Deliverable Report

Deliverable No:	D11.4		**Delivery Month:**	36
Deliverable Title	Report on the nutrient retention and digestive physiology in Atlantic halibut larvae reared in RAS vs FTS			
WP No:	11		**WP Lead beneficiary:**	P17. NIFES
WP Title:	Nutrition – Atlantic halibut			
Task No:	1.1		**Task Lead beneficiary:**	P17. NIFES
Task Title:	Comparison of nutrient retention in Atlantic halibut larvae reared in RAS vs FTS			
Other beneficiaries:	P7. IMR	P15. ULL		
Status:	Delivered		**Expected month:**	36
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Objective: The objective of this Deliverable was to better understand the effects of recirculation (RAS) vs flow-through (FTS) water management systems on Atlantic halibut larval nutrient utilization.

Introduction

Atlantic halibut larvae kept in a recirculated aquaculture systems (RAS) will encounter matured water, which can affect their gut flora (Nayak, 2010) in a way that may have a positive effect on intestinal health. Gnotobiotic and conventional studies indicate the involvement of gut microbiota in nutrition and epithelial development (Nayak, 2010). Gastrointestinal bacteria may also produce essential nutrients such as vitamins and polyunsaturated fatty acids, and enzymes that can aid digestion (Ray et al., 2012). These considerations favour the hypothesis that the general nutrient absorption and retention in the fish is affected by RAS. Iodine retention must have an extra focus, since NO_3^- at levels found commonly in recirculation systems block iodide uptake by the sodium iodide symporter and may cause goiter in the fish (Morris et al., 2011; Ribeiro et al., 2011).

This study aimed to determine the potential effects of RAS vs FTS on water chemistry, water microbiology, microbial colonisation of larvae and fish performance. One group of Atlantic halibut was held in a flow-through system while another group was held in a RAS system. Larval rearing methodology, larval performance and microbiology is described in ***D17.2 Determine if RAS is a more effective protocol than FT for Atlantic halibut larvae.*** In the present task, analyses of the nutritional profile of the larvae at days post first-feeding (38 dpff), as well as the main digestive enzyme activities at 30 dpff were measured, in order to compare nutrient retention and digestive system physiology between the two fish groups.

Materials and methods

Atlantic halibut larvae were fed *Artemia* nauplii enriched with Multigain from 1 until 38 dpff. One group of larvae was held in a RAS system and the other group in a FTS in triplicate tanks. Both groups showed good survival, pigmentation and eye migration, however the growth was significantly different, final weights being 0.054±0.003 and 122±0.010 g in RAS and FTS, respectively (**Table 4**, see ***Deliverable D17.4 Comparison of feeding on-grown Artemia versus Artemia nauplii on Atlantic halibut larval performance*** for details on larval rearing and performance).

Samples (0.2-1g per analyses) were taken for nutrient analyses at the end of the experiment (38 dpff) and samples for analyses of selected digestive enzymes from the pancreas and stomach were taken at 30 dpff. The nutrients were analyzed by ISO accredited methods at NIFES (**Table 1**).

Table 1. Analytical methods: Principles and references

Analyte	Principle	Reference
Dry matter	Gravimetric after freeze drying	Hamre & Mangor-Jensen 2006
Protein	N x 6.25 Leco N Analyzer	Hamre & Mangor-Jensen 2006
Free amino acids	HPLC and post column derivatization	Srivastava et al. 2006
Total lipids	Gravimetric after acid hydrolyses	EU directive 84/4 1983
Fatty acids	Transmethylation extraction and GC/FID	Lie & Lambertsen 1991
Lipid classes	HPTLC	Jordal et al. 2007
Thiamine	HPLC	CEN 2003b
Vitamin C	HPLC	Mæland & Waagbø 1998
Vitamin A	HPLC	Moren et al. 2002
Vitamin D	HPLC	CEN 1999
Vitamin E	HPLC	Hamre et al. 2010
Sum vitamin K[3]	HPLC	CEN 2003a
Microminerals	ICP-MS	Julshamn et al. 2004
Iodine	ICPMS	Julshamn et al. 2001

