New species for EU aquaculture

Deliverable Report

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Deliverable Title	Effect of probiotics on Atlantic halibut larval microbiota and survival		
WP No:	17	**WP Lead beneficiary:**	P7. IMR
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Task Title:	The effect of probiotics on Atlantic halibut larval microbiota, survival and development of an industrial protocol		
Other beneficiaries:			
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Objective: The objective of this deliverable was to investigate the effect of probiotics on Atlantic halibut larval microbiota and survival.

Introduction:

Infections with opportunistic bacteria are a severe problem in aquaculture, especially in marine larviculture used for the production of juvenile fish for commercial fish farming or for re-stocking of natural populations. While at later life stages the frequency of bacterial infections can be reduced by preventive measures such as vaccination and good management practices, the very young larvae and small fish have an immature immune system and cannot be protected by vaccination. Very often infections in larviculture are treated by antibiotics. However, this is not a sustainable practice since bacterial antibiotic resistance will develop and antibiotic-contaminated effluents are deleterious to marine ecosystems. Therefore, alternative strategies for preventing bacterial infections in fish larvae, such as pathogen-reducing probiotic bacteria or bacteriophages are highly needed.

The commercial production of Atlantic halibut (*Hippoglossus hippoglossus*) fry is currently carried out in flow through systems (FT), while there is a growing consensus that Recirculating Aquaculture Systems (RAS) would offer more stable environmental and chemical water parameters that would lead to improved larval performance. In this Deliverable, we have carried out a metagenomic analysis of the bacteriological composition of water and larvae in RAS and FT systems for both yolk sac and first feeding stages. This will provide a basis for selection of candidate probiotic bacteria for use in Atlantic halibut larviculture.

Materials and methods:

Yolk sac stage:

The yolk sac stage lasts for 43 days at 6°C in Atlantic halibut. Fertilised eggs are transferred to silos approximately 3 days prior to hatch. At this time, a salinity gradient has been established in the upper part of the silo by use of freshwater. Hatching is synchronized by use of light, which arrests hatching, and thereafter darkness to induce hatching. The salinity gradient is present during hatching and for one or two more days, depending of the buoyancy of the larvae. Recirculation is not used in this period. The silos used for water treatment and for larval rearing, are 5000 litres in volume (**Fig. 17.3.1**). Approximately 1 to 2 litres of eggs (40 000-80 000 eggs) are normally incubated in one silo, depending on the size of the egg batch. There is no feeding or any addition of organic material during this period.

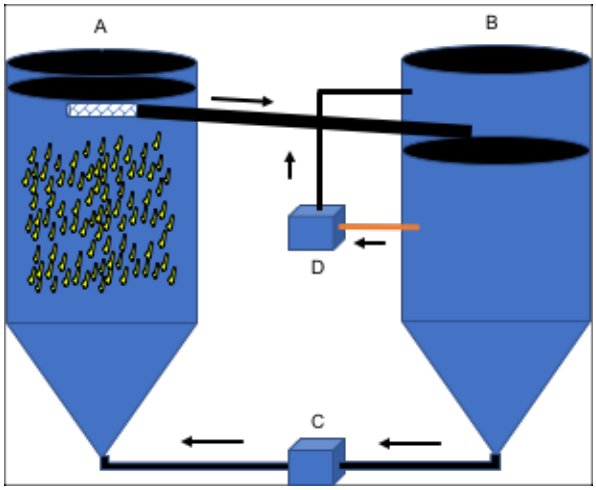

Figure 17.3.1. Illustration of the RAS used for yolk sac larvae. A= silo with larvae, B=water treatment, C= water pump including flowmeter. D= water cooler.

First feeding stage:

A RAS system from Tropical Marine Centre (TMC) (**Fig. 17.3.2**) has been used by the IMR for research on several cold-water and warm-water marine species. In this set up three first feeding tanks were connected to the system (**Fig. 17.3.3**).

