PAPER II

Errata

Paper II.

Table 1:

The ingredient list for the heat coagulated, protein bound diet should be like this:

Heat coagulated, protein bound feed (A)	
Extruded wheat	37
Saithe fillet	612
Squid mantle	68
Hydrolysate[3]	**100**
Amino acids [4]	**41**
Marine phospholipids [5]	50
Marine PUFA oil [6]	50
Vitamins [7]	10
Minerals [7]	30
Antioxidants and pigments[8]	2
	1000

The errata are in bold.

Table 4:

Table 4 should be like this:

Cutoff fraction	Algae extract	Pepsin hydrolysed algae extract
<3k	41.6 ± 0.8 [b]	65.6 ± 0.6 [a]
3-10k	3.1 ± 2.8	**0.8** ± 0.8
10-30k	4.7 ± 0.8	10.4 ± 4.0
30-100k	4.2 ± 3.2	4.2 ± 4.8
>100k	1.7 ± 1.0 [b]	7.4 ± 0.9 [a]
Sum recovered	55.4 ± 1.3 [b]	88.4 ± 1.5 [a]
Unrecovered	44.7 ± 1.3 [a]	11.6 ± 1.5 [b]

The erratum is in bold.

Page 410, lines 26-29:

"..81.0 and 65.6% … <3k and <100k fractions, respectively .." should read
"..81.0 and 65.6% … <100k and <3k fractions, respectively .."

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Leaching properties of three different micropaticulate diets and preference of the diets in cod (*Gadus morhua* L.) larvae

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Abstract

Leakage of water soluble nutrients from larval microparticulated feeds is probably extensive and needs to be further investigated. Leaching rates of ^{14}C-labelled serine, pepsin hydrolysed, protein enriched ^{14}C-algae extract and intact protein enriched ^{14}C-algae extract were measured from three microparticulated feeds for marine fish larvae (heat coagulated, protein bond feed; agglomerated feed; protein encapsulated feed). The effects of particle size (<0.3 mm, 0.3–0.6 mm; 0.6–1.0 mm) and immersion time (1–60 min) in salt water were also tested. Leaching increased by decreasing molecular weight of leaching component ($P<10^{-5}$), by the feeds in order encapsulated, heat coagulated and agglomerated feeds ($P<10^{-5}$), by longer immersion time ($P<10^{-5}$), and by decreasing feed particle size ($P<10^{-5}$). Due to low protein content of the algae extract, the leaching rates of intact and hydrolysed algae extract did not represent absolute estimates for protein and hydrolysed protein leaching. A new estimate for leakage of hydrolysed protein was calculated based on molecular weight distribution of the hydrolysed algae extract analysed by cutoff centrifugation of the extract. Assuming that molecules<300–600 or <9–18 kD would leak, leakage of hydrolysed protein from the smallest feed particles after 5 min immersion would be 80–98%, 43–54% and 4–6% of the agglomerated, heat coagulated and protein encapsulated feeds, respectively. The feeds were also tested for preference in cod larvae of two different sizes (5.6 ± 2.5 mg and 15.8 ± 7.2 mg). The preference was highest for the heat coagulated feed in the first experiment (feed intake 0.32 ± 0.06 mg dry feed larvae^{-1}) and the agglomerated in the second (2.04 ± 0.32 mg dry feed larvae^{-1}), while the protein encapsulated feed was preferred at lower rates in both experiments (0.11–0.14 mg dry feed larvae^{-1}).

Keywords: Microparticulated diet; Hydrolysed protein; Leaching; Atlantic cod; Larvae

1. Introduction

In start feeding of marine fish larvae, feeding with live prey is still the only alternative for most species.

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Furthermore, when weaning at early stages, formulated diets usually give lower larval performances than live feed. To substitute live feed, a range of different formulated feeds have been developed (Langdon, 2003) and extensively tested. The most widely used diets seem to be microbound particles with gelatine, zein, alginate, carboxymethyl-cellulose or carrageenan as binding components (López-Alvarado et al., 1994; Lee et al., 1996; Baskerville-Bridges and Kling, 2000; Guthrie et al., 2000; García-Ortega et al., 2001), protein encapsulated particles (Walford et al., 1991; López-Alvarado et al., 1994; Ozkizilcik and Chu, 1996; Önal and Langdon, 2000; Yúfera et al., 2000), lipid coated particles, usually embedded in microbound particles (López-Alvarado et al., 1994; Baskerville-Bridges and Kling, 2000; Önal and Langdon, 2004) and mechanically mixed or agglomerated particles (Cahu et al., 1999; Guthrie et al., 2000; Engrola et al., 2001; Rueda-Jasso et al., 2001).

The success of a larval feed depends on a complex composition of characteristics of the feed. The particle has to be visual and catchable for the larvae. This means that colour, shape, size, sinking properties and release of attractants are of importance. The larvae have to be able to digest the particles and be able to absorb and assimilate the nutrients, i.e. digestibility, binding properties of the feed, leakage and bioavailability of the nutrients play major roles. At last, the nutrient composition of the feed must fit the requirement of the larvae.

Leaching of small, water soluble components are of particular concern in larval feeds due to the short diffusion distance from the core of the particle to the surface. Leaching of amino acids and water soluble vitamins have been measured in some studies (López-Alvarado et al., 1994; Ozkizilcik and Chu, 1996; Marchetti et al., 1999; Baskerville-Bridges and Kling, 2000; Yúfera et al., 2002; Önal and Langdon, 2004), and these studies show that leaching may be extensive and that there are large differences between feed types and between different components that leach from the feeds.

Zambonino Infante et al. (1997) and Cahu et al. (1999) showed that hydrolysed protein supplementation of larval formulated feeds increased growth, accelerated gut and pancreas maturation, decreased incidence of spinal deformities and increased survival rates. Also Day et al. (1997) found positive correlation between level of hydrolysed protein in the weaning diet and survival. Other experiments performed with either larvae or juveniles show no advantageous effects of dietary protein hydrolysate (Oliva-Teles et al., 1999; Kolkovski and Tandler, 2000; García-Ortega et al., 2001). The contradictions may be caused by the need of an optimal level of dietary hydrolysate to obtain enhancement, while in the mentioned studies a wide range of hydrolysate quantities were used. Also the quality of the hydrolysate (peptide length and distribution, amino acid composition) may be of significance, as well as the developmental stage of the fish tested. Another problem is leakage. Feeds containing high level of hydrolysed protein will probably have high protein leaching rates which may result in protein levels below requirement in the feed ingested by the larvae.