Samples of larvae were collected for comparisons of main digestive enzyme activities, which were undertaken by P15. ULL. A number of 7-17 larvae at 30 dpff from each stock were pooled according to their age-size and replicate availability, after previous dissection and discard of heads and tails. The samples were completely homogenized (Ultra-Turrax T8, IKA©-Werke, Germany), in 5 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3300 x g for 3 min at 4ºC, the supernatant removed for enzyme quantification and kept at -80ºC until further analysis. Enzymatic determinations for total amylase, lipase, alkaline protease and pepsin activities were based on methods performed and described by Gisbert et al. (2009). In brief, total alkaline proteases were measured using azocasein (0.5%) as substrate in Tris-HCl 50 nmol.l-1, and pH 9. Alkaline protease activity is measured in nmoles azo dye per minute and per ml of tissue homogenate at 366 nm. Alpha-amylase (E.C. 3.2.1.1) activity was determined according to Métais and Bieth (1968) using 0.3% soluble starch. Amylase

activity was defined as the amount of starch (mg) hydrolyzed during 30 min per ml of tissue homogenate at 37ºC at 580 nm. Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30ºC using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6080 x g and 4ºC and the increase in absorbance of the supernatant read at 405 nm. Bile salt-activated lipase activity was defined as the amount (nmol) of substrate hydrolyzed per min per ml of enzyme extract (Iijima et al., 1998). Finally pepsin activity was defined as the nmol of tyrosine liberated per min at 37ºC per ml of tissue homogenate at 280 nm (Worthington, 1991).

All enzymatic activities were expressed as specific activity defined as units per milligram of protein. Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate from each pool of larvae and absorbance read using a spectrophotometer (Beckman Coulter DU800, Fullerton, CA).

Statistical analysis
Nutrient concentrations are given (mean±SD) on wet weight or as % of total fatty acids (TFA). Differences between larvae held in RAS or FTS were identified using a t-test after checking for homogenous variances by Levenes test, software Statistica (ver 12, Statsoft Inc, Tulsa, OK).

Enzyme activities are expressed as mean ± SD. Normal distribution was checked for all data with the one-sample Kolmogorov–Smirnoff test and homogeneity of the variances with the Levene test. The group data were statistically tested using one-way ANOVA followed by the Tukey test. When variances were not homogeneous, a non-parametric Kruskal-Wallis test was applied. The significance level for all the analysis was set at 5%. All the data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com).

Results and discussion

In *Artemia*, the protein concentration was 43±4 g/100g dry wt., (**Table 2**), considerably lower than that measured in copepods (63±9 g/100g), which is the natural prey of marine fish larvae (Hamre et al., 2013). A protein/N factor of 5.25 (Hamre et al., 2013) was used to calculate the protein content of *Artemia*. A similar protein level in *Artemia* was found by Karlsen *et al.* (2015). The lipid level of *Artemia* was more in line with copepod concentrations, and the micronutrient composition was similar to what has been found before (Hamre et al., 2013). Thiamine and iodine have been the only micronutrients suspected to be too low in *Artemia*, but these nutrients are probably added to Multigain (Hamre, unpublished analytical results) and are present at above requirement levels in *Artemia* in this experiment. The fatty acid composition of *Artemia* is characterized by relatively low levels of long chain n-3 fatty acids, DHA comprising only 7.7% of total fatty acids. This is just above the requirement for sustaining good growth and survival (6% of TFA, (Hamre and Harboe, 2008a)) and far below the requirement of above 13% of TFA for good pigmentation (Hamre and Harboe, 2008b).

