicate that *Artemia* masticated captured beads before
395 ingestion. Effects of particle size and concentration on ingestion rates of *Artemia* are
396 described by several authors (Reeve, 1963ab; Gelabert Fernandez 2001,2003; Han et al.,
397 2005), but literature on *Artemia*'s capability to masticate particles was unfortunately not
398 found. Alternatively, the beads could have been ruptured by rapid enzymatic breakdown.
399 There was no significant increase in riboflavin concentration in *Artemia* exposed to riboflavin
400 dissolved in culture water compared with that of unfed controls, indicating that drinking the
401 culture medium was not sufficient to enrich *Artemia* with riboflavin. A concentration of
402 approximately 50 mg kg $^{-1}$ riboflavin in unenriched *Artemia* is similar to that reported by van
403 der Meeren (2003). A seven-fold increase in riboflavin concentration was obtained after
404 feeding *Artemia* on LSB containing particulate riboflavin.
405 Enrichment with LSB containing 6.4 % riboflavin increased the concentration of
406 riboflavin in *Artemia* with 281 mg kg $^{-1}$ (48 to 329 mg kg $^{-1}$). This was equivalent to *Artemia*
407 ingesting an LSB ration equivalent to 4.4 % of total body dry weight, although the actual
408 ingested ration might have been higher because losses of riboflavin from the beads and
409 *Artemia* were not considered. The lipid content of newly hatched *Artemia* (*A. franciscana*) is
410 145 mg g $^{-1}$ dry weight (Evjemo et al., 2001) while the lipid content of enriched *Artemia* is
411 reported to be 220-250 mg g^{-1} dw (Evjemo et al., 2001; Vander Meeren, 2003). Therefore,
412 lipid in LSB ingested to increase the riboflavin content with 281 mg kg^{-1} would represent 18
413 to 30 % of the total lipid content of the *Artemia* (dw).
414 LSB appears to be a good tool for delivering riboflavin to *Artemia*. Even with high
415 leaching losses, LSB retaining 20 % of its core material should be sufficient to increase the

416 concentration of most micronutrients in *Artemia*. Delivery of sufficient amounts of nutrients
417 that are needed in larger quantities, such as free amino acids, would be more difficult using
418 LSB types described in this study.

419 Liposomes have also proved to be a successful tool for enriching *Artemia* with lipid
420 and water-soluble nutrients (Hontoria et al., 1994; Ozkizilcik and Chu, 1994; McEvoy et al.,
421 1996; Tonheim et al., 2000; Monroig et al., 2003, 2006) and with good retention of
422 incorporated water-soluble nutrients (Hontoria et al., 1994; Monroig et al., 2003).
423 Improvement in the proportion of unsaturated fatty acids is possible by preparing liposomes
424 with krill phospholipid extracts (McEvoy et al., 1996; Monroig et al., 2003 and 2006);
425 however, an increased proportion of phospholipids led to reduced retention of
426 carboxyfluorescein (Monroig et al., 2003). In contrast to LSB, the core material of liposomes
427 has to be delivered in aqueous solution and the volumetric proportion of liposomes made up
428 of aqueous core solution is usually low (Touraki et al., 1995). The benefits of using LSB
429 rather than liposomes include flexibility in choice of the physical nature of the core material,
430 high core incorporation rates and choice of hydrophobic material (TAG, phospholipid,
431 paraffin wax etc.) for the bead matrix.

432 In summary, this study demonstrates that LSB containing high concentrations of
433 lecithin can be dispersed easily in water and that addition of lecithin does not affect retention
434 of glycine compared to LSB prepared with triacylglycerol alone. After a high initial loss, 20
435 % of initially incorporated particulate glycine and OTC was retained for up to an h. LSB
436 containing paraffin wax in the hydrophobic matrix were rapidly ingested and digested by
437 *Artemia* and the concentration of riboflavin increased seven-fold within one h of enrichment.