Figure 17.3.2. The RAS system P5000P MARINE from Tropical Marine Centre used in the study.

Figure 17.3.3. First feeding tanks attached to the RAS system.

The first-feeding tanks were flat bottomed, with a volume of 1100 l and a water flow of 5 l per minute. Water temperature was 12 ± 0.3°C during the whole period. The tanks had shadow frames to avoid illumination of the walls and fluorescent (daylight) light sources placed 70 cm above the water surface, giving a light intensity of approximately 400 lux at the surface. The tanks had central aeration near the bottom. Dead material was removed by a siphon. The water volume that was removed daily by siphoning represented the water exchange in the RAS system. The recirculating volume was calculated to 97%. Water turbidity was created by use of dissolved clay (Sibelco, Vingerling K148, white) to an initial turbidity of 2 NTU. Approximately 10g of clay was dissolved in one liter of freshwater and added to each tank twice a day. Before the water returned to the RAS unit it was filtered to remove *Artemia* and part of the clay. The antibiotic florfenicol was added to the first four meals of *Artemia* in both FT and RAS tanks, in order to remove any pre-existing bacteria in the larval guts.

Metagenomic analysis of bacterial composition:
Sampling of bacteria from water: Water samples of 45 ml were taken from the silos or the tanks (n=3 from each unit per sampling) and centrifuged at 3200 g for 30 minutes at 4°C. The pellets were resuspended in 1 ml SLB (sucrose lysis buffer: 20 mM EDTA, pH 8.0; 400 mM NaCl; 0.75 M sucrose; 50 mM Tris-HCl, pH 9.0) and kept at – 20°C until further processing for DNA isolation.

Sampling of larvae: Individual larvae (n=4 from each unit per sampling) were transferred to 2 ml Eppendorf tubes (omitting carrying over seawater) and frozen at – 20 °C until further processing for DNA isolation. For larger larvae at the end of start-feeding, individual larvae were homogenized using a Kontes pestle, and 200 µl of the homogenates were kept at – 20 °C.

DNA was isolated from the samples by using the CTAB (hexadecyltrimethylammonium) method as described by Zhou et al 1996. Briefly, starting with 200 µl samples, 2 volumes of 1% CTAB buffer (1% CTAB, 0.75 M NaCl, 50 mM Tris pH 8, 10 mM EDTA) and proteinase K (final concentration 100 mg per ml) were added to the SLB preserved samples and incubated for one hour at 60 °C. Then SDS (final concentration 2%) was added and incubated further for one hour at 60 °C, before extraction once with phenol/chloroform, then twice with chloroform and finally precipitation of the DNA with ethanol and resuspension in 30 µl pure water.

The 16S rRNA sequencing was performed according to the Illumina protocol. Briefly, starting with 5 µl of isolated DNA, the following forward and reverse primers respectively TCGTCGGCAGCGTCA-

GATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and GTCTCGTGGGCTCGGAGATGTGTAT-AAGAGACAGGACTACHVGGGTATCTAATCC were used to amplify the V3 and V4 region of the 16S ribosomal RNA gene. The PCR products were used as templates for a second PCR amplification in order to add index sequences at the ends of the products. The generated library of 16S rRNA PCR products was normalized, pooled and sequenced with an Illumina Miseq. The sequence data were analysed using the Illumina BaseSpace sequencing Hub.

Results and discussion:
For some of the samples the first round of PCR did not generate products that gave a visible band on an agarose gel, most probably due to a low amount of template DNA. These samples were not included in further processing.

Of the 27 water samples that were analyzed, we got in average 140 000 reads per sample, varying between 83 000 and 200 000 reads, and in average 550 bacterial species were identified per sample.
Of the 69 larval samples that were analyzed, we got in average 106 000 reads per sample, varying between 718 and 163 000 reads, and in average 477 bacterial species were identified per sample.