The larval final weight was 2.3 fold higher in larvae held in FTS compared to larvae held in RAS, e.g. 0.122 and 0.054 g, respectively (**Table 3**). Both lipid, measured as total fatty acids, and protein content was also higher in the FTS larvae. This is probably a result of the size difference, since dry matter, protein and lipid levels increase with increasing size in halibut larvae and juveniles, at least up to a body weight of 4 g (K. Hamre, unpublished). There were differences between the groups in a few micronutrients, the most interesting being Vitamin K (MK6), which was higher in RAS larvae than in FTS larvae, possibly because it was produced by microorganisms in the system. Iodine was not lowered in fish from the RAS system, although recirculation is known to block iodine uptake from the water (Morris et al., 2011; Ribeiro et al., 2011). In this experiment, sufficient iodine appears to have

been supplied by the diet. Differences in fatty acid composition were small and probably biologically insignificant (**Table 4**). The concentrations of most of the free amino acids were higher in RAS larvae than in FTS larvae (**Table 5**), however, the concentration of glycine was lower in the FTS larvae. A cause of the lowered growth of the RAS larvae could therefore be a lack of glycine for protein synthesis. Glycine is a nonessential amino acid, so there may have been an inhibitory effect on glycine synthesis or increased breakdown of glycine caused by some component in the RAS system. Glycine is also an inhibitory neurotransmitter. There were no effects of the treatments on other metabolites identified by the FAA method used, such as neurotransmitters, metabolites of the Urea cycle, ammonia, ethanolamine and taurine.

The average enzymatic activities expressed per mg protein are shown in Figure 1. In general terms, activities measured are within the range of previous studies performed at 28 dpff (see Deliverable 11.3) and confirms that, as reported for other fish species, functional development of the digestive system is a well-conserved process that generally occurs within a range of body sizes regardless larval age or rearing conditions (Solovyev et al., 2016). In fact, at 30 dpff and under the present rearing conditions only a significantly higher total amylase activity was found in larvae reared under the FTS system. Although an apparent trend for a lower alkaline protease and pepsin activities seem to be present in the FTS larvae compared to those of RAS larvae, these trends are diluted when taking into account that larval total protein and larval size were significantly higher under the FTS water system rearing conditions. This higher amylase activity patterns might be due to differences in feeding rates and or growth performance since carbohydrate and glycogen contents provided by the feeding regime were the same in both rearing systems.

Conclusion

In the present experiment RAS gave considerably worse larval performance than FTS. Except for a higher level of MK6 in the RAS larvae, there was no indication that the general water conditions including bacterial community of the RAS system had any positive effect on nutrient digestion and retention capacities in the larvae. On the contrary, low free glycine levels in the larvae may have inhibited protein synthesis. The mechanism(s) by which RAS inhibited growth will be further investigated by characterization of water quality and microbial community in task 17.2.

Deviations: There were no deviations from the approved DOW.

Table 2. Nutrient composition of *Artemia* nauplii used in the experiment (mean±SD, n=2; DM, dry matter, TOH, tocopherol; MK, menaquinone; TFA, total fatty acids; PL phospholipids; WW, wet weight)

Nutrient	Unit/DM		Fatty acid	%TFA
Lipid	g/100g	18±3	14:0	1.8±0.0
Protein	g/100g	43±4	16:0	15.3±0.6
Thiamine	mg/kg	8.3±0.4	16:1n-7	1.5±0.0
Vitamin C	mg/kg	274±149	18:0	5.2±0.4
Vitamin D3	mg/kg	0.36±0.02	18:1n-9	14.0±0.1
Vitamin E (α-TOH)	mg/kg	773±45	18:1n-7	5.2±0.1
Vitamin E (β-TOH)	mg/kg	0.60±0.15	18:2n-6	5.1±0.1
Vitamin E (γ-TOH)	mg/kg	1.31±0.07	18:3n-3	22.0±0.1
Vitamin K (MK4)	mg/kg	1.3±0.2	18:4n-3	3.8±0.2
Vitamin K (K1)	µg/kg	11.7±3.2	20:4n-6 (ARA)	2.4±0.1
Total vitamin K	mg/kg	1.3±0.2	20:5n-3 (EPA)	4.0±0.2
Iodine	mg/kg	7.0±1.0	22:5n-6	2.7±0.3
Manganese	mg/kg	6.9±0.8	22:6n-3 (DHA)	7.7±1.1
Iron	mg/kg	178±25	Saturated	23.1±1.2
Copper	mg/kg	19±1	Monounsat.	22.5±0.1
Zinc	mg/kg	167±8	Sum n-3	39.4±1.2
Selenium	mg/kg	2.7±0.1	Sum n-6	10.9±0.2
			n-3/n-6	3.7±0.1
			TFA (mg/g WW)	12.9±0.4
			PL (mg/g WW)	4.5±0.1