Leakage of dietary hydrolysed protein, or peptides, from marine fish feed particles has so far not been investigated. The aim of this study was to examine leaching rates of proteineous components of different molecular size from three different formulated diets. The following dietary components were selected for leaching measurements: a) serine, as a representative amino acid, b) a pepsin hydrolysed, protein enriched algae extract, and c) a protein enriched algae extract. The components were labelled with ^{14}C. The diets were: a heat coagulated, proteinbound diet (A) that is based on a fish batter gel set by the action of micro waves. The dried product is crushed into appropriate particle sizes that were irregular in shape (Hamre et al., 2001). The next diet was an agglomerated one (B) based on micronised fish meal and glue water. These ingredients were gently processed to preserve the collagen in native conformation. Upon agglomeration, the low temperature gelating properties of the protein is utilized to bind the particles. In the agglomeration process, spherical and soft particles were formed. (Hoestmark and Nygaard, 1992). The last diet was based on protein encapsulation (C) where the cross linking of casein by 1,3,5-benzenetricarboxyl chloride at the surface of the capsules envelopes the core. The capsules were regarded as soft-walled and spherical with some depressions on the surface (Jones, 1980; Fernández-Díaz and Yúfera, 1995).

We also tested the preference of the three diets in cod larvae by adding unique inert markers (yttrium, ytterbium or lanthanium) in the diets and feed the

diets simultaneously in the fish tanks. The results of the leakage and preference studies will be the basis of our choice of diet for experiments with hydrolysed protein to Atlantic halibut and Atlantic cod larvae.

2. Materials and methods

2.1. The feeds

Feed A (Tables 1 and 2) was a heat coagulated, protein bound diet produced at the National Institute of Nutrition and Seafood Research, Bergen, Norway (Hamre et al., 2001). Minced saithe fillets and squid mantles were finely comminuted and thoroughly mixed in a food processor with all the other ingredients, pressed into diet strings, heat coagulated in a microwave oven, dried and then crushed and sieved. Feed B (Tables 1 and 2) was an agglomerated feed produced by the Norwegian Institute of Fisheries and Aquaculture Research, Bergen, Norway (Hoestmark and Nygaard, 1992). The feed was produced mainly of fine-milled (particle size < 50 pm), high quality fish meal and fish glue water that were high in low tem-

Table 1
Ingredient lists for the diets used in the experiments

Heat coagulated, protein bound feed (A)		Agglomerated feed (B)		Protein encapsulated feed (C)	
Extruded wheat	37	Mixture of:	1000	Micronised fish meal[i]	280/240
Saithe fillet	612	Micronised fish meal		Casein[j]	260/300
Squid mantle	68	Marine/vegetable oil		Dextrin	40
Hydrolysate[c]	41	Glue water		Fish lipids and vitamin emulsion[k]	120
Amino acids[d]	100	Minerals and vitamins		Hydrolysate[c]	120
Marine phospholipids[e]	50	Hydrolysate[c] (6 g kg^{-1};		Cuttlefish meal	100
Marine PUFA oil[f]	50	Only In the leaching exp.)		Premix C[l]	30
Vitamins[g]	10			Soy lecithin	30
Minerals[g]	30			Vitamin complex[m]	10
Antioxidants and pigments[h]	2			Vitamin E[n]	10
	1000		1000		1000

The diets are added a radioactively labelled component in the leaching experiment[a] and a tracer in the preference experiment[b]. Ingredients are mg (g wet wt.)$^{-1}$.

[a] In the leaching experiment one batch of each feed was added hydrolysed casein mixed with a ^{14}C-labelled component (^{14}C-algae extract, hydrolysed ^{14}C-algae extract or ^{14}C-serin) as explained below (3) and in Materials and methods.

[b] In the feed preference experiment the feeds A, B and C were added 5 mg g^{-1} Y_2O_3, La_2O_3, and Yb_2O_3 (Sigma-Aldrich Corp., St.Louis, Mo, USA), respectively, in addition to the ingredients listed in the table.

[c] In the leaching experiment this ingredient was pepsin hydrolysed casein + a ^{14}C-labelled component as explained in Materials and Methods. In feed A, 60% of the hydrolysate was saithe and squid hydrolysate as in the preference study. In the preference study, this ingredient was pepsin hydrolysed saithe fillet and squid mantle (9:1). Method as explained for hydrolysation of casein and ^{14}C-algae extract.

[d] Amino acids (g kg^{-1} premix): Asp, 19.6; Glu, 20.8; Asn, 14; Ser, 28.4; His, 14.4; Gln, 28.8; gly, 122; Thr, 17.6; Arg, 122; Ala, 57.2; tau, 289.2; Tyr, 19.2; Val, 31.6; Met, 49.6; Try, 8.8; Phe, 24.4; Ile, 22.4; Leu, 41.6; Lys, 68.4 (Sigma-Aldrich Corp., St.Louis, MO, USA).

[e] Marine phospholipids extracted from fish meal as described by Sola et al. (1994).

[f] Epax 2050 TG (Pronova Biocare, Lysaker, Norway).

[g] NRC 1993, except for α-tocopheryl acetate: 200 mg kg^{-1} and vitamin C: 400 mg kg^{-1}. Vitamins, Roche (Basel, Switzerland); minerals, Merck (Darmstadt, Germany).

[h] Tocoferolmix 750 (Nutrilo GmbH, Cuxhaven, Germany); Ascorbic acid 1200 (Norsk Medisinaldepot, Bergen, Norway); Astaxanthin 8% 20 (Roche, Basel, Switzerland); betacaroten 8% 20 mg kg^{-1} (Roche, Basel, Switzerland).

[i] AgloNorse® Microfeed (Ewos AS, Bergen, Norway): Leaching experiment: 280 mg g^{-1}; Preference experiment: 240 mg g^{-1}.

[j] ICN 901293, MP Biomedicals (Irvine, Ca, USA). Leaching experiment: 260 mg g^{-1}; Preference experiment: 300 mg g^{-1}.

[k] Kurios (Libourne, france) lipid emulsion: Fatty acids (67%), W3 HUFA (38.87%), DHA+EPA (32.2%); Vitamins (IU mL^{-1}): Vitamin A 100, vitamin D 50, vitamin C 250; Minerals and pigments; Emulsifiers (5%).

[l] Rovimix Stay C-35 (Roche, Basel, Switzerland).