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541 **Legend to figures**

542 Figure 1: Concentration of incorporated particulate glycine in LSB prepared with different
543 concentrations of soy lecithin and menhaden stearine. Concentrations of glycine are given as
544 % dry weight. Data are means of three replicates. Error bars represent standard deviations.
545 LSB types sharing the same letter are not significantly different from each other (ANOVA,
546 followed by Tukey HSD, $p < 0.05$).
547
548 Figure 2: Percent losses of glycine due to rinsing LSB with distilled water, for LSB
549 containing particulate glycine and different concentrations of soy lecithin and menhaden
550 stearine. Losses are given as % lost (dw) based on the initial glycine concentration of each
551 LSB preparation. Data are means of three replicates. Error bars represent standard deviations.
552 LSB types sharing the same letter are not significantly different from each other (ANOVA,
553 followed by Tukey HSD, $p < 0.05$). (% loss = 0.35 + 0.946 x Lecithin %, R^2= 0.89, P<
554 0.00001))
555
556 Figure 3: Percent loss of glycine after 20 min leaching in distilled water for LSB containing
557 particulate glycine and different concentrations of soy lecithin and menhaden stearine. Losses
558 are given in terms of % initially incorporated glycine (dw). Data are means of three replicates.
559 Error bars represent standard deviations. LSB types sharing the same letter are not
560 significantly different from each other (ANOVA, followed by Tukey HSD, $p < 0.05$).
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566 Figure 4: Percent loss of glycine after 10 min leaching of LSB in either distilled water or
567 water containing 0.1 % SDS. LSB initially contained 16 % particulate glycine and either 0 %
568 or 40 % w/w soy lecithin. Data are means of three replicates. Error bars represent standard
569 deviations. LSB types sharing the same letter are not significantly different from each other
570 (ANOVA, followed by Tukey HSD, $p < 0.05$).

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572 Figure 5: *Left:* LSB containing 840 g kg^{-1} lipid (100 % menhaden stearine) and 160 g kg^{-1}
573 crystal glycine suspended in seawater. Magnification x 100 *Right:* LSB containing 840 g kg^{-1}
574 lipid (60 % menhaiden stearine and 40 % soy lecithin) and 160 g kg^{-1} particulate glycine
575 suspended in seawater. Magnification x 400. The pictures were taken using differential
576 interference contrast (DIC) (Olympus BX 51).

577

578 Figure 6: Percent loss of oxytetracycline (OTC) from LSB prepared with 545 g kg^{-1} soy
579 lecithin, 227 g kg^{-1} paraffin wax, 160 g kg^{-1} cod liver oil and 64 g kg^{-1} oxytetracycline.
580 Leaching was carried out in distilled water and losses were expressed in terms of % loss of
581 initially incorporated OTC. Data are means of three replicates. Error bars represent standard
582 deviations.

583

584 Figure 7: LSB prepared with paraffin wax, soy lecithin and cod liver oil (227 g kg^{-1}, 545 g kg^{-}
585 ^{1} and 164 g kg^{-1}) and 64 g kg^{-1} riboflavin (Table 3, LSB type 1). The picture was taken using
586 an epifluorescent microscope (Leica DM1000, Leica inc) fitted with a UV light source
587 (excitation of 450 - 490nm and emission of 515 - 528nm). LSB were dispersed in seawater for
588 one h and 55 min and riboflavin particles were still visable within the beads.

589

590 Figure 8: Left: *Artemia* enriched with LSB containing riboflavin after one h enrichment; a string of
591 faeces can be seen (Leica DM 1000)
592 Right: Digestive tract of *Artemia* fed on riboflavin-containing LSB, filled with fluorescent riboflavin,
593 shows that *Artemia* ingested LSB. Whole LSB are not visible, indicating that they were broken down.
594 The picture was taken using an epifluorescent microscope (Leica DM1000, Leica inc) fitted
595 with a UV light source (excitation of 450 - 490nm and emission of 515 - 528nm).
596
597 Figure 9: Concentration of riboflavin (µg g^{-1} dry weight) in *Artemia* either enriched for 1 h
598 with LSB containing riboflavin, fed on a combination of empty LSB and riboflavin dissolved
599 in the culture water or unfed *Artemia*. Data are means of three replicates except for unfed
600 *Artemia* that were tested in duplicate. Error bars represent standard deviations for the two
601 enriched *treatments* and mean ± range for the unfed *Artemia*. Treatment means sharing the
602 same letter are not significantly different from each other (Student t-Test, $p = 0.002$).
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614 **Tables**

615

616 Table 1: Composition of LSB containing particulate glycine. Each bead type was prepared in
617 triplicate.

Lecithin content % (w/w)	Menhaden stearine[a] g kg^{-1}	Soy lecithin[b] g kg^{-1}	Glycine[c] g kg^{-1}
0%	840	0	160
1%	831.6	8.4	160
5%	798	42	160
10%	756	84	160
20%	672	168	160
40%	504	336	160

618

619 [a] Crude menhaden stearine (Omega protein, USA).