Table 3. Weight and nutrient composition of whole body of Atlantic halibut larvae fed *Artemia* nauplii and held in recirculation (RAS) or flowthrough (FTS) water management systems for 38 days from first-feeding. (mean±SD, n=3, significant differences are shown in red (t-test); WW, wet weight; TOH, tocopherol; MK, menaquinone).

	Unit/WW	RAS	FT	p
Wet weight	g	0.054±0.003	0.122±0.010	<0.001
Dry Matter g/100g)	g/100g	14.8±0.6	16.1±1.4	0.192
Protein	g/100g	10.0±0.5	11.1±0.3	0.029
Fatty acids	g/100g	1.82±0.10	2.50±0.03	0.002
Vitamin A	mg/kg	0.97±0.12	1.00±0.10	0.725
Thiamine	mg/kg	0.83±0.06	0.70±0.10	0.116
Vitamin C	mg/kg	64±5	67±3	0.361
Vitamin E (α-TOH)	mg/kg	25±2	27±1	0.101
Vitamin E (β-TOH)	mg/kg	0.13±0.02	0.09±0.01	0.030
Vitamin E (γ-TOH)	mg/kg	0.09±0.01	0.09±0.01	0.695
Vitamin K (MK4)	µg/kg	29±3	29±5	0.796
Vitamin K (K1)	µg/kg	0.79±0.13	0.86±0.19	0.644
Vitamin K (MK6)	µg/kg	15±6	2.2±1.3	0.025
Vitamin K (MK7)	µg/kg	9.2±4.8	3.8±3.1	0.174
Vitamin K (MK8)	µg/kg	0.47±0.19	0.55±0.21	0.659
Total vitamin K	µg/kg	54±14	37±10	0.162
Iodine	mg/kg	0.44±0.04	0.29±0.01	0.004
Manganese	mg/kg	0.43±0.04	0.52±0.07	0.128
Iron	mg/kg	5.1±0.7	6.6±1.5	0.188
Copper	mg/kg	0.50±0.03	0.37±0.06	0.024
Zinc	mg/kg	24±1	20±1	0.024
Selenium	mg/kg	0.28±0.02	0.28±0.04	1.000

Table 4. Fatty acid composition in whole body of Atlantic halibut larvae fed *Artemia* nauplii and held in recirculation (RAS) or flowthrough (FTS) water management systems for 38 days from first-feeding. (mean±SD, n=3, significant differences are shown in red (t-test))

% total fatty acids	RAS	FT	p
16:00	13.2±0.4	12.1±0.1	0.007
16:1n-9	1.3±0.1	1.3±0.1	1.000
16:1n-7	1.6±0.1	1.4±0.0	0.067
18:00	7.4±0.3	7.0±0.2	0.105
18:1n-9	15.7±0.3	16.3±0.2	0.043
18:1n-7	6.2±0.1	6.5±0.1	0.021
18:2n-6	4.8±0.2	5.1±0.1	0.018
18:3n-3	12.9±0.6	14.5±0.8	0.050
18:4n-3	1.8±0.2	2.0±0.1	0.124
20:4n-6 (ARA)	6.4±0.2	6.4±0.2	0.815
20:5n-3 (EPA)	6.8±0.1	6.7±0.2	0.417
22:5n-6	2.1±0.1	2.1±0.1	1.000
22:5n-3 (DPA)	0.5±0.1	0.5±0.0	0.374
22:6n-3 (DHA)	8.9±0.2	7.6±0.3	0.002
Saturated	21.9±0.6	20.3±0.3	0.013
Monunsaturated	25.8±0.4	26.5±0.4	0.086
Sum n-3	32.5±0.8	33.0±0.9	0.475
Sum n-6	14.1±0.0	14.5±0.1	0.002
n-3/n-6	2.3±0.1	2.3±0.1	0.230