[m] Kurios (Libourne, France) polyvitamin fish complex (mg ml^{-1} complex): thiamin 3.75; riboflavin 1.5; pyridoxine 1.25; pantothenic acid 11.25; methionine 1; choline 10; vitamin B_{12} 0.01; vitamin C 5; vitamin E 7.5; vitamin K_3 0.75; vitamin A 10 000 IU ml^{-1}; vitamin D_3 7500 IU ml^{-1}.

[n] DL-alpha-tocopherol, ICN 100555, MP Biomedicals (Irvine, CA, USA).

Table 2
Composition of the diets used in the experiments

	Heat coagulated, protein bound feed (A)	Agglomerated feed (B)	Protein encapsulated feed (C)
Dry matter (mg g^{-1} wet wt)	924.2 ± 0.8	927.5 ± 0.6	937.5 ± 2.0
Total lipids (mg g^{-1} dry wt)	113.4 ± 0.2	209.4 ± 1.6	160.6 ± 1.9
Crude protein (mg g^{-1} dry wt)	786.3 ± 2.2	662.3 ± 3.9	675.6 ± 18.8
Protein solubility (meq Leu-α-NH_2 g^{-1} protein)	0.94 ± 0.00	0.60 ± 0.01	<0.09 ± 0.02
Radioactivity (kBq g^{-1})[a]			
– Algae extract	11.55 ± 0.90	7.86 ± 0.34	0.131 ± 0.006
– Hydrolysed algae extract	12.91 ± 0.42	7.22 ± 0.16	103.81 ± 10.11
– Serine	7.29 ± 0.24	3.84 ± 0.13	22.56 ± 0.81

[a] Only in the diets used in the leaching experiment.

perature gelating protein that bound the particles together. The ingredients were mixed in an agglomerator to produce roe-like particles. All fish based ingredients were made of fresh raw materials that were processed gently to avoid heat denaturation and deterioration of the nutrients to keep high biological digestibility, high acceptance in fish larvae and to utilize the cold gel setting property of collagen during feed manufacturing. Both feed A and B were sieved into three particle size fractions: <0.3 mm, 0.3–0.6 mm and 0.6–1.0 mm. Feed C (Tables 1 and 2) was protein microcapsules produced at the Instituto de Ciencias Marinas de Andalucía (CSIC), Cádiz, Spain as described by Yúfera et al. (1999, 2000). The ingredients, dispersed in alkaline Tris–HCl buffer, were homogenised with lecithin-cyclohexane (2% w/v) for 16 min at 1000 rpm. A cross linking agent, 1,3,5-benzenetricarboxyl chloride (trimesoyl clorid), was added in the middle of the process. The diet particles formed were allowed to settle and the lecithin:cyclohexane solution was decanted. After washing with cyclohexane, the particles were dispersed in gelatin (15%w/v), and then repeatedly washed in fresh water and finally in buffered saline (pH8) and then freeze-dried. The particle size of the feed was 0.12–0.32 mm. For the leaching experiment, different batches of all three diets were added a radioactively labelled components: ^{14}C-serine (ARC, St. Louis, MO, USA), hydrolysed or intact ^{14}C-algae extract enriched in protein as described below. The diets for the preference experiment were added different tracers (yttrium, Y_2O_3; lanthanum, La_2O_3; and ytterbium, Yb_2O_3; Sigma-Aldrich Corp., St. Louis, MO, USA) in quantities of 5 mg kg^{-1} so that feed intake could be quantified.

2.2. Production, hydrolysation and characterisation of a ^{14}C-labelled algae extract enriched in protein

The wall-less mutant *Chlamydomonas reinhardtii* (CCAP 11/32CW15+) was grown at 28 °C, at an irradiance of 150 μmol m^{-2} s^{-1} (PAR – Photosynthetic Active Radiance) in a modified Sueoka nutrient medium (Sueoka et al., 1967) as described by Lien and Knutsen (1976). Assimilation of ^{14}C was based on the method of Steemann Nilsen (1952). A dense culture of *C. reinhardtii* was incubated with 5 mCi $H_2^{14}CO_3$ (Carbon 14 Centralen, Denmark) in a closed system to prevent loss of activity in form of $^{14}CO_2$. A gentle mixing of the culture was obtained with a magnetic stirrer. After incubation for 17 h, the culture was centrifuged and the algae pellet was resuspended in a PBS buffer. The cells were transferred to a cell disruption bomb (Parr Instrument Company, Moline, IL, USA) and pressurized to 200 bar for 45 min. The cells were lysed when the pressure was released.

The opened cells were added DNase and RNase and incubated in room temperature for 4 h. Cell remnants were removed by centrifugation and the protein salted out with $(NH_4)_2SO_4$ (450 g l^{-1}). The precipitate was centrifuged, resuspended in PBS-buffer and dialysed (benzoylated dialysis tubing, Mw>2000 and Mw<1200; Sigma-Aldrich Corp., St.Louis, MO, USA). The dialysed extract was freeze-dried.

The protein enriched ^{14}C-algae extract was used to simulate leaching of intact and hydrolysed protein. To make the hydrolysed preparation, part of the ^{14}C-algae extract (60,320 and 100 mg for diet A, B and C, respectively) was mixed with casein (1.5–2 g; Tine, Norway) and dispersed in distilled water. The pH was adjusted to 4.2 with addition of 3 M HCl before pepsin

(37 mg g^{-1} casein; Sigma, St.Louis, MO, USA) was added. After incubation overnight with gentle stirring, the samples were neutralised with 3 M NaOH and freeze-dried. To make the simulated protein and free amino acid preparations, pepsin hydrolysed casein was mixed with the ^{14}C-algae extract and ^{14}C-serine (ARC, St.Louis, MO, USA), respectively.

Molecular weight distribution of labelled algae extract and labelled hydrolysed algae extract was analysed by cut-off centrifugation. Dilute solutions (0.51 mg ml^{-1} in 0.02 M phosphate buffer, pH7.6) of the labelled components were successively centrifuged in 100, 30, 10 and 3 k cutoff vials (Microsep™, Pall Life Sciences, Ann Arbor, MI, USA). The samples were centrifuged twice in all cutoff vials with addition of buffer in-between. Radioactivity was measured in all fractions. The expected molecular weight of components retained by the different cutoff vials are shown in Table 3.

2.3. The leaching experiment

Leaching rates were measured in 50 ml nunc boxes (Nalge nunc Int., Rochester, NY, USA) lined up in an agitating water bath (Heto, Birkerød, Denmark). Triplicate feed samples of 0.2 g were immersed in 10 ml 3% NaCl (12 ± 3 °C) and stirred (120 rpm, 3 cm stroke distance) for 1, 5 or 60 min. After filtration (597½ S and S folded filters, Schleicher and Schuell GmbH, Dassel, Germany), the eluate was transferred to plastic LSC vials (Packard Bioscience, Groningen, The Netherlands) and analysed for radioactivity. Leaching was quantified as amount of radioactivity in the eluate in percent of initial total radioactivity of the feed.