620 [b]Soy lecithin, refined (M.W= 327.27; Cat no. 102147 Biomedicals).

621 [c]Glycine (minimum 99 %; Sigma).

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635 Table 2: Composition of LSB containing an aqueous slurry of glycine. Each bead type was

636 prepared in triplicate.

Lecithin content % (w/w)	Menhaden stearine g kg^{-1}	Soy lecithin g kg^{-1}	Glycine g kg^{-1}	Water g kg^{-1}
0%	552	0	160	288
1%	546	6	160	288
5%	522	30	160	288

637 [a] Crude menhaden stearine, Omega protein, USA.

638 [b]Soy lecithin, refined (M.W= 327.27; Cat no. 102147 Biomedicals).

639 [c]Glycine (minimum 99 %; Sigma).

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643 Table 3: Composition of LSB containing paraffin wax.

LSB type	Paraffin wax[a] g kg^{-1}	Soy lecithin[b] g kg^{-1}	Cod liver oil[c] g kg^{-1}	OTC[d] g kg^{-1}	Riboflavin[e] g kg^{-1}
1	227	545	164		64
2	227	545	164	64	-
3	240	580	180	-	-

644

645 [a]Paraffin wax mp 56-60 °C (76231 Fluke chemica)

646 [b] Soy lecithin, refined (M.W= 327.27; Cat no. 102147 Biomedicals).

647 [c]Cod liver oil (Twinlab, USA).

648 [e]Oxytetracycline hydrochloride (minimum 95 %;, Sigma).

649 [e]Riboflavin (Sigma).

650

651 Table 4: Encapsulation efficiency of glycine and water and losses of glycine by rinsing and
652 leaching from LSB with 0 %, 1 % and 5 % lecithin and a core of an aqueous slurry of glycine.
653 The encapsulation efficiencies for both glycine and water are given as % of added
654 concentrations. The losses of glycine by rinsing and leaching (20 min) are given as % of
655 incorporated glycine. Data are means of three replicates. LSB types sharing the same letter are
656 not significantly different from each other (ANOVA, followed by Tukey HSD, $p < 0.05$).

	0 % lecithin	1 % lecithin	5 % lecithin
Encapsulation efficiency of glycine (%)	101 ± 3^a	108 ± 6^a	106 ± 1^a
Encapsulaton efficiency of water (%)	102 ± 3^a	95 ± 3^a	99 ± 4^a
Loss of glycine by rinsing (%)	25 ± 4^a	23 ± 4^a	27 ± 2^a
Loss of glycine by leaching (20 min) (%)	98 ± 8^a	101 ± 4^a	97 ± 2^a

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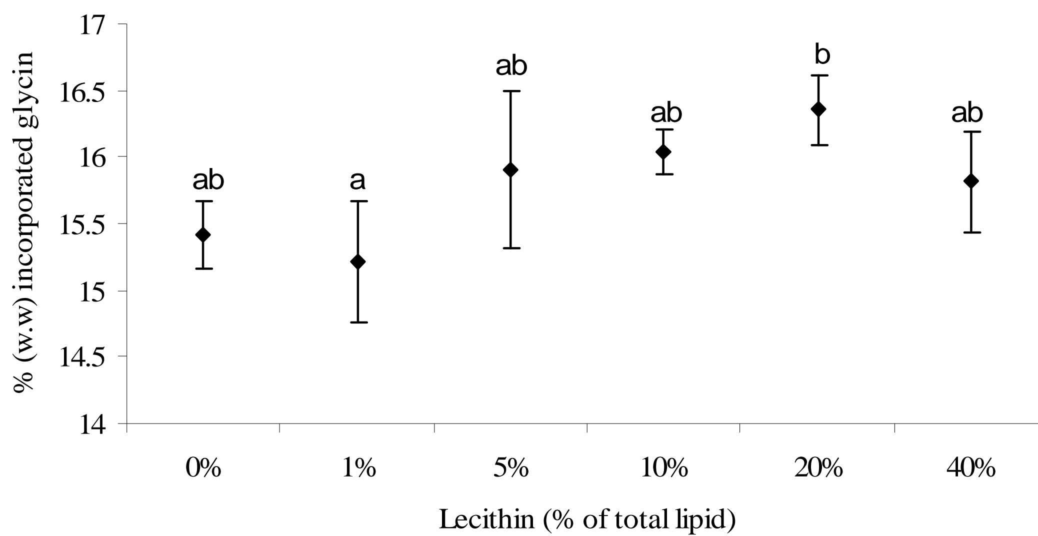