Table 5. Free amino acid concentrations in whole body of Atlantic halibut larvae fed *Artemia* nauplii and held in recirculation (RAS) or flow through (FTS) water management systems for 38 days from first-feeding. (mean±SD, n=3, significant differences are shown in red (t-test))

g/kg WW	RAS	FT	p
Aspartic acid	0.13±0.01	0.10±0.01	0.008
Threonine	0.12±0.01	0.11±0.02	0.561
Serine	0.18±0.01	0.14±0.01	0.014
Asparagine	0.03±0.01	0.05±0.02	0.073
Glutamic acid	0.25±0.02	0.22±0.02	0.116
Glutamine	0.19±0.00	0.12±0.02	0.002
Proline	0.11±0.01	0.09±0.01	0.047
Glycine	0.11±0.00	0.16±0.01	0.002
Alanine	0.33±0.02	0.30±0.04	0.329
Valine	0.14±0.01	0.09±0.02	0.011
Methionine	0.09±0.01	0.06±0.01	0.008
Isoleucine	0.10±0.01	0.07±0.01	0.018
Leucine	0.20±0.01	0.13±0.02	0.005
Tyrosine	0.16±0.02	0.10±0.01	0.006
Phenylalanine	0.14±0.01	0.09±0.02	0.012
Lysine	0.32±0.03	0.22±0.02	0.005
Histidine	0.08±0.01	0.10±0.02	0.230
Tryptophan	0.03±0.00	0.02±0.01	0.016
Arginine	0.32±0.02	0.21±0.04	0.009
SUM	3.04±0.17	2.36±0.25	0.018

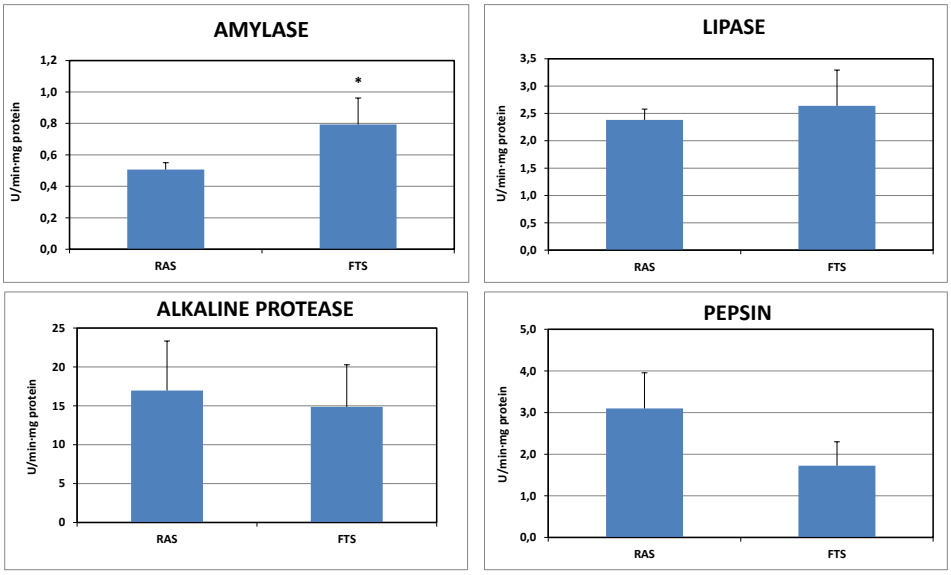

Figure 1. Activity of digestive enzymes (units/min*mg protein) in Atlantic halibut fed *Artemia* nauplii and held in recirculation (RAS) or flow-through (FTS) water management systems for 30 days from first-feeding. (mean±SD, n=3). Significant differences are shown with an asterisk (t-test).

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