Table 3
Expected molecular weight of components retained by the different cutoff vials and the corresponding estimate of number of amino acid (AA) in retained peptides

	Molecular weight (kD)	Estimated peptide length[a] (AA)
Dialysis[b]	1.2–2.0	10–17
Cutoff fraction[c]:		
3 k	9–18	75–150
10 k	30–60	250–500
30 k	90–180	750–1500
100 k	300–600	2500–5000

[a] Estimated from a weighted averagely molecular weight of 119.4 D per amino acid (Creighton, 1992).
[b] The extract was dialysed before it was included in the diet.
[c] According to the supplier (Pall Life Sciences, Ann Arbor, MI, USA), the cutoff vials are expected to retain molecules with weights 3-6 times higher than the noted cutoff.

2.4. The preference experiment

The experiment was performed at Institute of Marine Research (Storebø, Norway). Eggs were collected from one group of Atlantic cod (*Gadus morhua*) broodstock, and treatment of eggs and pre-weaned larvae was in accordance with van der Meeren and Ivannikov (2001). The larvae were first-fed at day 5 after hatching (dph) on Rotimac (Biomarine Aquafauna Inc.,Hawthorne, CA, USA) enriched rotifers (*Brachionus plicatilis*) in 500 l tanks. From 27 dph they were fed DC-DHA-Selco (INVE Aquaculture NV, Dendermonde, Belgium) enriched *Artemia* (Artemia Systems Inc., Gent, Belgium).

At 39 dph the larvae (wet weight 5.6 ± 2.5 mg) were distributed into triplicate 50-l tanks (700 larvae $tank^{-1}$; 11.5 ± 0.3 °C; water flow 0.64 ± 0.05 l min^{-1}; siphoned twice day^{-1}). They were fed *Artemia* on the day of transfer (3 × 1000 *Artemia* l^{-1}). The following two days the larvae were manually fed 8 rations of a mixture (1:1:1) of the three experimental feeds, in total 4 g feed per day of each feed. The third day they were given the whole day ration in the morning and sampled an hour later.

Ten days later (49 dph), the experiment was repeated with 290 larvae $tank^{-1}$ (wet weight 15.8 ± 7.2 mg). Environmental conditions were similar as mentioned above except that water flow was increased to 0.88 ± 0.04 l min^{-1}.

At sampling, all larvae from each tank were collected, anaesthetized with metomidate (0.5 g l^{-1} in 50:50 distilled water:seawater) and pooled in two samples per tank. The samples were immediately frozen on dry ice and stored at −80 °C until analysed.

2.5. Chemical analysis

Content of dry matter in the feeds was measured gravimetrically after 24 h at 104 °C, and total lipid was extracted with ethyl acetate:isopropanol (7:3) followed by gravimetrical measurement (Norsk Standard, 1996). Crude protein (N × 6.25) of the feeds and of the ^{14}C-labeled algae extract was determined by Kjeldahl digests (Crooke and Simpson, 1971). Protein

solubility was estimated by determining the free α-amino groups reacting with trinitrobenzenesulphonic acid (TNBS). The binding of TNBS to free α-amino groups was determined colorimetrically using L-leucine as a standard (Adler-Nissen, 1979). All these diet analyses were performed on the diets used in the preference experiment.

Radioactivity was measured in a Tricarb® Liquid Scintillation Analyzer (Packard Instrument Co, Meriden, CT, USA) using Hionic Fluor or Ultima Gold XR (Packard Bioscience, Groningen, The Netherlands) as scintillation cocktail. To measure total radioactivity the feed was treated with a solubilizer (Solvable, Packard Bioscience) before added to the cocktail.

Lanthanium, yttrium and ytterbium were determined in feed and larvae from the preference study by inductively coupled plasma mass spectrometry (Perkin Elmer SCIENX Elan 5000A ICP-MS) as described by Otterå et al. (2003).

2.6. Statistical analyses and calculations

Statistical analyses were performed with the software Statistica (version 6.1; StatSoft Inc., Tulsa, OK, USA). Leakage was statistically analysed by ANCOVA with feed, particle size and leaching component as categorical variables and immersion time as a continuous variable (i.e. immersion time was the covariate). As one of the feeds in the leaching experiment only was in one feed particle size in contrast to three particle sizes of the other two feeds, statistical analysis was performed on two sub sets of the leaching data to fulfil the requirement of balanced design. Each of the data sub sets were also full factorial designs. The statistical model of the first sub set (Model 1) included feed A (heat coagulated, protein bound diet) and B (agglomerated diet) and all three feed particle sizes, and the model of the second sub set (Model 2) included feed A, B and C (protein encapsulated diet), but only feed particle size < 0.3 (i.e. feed C of particle size 0.12–0.32 mm). Leaching rates were $\log_{10}$ transformed and immersion times were 1^{*} time^{-1} transformed. The latter transformation reverses the order of immersion time, so negative gradients will mean increasing rate of leaching. This makes up the following statistical models on leaching:

Model 1: Log_{10} (leaching + 1) = Feed (A; B) + particle size (< 0.3 mm; 0.3–0.6 mm; 0.6–1.0 mm) + leaching component (protein enriched algae extract; hydrolysed, protein enriched algae extract; serine) + immersion time ($60^{-1} - 1^{-1}$).

Model 2: Log_{10} (leaching + 1) = Feed (A; B; C) + leaching component (protein enriched algae extract; hydrolysed, protein enriched algae extract; serine) + immersion time ($60^{-1} - 1^{-1}$).

The feeds are: A=Heat coagulated, protein bound; B=Agglomerated; C=Protein encapsulated.

Estimates for leaching rates of hydrolysed protein (Table 6) were analysed by one way ANOVA followed by Tukey HSD for difference between the feeds at different immersion intervals and different size of molecules (9–18 and 300–600 kD) that are considered to have potential to leach.

In the preference study, ingestion of the different diets was tested by ANOVA after square root transformation of feed intake. Statistical differences between groups were analysed by Tukey's HSD in both leaching and preference experiments. Statistical differences between the molecular weight fractions of intact and hydrolysed algae extract separated by cutoff centrifugation were analysed by *t*-test. The analyses are based on means ± SD of 3 replicates in the leaching and preference studies and of 2 replicates in the molecular weight distribution measurement. The data were considered significantly different when $P < 0.5$.