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676 Figure 1:

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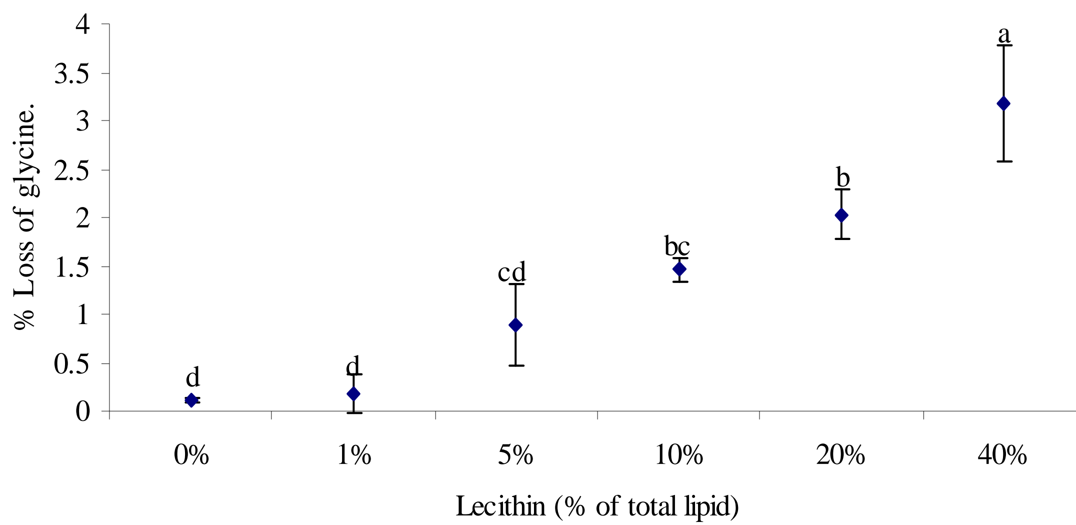

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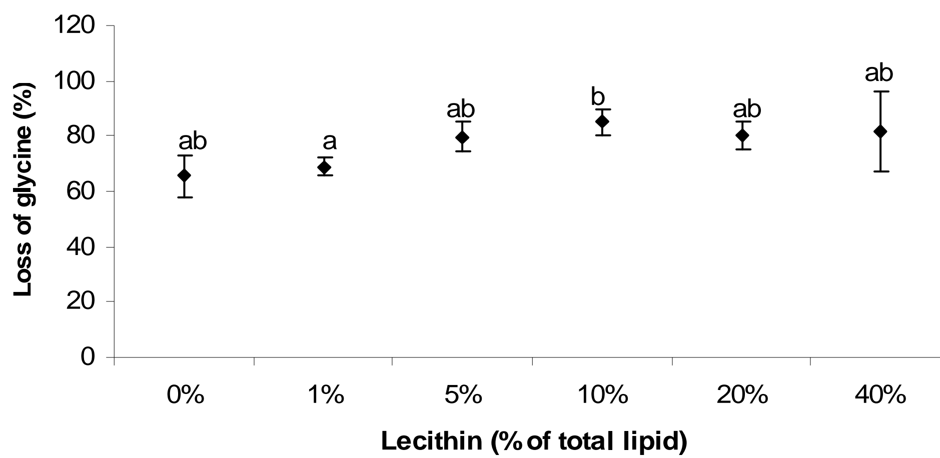

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712 Figure 3:

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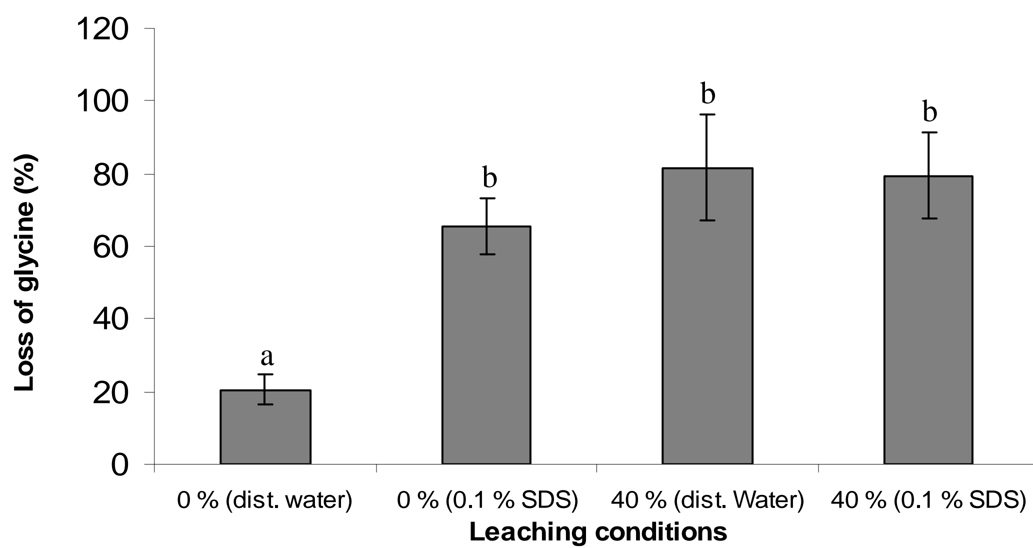

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730 Figure 4:

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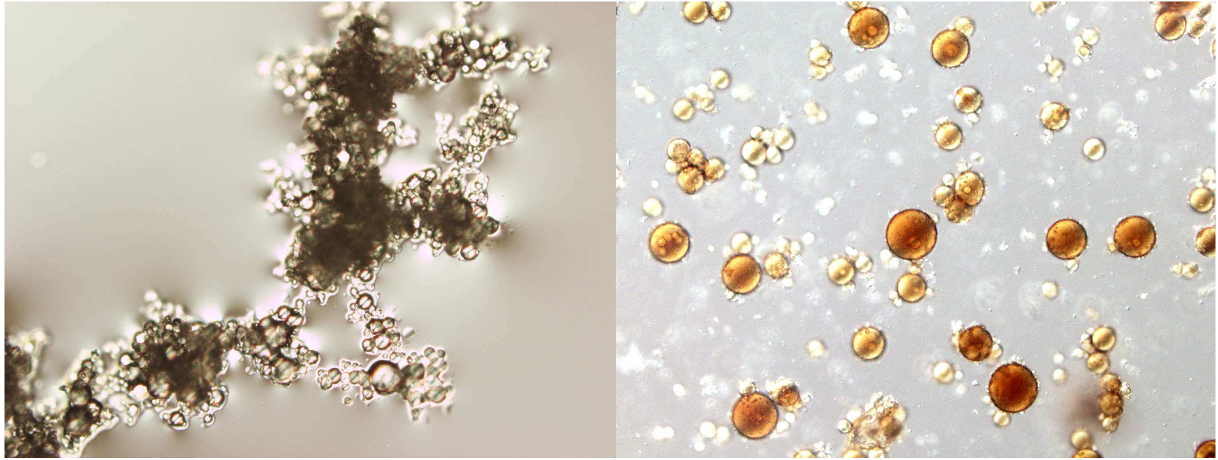

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749 Figure 5:

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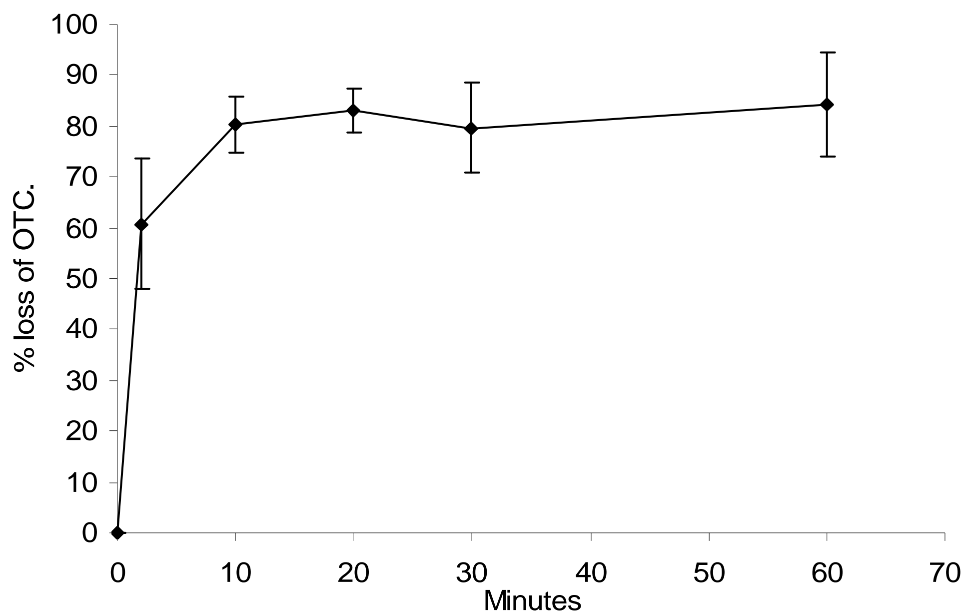

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767 Figure 6:

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784 Figure 7:

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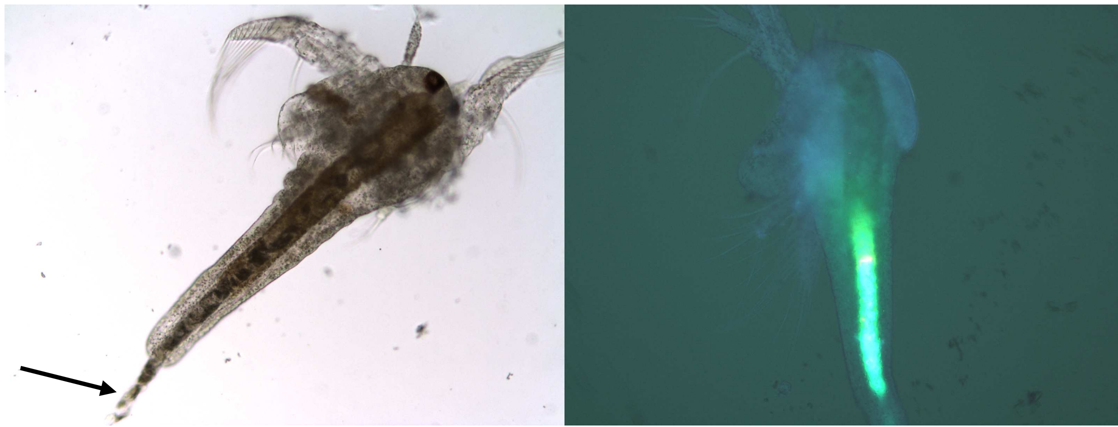

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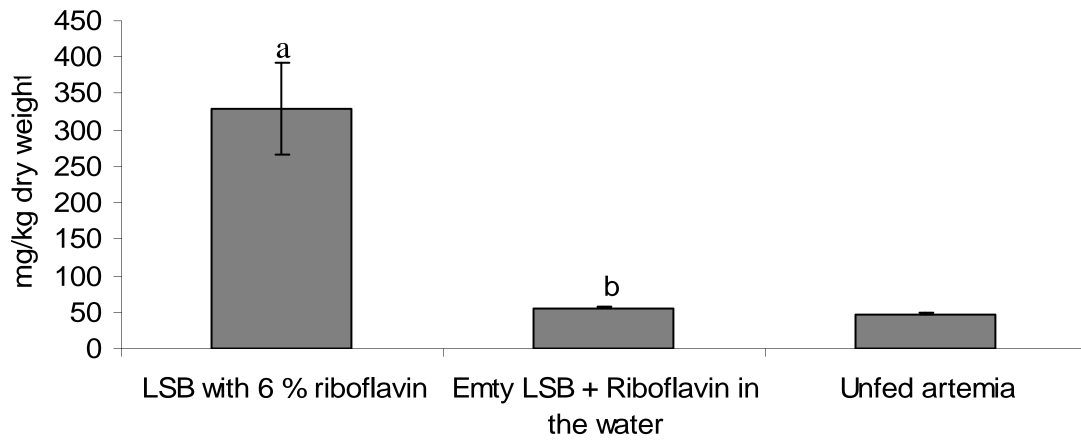

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822 Figure 9:

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