Feed intake in the preference experiment was calculated as: Mg diet (larvae)$^{-1}$ = Tr_L * Tr_D^{-1} * Wt_L *1000 where: Tr_L=Amount of tracer in the larvae (μg (g lavae)$^{-1}$) Tr_D=Amount of tracer in the diet (μg (g diet)$^{-1}$) Wt_L=mean wet weight of the larvae (g; $n = 32$–36).

3. Results

3.1. Intact and hydrolysed, protein enriched ^{14}C-algae extract

Crude protein content of the protein enriched extract of ^{14}C-labelled algae was measured as 8.5 ± 0.1% and specific activity was 29.8 kBq g^{-1} dry weight. Cutoff centrifugation left 45 and 12% of the radioactivity unrecovered in the algal extract and

Table 4
Cutoff fraction distribution of ^{14}C-labelled algae extract and pepsin hydrolysed ^{14}C-labelled algae extract

Cutoff fraction	Algae extract	Pepsin hydrolysed algae extract
<3 k	41.6 ± 0.8[b]	65.6 ± 0.6[a]
3–10 k	3.1 ± 2.8	8 ± 0.8
10–30 k	4.7 ± 0.8	10.4 ± 4.0
30–100 k	4.2 ± 3.2	4.2 ± 4.8
>100 k	1.7 ± 1.0[b]	7.4 ± 0.9[a]
Sum recovered	55.4 ± 1.3[b]	88.4 ± 1.5[a]
Unrecovered	44.7 ± 1.3[a]	11.6 ± 1.5[b]

Values are radioactivity in fraction in percent of radioactivity in the applied sample. Values in the same row are significantly different when marked with different superscript (Student *t*-test).

hydrolysed algal extract, respectively ($P=0.0018$; Table 4), and thus 30% more of the total radioactivity was unrecovered in the intact algal extract compared to the hydrolysed. The unrecovered radioactivity was probably high molecular components associated with the membranes of the cutoff vials. The <3 and >100 k fractions were higher in the pepsin hydrolysed extract than in the intact algal extract ($P=0.0008$, $P=0.026$, respectively), and the sum of radioactivity in these two fractions increased with 33% after pepsin hydrolysis. In the other fractions there were no significant differences.

3.2. Leaching

To cover the whole data set of leaching rates, statistical analysis was performed on two sub set of the leaching data as described in material and methods. The statistical results of the two sub set were similar as shown in Table 5. All the experimental factors (feed, particle size, leaching component and immersion time) significantly affected leaching (Table 5; Figs. 1, 2 and 3). Leaching increased by the feeds in order protein encapsulated (C), heat coagulated (A) and agglomerated (B; $P<10^{-5}$). It also increased by longer immersion time of the feed particles in salt water ($P<10^{-5}$), but in the model of the data sub set where only the smallest feed particle size was included (model 2), the rate of increase

Table 5
Statistical analysis (ANCOVA) of leaching where the analysis is performed on two sub set of the total data set

Leaching as function of:	Model 1		Model 2	
	Significant differences	*P*	Significant differences	*P*
Main effects:				
• Feed type[b]	B>A	$<10^{-5}$	B>A>C	$<10^{-5}$
• Particle size	(<0.3)>(0.3–0.6)>(0.6–1.0)	$<10^{-5}$	Only (<0.3) included	
• Leaching component[c]	Ser> hp> p	$<10^{-5}$	Ser>hp>p	$<10^{-5}$
• Immersion time[d]	$Y=1.61-0.29X$	$<10^{-5}$	$Y=1.42-0.10X$	$<10^{-5}$
Interaction effects:				
• Feed x leaching component		$<10^{-5}$		$<10^{-5}$
• Particle size x immersion time[d]	<0.3: $Y=1.68-0.10X$ 0.3–0.6: $Y=1.69-0.26X$ 0.6–1.0: $Y=1.54-0.46X$	$<10^{-5}$	Only (<0.3) included	
Adjusted R^2	93.7%		98.3%	

The two data set were balanced full factorial designs, and the first one (model 1[a]) included the heat coagulated (A) and agglomerated (B) feeds. The other (model 2[a]) included all three feeds[b], but only the smallest particle size (<0.3 mm). The table presents statistical differences between groups within each explanatory variables (Tukey HSD), significance levels of the variables, regression lines for leaching rates, and the adjusted coefficients of determinations (adjusted R^2) for the models.

[a] Model 1: Log_{10} (leaching+1)=Feed (A; B[2])+particle size (<0.3 mm; 0.3–0.6 mm; 0.6–1.0 mm)+leaching component (serine; pepsin hydrolysed, protein enriched algae extract; protein enriched algae extract)+immersion time (60^{-1}–1^{-1}). Model 2: Log_{10} (leaching+1)=Feed (A; B; C[2])+leaching component (serine; pepsin hydrolysed, protein enriched algae extract; protein enriched algae extract)+immersion time (60^{-1}–1^{-1}).

[b] A=Heat coagulated, protein bound diet; B=Agglomerated diet; C=Protein encapsulated diet.

[c] Ser=serine; hp=pepsin hydrolysed, protein enriched algae extract; p=protein enriched algae extract.

[d] The back transformed lines will be: $10^{y}=10^{a+bx}$, where $y=\log_{10}(\text{leaching}+1)$; x=(immersion time)$^{-1}$, thus 60^{-1}, 5^{-1} and 1^{-1}; a=intercept; b=gradient. Note that the regression lines start at 1 min of water immersion and not at 0 as the curves in Figs. 1, 2 and 3.

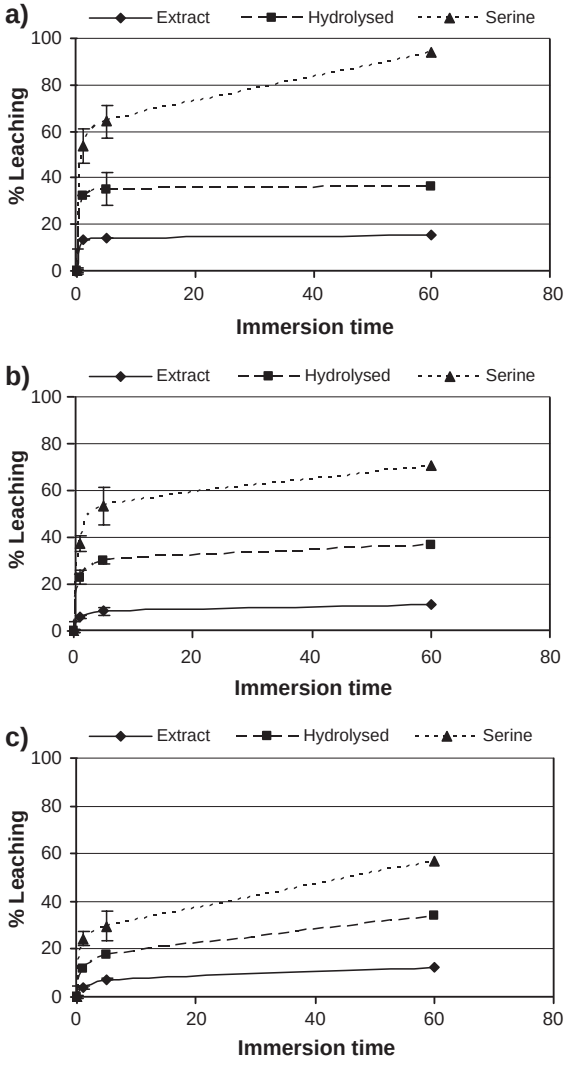

Fig. 1. Leaching pattern of heat coagulated, protein bound feed (A) of particle size a) <0.3 mm, b) 0.3–0.6 mm and c) 0.6–1.0 mm during 60 min immersion in salt water (3% NaCl). The leaching components were: Extract=protein enriched algae extract; Hydrolysed=pepsin hydrolysed, protein enriched algae extract; Serine.

between 1 and 60 min immersion time was low. The full leaching potential had almost been reached before the first point in time of analysis which was after 1 min water immersion. Note that the negative gradients of the regression lines mean increasing rate of leaching due to the transformation of immersion time ($60^{-1} - 1^{-1}$). Furthermore, leaching decreased with increasing feed particle size ($P<10^{-5}$) and molecular weight of leached component (serine> hydrolysed, protein enriched algae extract>protein enriched algae extract; $P<10^{-5}$).

The interaction effects between immersion time and particle size and between leaching component and feed were significant (Table 5; $P<10^{-5}$). The small particles showed high leaching rates at all measured leaching intervals (1–60 min), as can be seen from the relatively high intercept and slow gradient of the regression line in Table 5. The bigger particles

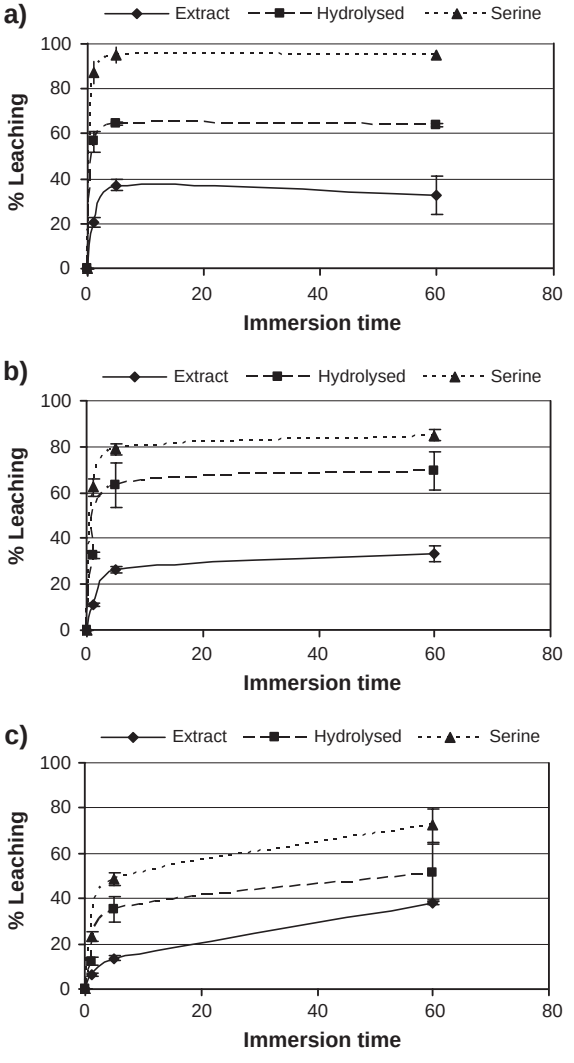

Fig. 2. Leaching pattern of the agglomerated feed (B) of particle size a) <0.3 mm, b) 0.3–0.6 mm and c) 0.6–1.0 mm during 60 min immersion in salt water (3% NaCl). The leaching components were: Extract=protein enriched algae extract; Hydrolysed=pepsin hydrolysed, protein enriched algae extract; Serine.

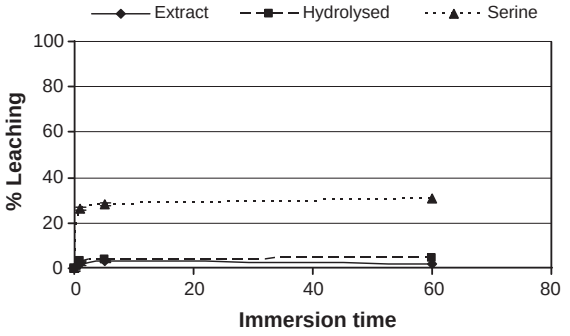

Fig. 3. Leaching pattern of the protein encapsulated feed (C) of particle size 0.12–0.32 mm during 60 min immersion in salt water (3% NaCl). The leaching components were: Extract=protein enriched algae extract; Hydrolysed=pepsin hydrolysed, protein enriched algae extract; Serine.

clearly increased in leaching rates after prolonged immersion time. The steeper gradients of the regression lines illustrate this. Regarding leaching component and kind of feed, leaching of intact algae extract was more differentiated between the feeds while leaching of serine was high in all feeds ($P<10^{-5}$).

Due to the low protein content of the algae extract, the leaching rates of intact and hydrolysed algae extract cannot be considered to represent true estimates of protein and hydrolysed protein leaching, respectively. New estimates of leaching of hydrolysed protein was calculated assuming that only the labels in the <3 or <100 k cutoff fractions could leak and that non-protein labels in these fractions would have similar leaching rates as protein (Table 6). In these estimates, the molecules that did not pass the 100 k membrane (300–600 kD) in the cutoff centrifugation are considered unable to leak, and the molecules that passed the 3 k membrane (9–18 kD) are considered to have very high potential to leak and to typically represent hydrolysed protein as this fraction increased by hydrolysation. The leaching potential of intermediate molecules (18–300 kD) is more uncertain. Thus, one may assume that the true leaching potential of pepsin hydrolysed protein will be in the range between the estimated ones. The estimated leaching potentials will be 81.0 and 65.6% of initial total radioactivity from the hydrolysed algae extract in <3 and <100 k fractions, respectively (Table 4). According to these estimates leakage of hydrolysed protein from the smallest feed particles tested after 5 min immersion would be 80–98%, 43–54% and 4–6% for the agglomerated, heat coagulated and protein encapsulated feeds, respectively (Table 6). The estimate for the agglomerated feed is tangent to serine leakage in Fig. 2.

Table 6
Leaching rates of hydrolysed algae extract from the experimental feeds, assuming that only components in the <100 or <3 k fractions have the potential to leak

Leaching potential	<300–600 kD			<9–18 kD		
Immersion time (min)	1	5	60	1	5	60
Agglomerated feed (B)	70 ± 5	80 ± 1	79 ± 1	86 ± 7	98 ± 1	98 ± 1
Heat coagulated, protein bound feed (A)	40 ± 1	43 ± 0	45 ± 9	50 ± 2	54 ± 0	55 ± 11
Protein encapsulated feed (C)	4 ± 0	4 ± 0	5 ± 0	5 ± 0	6 ± 0	7 ± 0

This corresponds to components of <300–600 and <9–18 kD, respectively. (Particle size: <0.3 mm for feeds A and B, and 0.12–0.32 for feed C).

3.3. Feed preference

Cod larvae at day 39 after hatching had the highest feed intake of the heat coagulated, protein bound diet (A) when fed the three experimental feeds simultaneously (Table 7). The second highest intake was of the agglomerated diet (B). When the experiment was repeated ten days later, the highest intake was of feed B followed by feed A. In both experiments the ingestion of the protein encapsulated diet (C) was lower than that of the two other feeds. In both experiments the particle sizes were <0.3 mm of feed A and B, and 0.12–0.32 mm of feed C.

Table 7
Feed intake (mg larvae^{-1}) fed in excess to cod larvae in a 1:1:1 mixture of the three diets

Age of larvae[a]	Heat coagulated, protein bound feed (A)	Agglomerated feed (B)	Protein encapsulated feed (C)
39 dph	0.32 ± 0.06^{a}	0.21 ± 0.07^{b}	0.14 ± 0.05^{c}
49 dph	1.15 ± 0.33^{b}	2.04 ± 0.32^{a}	0.11 ± 0.03^{c}

Values in the same row are significantly different when marked with different superscript.

[a] The wet weights of the larvae were: 39 dph, 5.6 ± 2.5 mg; 49 dph, 15.8 ± 7.2 mg.

4. Discussion

Leaching of components from formulated feeds for marine fish larvae may have both positive and negative consequences. A positive effect is release of attractants that may trigger the fish to ingestion, and thus may affect growth and survival (Kolkovski et al., 2000). Concerning larval feeds that consist of very small particles, some studies have revealed that leaching from a number of feeds is extensive (López-Alvarado et al., 1994; Baskerville-Bridges and Kling, 2000), and thus indicating that the negative consequences of leaching probably are dominating. By leaching, nutrients intended for the larvae are lost, but made easily available for micro organisms that deteriorate the water quality and may easily infect the larvae.

The main objectives of this study were to quantify and characterise leakage from three selected formulated feeds for marine fish larvae, and to investigate leaching of hydrolysed protein. Measurement of radioactivity was used to quantify leaching, and therefore a ^{14}C-labelled extract enriched in protein was produced from algae for use in this study. A vector of proteinous components with increasing molecular weight was made by analysing leaching of ^{14}C-serine, pepsin hydrolysed, protein enriched ^{14}C-algae extract and protein enriched ^{14}C-algae extract. The results show that leaching rates decreased with increasing molecular weight of the leaching components (Table 5). As seen in Figs. 1, 2 and 3, the effect of the leaching components upon leaching is clear in the heat coagulated, protein bound diet (Fig. 1) and the agglomerated diet (Fig. 2), while the leaching of hydrolysed and intact algae extract was similar in the protein encapsulated diet. In this diet too, leaching of the amino acid was distinctly higher than leaching of the other two analysed components.

The algae extract was planned to be highly enriched in protein, but the analysed crude protein content of the extract was very low (8.5%). This value covers both the labelled and unlabelled portion of the protein, while the leaching measurements only included the labelled portion. Transfer of label from the unrecovered cut off fraction to the <3 and >100 k fractions during hydrolysis indicates that the hot portion of the extract had a higher fraction of protein than the cold portion. Approximately 30% of the unrecovered fraction was hydrolysed by pepsin, indicating that at least 30% of the label was associated with protein in this fraction. It is uncertain how much of the label that was attached to protein in the other cut off fractions, but at least 30% of the 3 k fraction after hydrolysis was probably protein. The method used for isolation of protein, ammonium sulphate saltation, is simple and requires little investment in equipment, labour and raw materials, but failed in concentrating the protein.

Carbohydrate from the algae photosynthesis probably made up a large fraction of the extract. RNA and DNA were digested during processing of the extract and the nucleotides were probably removed during dialysis. Other components that could be present in the extract are glycolipids and glycoproteins. If we assume that these molecules are retained by the cutoff vials at similar molecular weights as protein and that their leaching rates are similar to proteins with similar molecular weights, the results of the leaching experiments may be taken as estimates of absolute leaching rates from the different feeds.

In this study, three types of feed produced with very different process technologies were tested. Protein is yet used as the matrix component in all three feeds. In the heat coagulated, protein bound feed (A) the ingredients are embedded in a heat set gel of finely comminuted fish muscles. The agglomerated feed (B) is cemented by collagen that has been preserved in the ingredients, and therefore forms a gel upon cooling (Hoestmark and Nygaard, 1992). In the protein encapsulated feed (C) the protein cross linker added during manufacturing, covalently binds to amine groups available on the surface of the precapsules. A continuous matrix of the protein and cross linker is thus made. The matrix has been thought to be only on the surface due to low permeability of the cross linker into the aqueous phase inside the capsules (Jones, 1980), keeping the protein in the core bioavailable, but further investigation has indicated that the matrix is uniform through the whole capsules (Nordgreen, pers.com.).

The clearly lowest leaching rates were measured from the protein encapsulated feed. The leaching of the intact and hydrolysed, protein enriched algae extract did not exceed 4% and the leaching of serine did not exceed 31% during an hour immersion in salt water. Also the studies by López-Alvarado et al. (1994) and Yúfera et al. (2002) showed low leakage

from the protein encapsulated feed compared to the microbound feeds. In the present study, there is a possibility that the labelled components were bound by the cross linker, or that the most easily leached compounds were lost from the feed particles in the washing procedures during manufacturing. This suggestion is also supported by the low protein solubility measured in the unlabelled diet (Table 2) and by Yúfera et al. (2002) who measured the encapsulation efficiency of free lysine to be 7.5%.

The heat coagulated, protein bound feed, and especially the agglomerated feed, showed far higher leaching rates than the protein encapsulated feed. In the agglomerated feed, 70–98% of serine leached from the particles within 60 min, while the leaching rates for hydrolysed and intact extract were 50–70 and 30–40%, respectively. The numbers for the heat coagulated diet were 60–98% for serine, less than 40% for hydrolysed extract and 10–20% for intact extract. Assuming that high molecular weight labels present in the algae extract retained by the <100 or <3 k cut off fractions, also would be more extensively retained by the feed particles than hydrolysed protein, new estimates of leakage were calculated. Total leaching of hydrolysed extract from the agglomerated feed (B) after 5 min constituted 98% of the radioactivity in the <3 k fraction and 80% of the sum of radioactivity in the fractions <100 k. Leaching from the heat coagulated feed (A) constituted 54 and 43%, respectively. Leaching of hydrolysed protein from feed A is thus approximately half of that from feed B.

Larger particles leached less than the smaller ones. This was expected, since the diffusion distance from the core of the particles is longer the larger the particles. The leaching curves plotted in Figs. 1, 2 and 3, showed increased leaching over time, but the rates of leaching between one and 60 min were low compared to the steep rate during the first few minutes of immersion in salt water. Leaching rates should have been measured at an earlier point than after one minute to produce more detailed leaching curves, but this introduces practical difficulties. A significant interaction effect was found between feed particle size and immersion time. Large feed particles continued to leak during 60 min of water immersion, while the smallest particles generally had reached final leaching level within one to five minutes. Several other properties of the feed particles may also have affected leakage from the three tested feeds. The particles differed in shape, being irregular (Diet A) or spherical (Diets B and C). The diets had also different ingredients and diet composition, which might have affected the results.

Although a single amino acid will not give the whole picture of amino acid leaching, only leaching of serine was measured for simplification purposes in this study. In addition, other studies have investigated amino acid leaching more thoroughly (López-Alvarado et al., 1994; Yúfera et al., 2002). Serine was chosen to represent the amino acids because of its position at the middle range of many hydrophobicity scales (Creighton, 1992).

A new method for analysing leakage was introduced in this study. By comparing the results on serine leaching with other amino acid leaching studies, the methods can be compared. Both López-Alvarado et al. (1994) and Yúfera et al. (2002) tested protein microencapsulated diets, and Yúfera et al. (2002) found, when using the same types of capsules as in the present study, that leaching of serine was 54.3–60.2% after 60 min of immersion, while in our study the result was $30.6 \pm 0.7\%$. López-Alvarado et al. (1994) found a leaching rate of $39.0 \pm 6.3\%$ from the microencapsulated diet after 2 min of immersion, while in our study comparable result would be $26.5 \pm 0.4\%$ and $28.4 \pm 0.7\%$ measured after 1 and 5 min, respectively. The results in the present study showed lower values than in the two mentioned studies, which may be due to differences in the diets, in the amino acid detection methods (amino acid analysis vs radioactivity), the immersion water (3% NaCl water vs 0.5 M borate buffer, pH8.5 vs distilled water), and/or the leaching models (stirring, volumes, shape of vials). When measuring leaching in laboratory scale, the purpose is to simulate the leaching rates of feed particles fed in fish tanks. In the latter situation leaching analysis is complicated due to low concentrations of feed particles and thus also of leaching component. How well a leaching model corresponds with what really happens when the feed particles are released in the fish tanks is of great importance. In the present study, stirring was kept high enough to avoid settling of particles in the leaching vials that could underestimate leakage due to local high concentration of leaching components.

In the preference study on cod larvae, the diets were added unique inert markers that have been successfully

used in digestibility and preference studies (Austreng et al., 2000; Otterå et al., 2003). The present study gives an idea of which of the three feeds that will give the highest ingestion rate in a long-term feeding experiment, and furthermore which feed that best will support growth and development of the fish larvae. Highest preference of the heat coagulated, protein bound diet was found in 39 dph old cod larvae, while the highest preference at the age of 49 dph was of the agglomerated diet. The amount of ingested protein encapsulated diet was lower in both experiments. Several factors might have affected the ingestion of the feeds and the preference of one type over the other. The tested feeds in this study differed in physical properties like colour, shape, sinking properties (results not presented) and leaching. The composition of ingredients and macronutrients differed a lot, and especially the different levels of hydrolysed protein, which may function as attractant, and the different ratios of protein and lipid may have affected ingestion (Kolkovski et al., 2000; Morais et al., 2005). Even though their size ranges were fairly similar, they surely differed in size profiles, and likely also in swelling properties that increase the differences in real particle size. Because the feeds were fed simultaneously to the larvae, one of the feeds might have triggered ingestion more than the others, and further might have increased the ingestion of one of the other feeds. To give a true picture of preference, the study should have been repeated by the feeds fed in separate tanks. It is possible that which feed that is the most optimal feed for the larvae, changes within short periods as the fish larvae grow very fast. This might have happened in the present study as the heat coagulated and the agglomerated diets each were most preferred in the first and second experiment, respectively.

In conclusion, measurement of leaching from formulated feeds intended for marine fish larvae indicated high rates of leaching during the first few minutes of water immersion, followed by a period of slow leaching rates before the total leaching potential is reached (Figs. 1, 2 and 3). Small feed particles have higher leaching potential than bigger one. As a total evaluation of the studied feeds, the agglomerated feed (B) showed very high leaching rates and thus low retention of water soluble components. The heat coagulated diet (A) showed half the leaching rates of hydrolysed protein compared to feed B. On the other hand, high amounts were ingested of these feeds compared to the third feed tested (Table 7). Based on mean ingestion in 39 and 49 dph old cod larvae, the agglomerated feed was the most preferred feed. The agglomerated feed has also given very good growth and survival in weaning experiment on cod and halibut compared to use of other formulated diets (unpublished data). The protein encapsulated feed (C) was the only feed that had acceptable low leaching rates. Because of the protein cross-linking technology, part of the added labelled component might have been covalently bound in the microcapsules and in that way reduced the real leaching potential of the feed. Unfortunately, the ingestion of this feed in cod larvae was very low at the tested ages of cod.

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