Taxonomic level: The method gives information about the ratio between different bacteria, down to species level. However, as one moves down the taxonomic levels (phylum, class, order, family, genus and species) the uncertainty of the classification will increase, as seen for a given analyzed larval sample in **Table 1**. For this reason, we chose to discuss our findings mostly at the genus level.

Table 1 Classification statistics

Classification Statistics

Taxonomic Level	Reads Classified to Taxonomic Level	% Total Reads Classified to Taxonomic Level
Kingdom	96,235	92.43 %
Phylum	95,605	91.82 %
Class	95,186	91.42 %
Order	94,453	90.71 %
Family	94,043	90.32 %
Genus	92,168	88.52 %
Species	50,053	48.07 %

Reproducibility of the quantification:

In **Table 2** (a, b, c d, e, f) the eight most abundant bacterial genera in each sample are given. **Tables 2a, 2b** and 2 c shows the results from 3 parallels water samples taken at the same unit (silo RAS) at the same time (day 20 after hatching). As one should expect for water samples, the parallels gave very similar results, which shows that the method is highly reproducible.

Tables 2d, 2e, 2f show the results from 3 larvae taken from the same unit and time as the water samples. Here we can observe that although the results from the 3 different larvae are quite similar, the variations are greater that for the water samples. This should not be unexpected, as the individual larvae can be regarded as a closed subunit compared to a water sample.

Table 2. Results from the parallels of the water samples (a,b,c) and the larval samples (d,e,f).

a

Top Genus Classification Results V10

Classification	Number of Reads	% Total Reads
Polaribacter	20,198	17.40 %
Unclassified at Genus level	17,282	14.89 %
Microbulbifer	16,678	14.37 %
Psychroflexus	13,802	11.89 %
Thalassobius	10,698	9.22 %
Sediminicola	4,499	3.88 %
Tenacibaculum	3,782	3.26 %
Methylotenera	2,664	2.29 %

Total Genus-level Taxonomic Categories Identified: 331. This table show

Water

Classification	Number of Reads	% Total Reads
Polaribacter	16,882	15.75 %
Unclassified at Genus level	16,165	15.08 %
Psychroflexus	15,061	14.05 %
Microbulbifer	13,038	12.16 %
Thalassobius	10,914	10.18 %
Tenacibaculum	3,531	3.29 %
Sediminicola	3,465	3.23 %
Flavobacterium	2,996	2.79 %

Total Genus-level Taxonomic Categories Identified: 336. This table shov

c

Classification	Number of Reads	% Total Reads
Polaribacter	19,850	16.84 %
Unclassified at Genus level	18,016	15.29 %
Psychroflexus	15,454	13.11 %
Microbulbifer	14,249	12.09 %
Thalassobius	10,996	9.33 %
Tenacibaculum	4,213	3.57 %
Sediminicola	4,067	3.45 %
Flavobacterium	2,916	2.47 %

Total Genus-level Taxonomic Categories Identified: 349. This table shov

d

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	52,083	45.15 %
Shewanella	14,181	12.29 %
Colwellia	6,489	5.63 %
Oceanospirillum	4,409	3.82 %
Oleispira	2,327	2.02 %
Hyphomicrobium	2,012	1.74 %
Bacillus	2,010	1.74 %
Tenacibaculum	1,612	1.40 %

Total Genus-level Taxonomic Categories Identified: 381. This table shov

Larvae

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	40,045	35.03 %
Colwellia	8,518	7.45 %
Oceanospirillum	4,902	4.29 %
Hyphomicrobium	4,552	3.98 %
Tenacibaculum	4,154	3.63 %
Fluviicola	3,363	2.94 %
Oleispira	2,910	2.55 %
Shewanella	2,629	2.30 %

Total Genus-level Taxonomic Categories Identified: 392. This table show

f

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	26,566	30.14 %
Shewanella	16,326	18.52 %
Oceanospirillum	3,561	4.04 %
Colwellia	3,404	3.86 %
Bacillus	2,518	2.86 %
Hyphomicrobium	2,296	2.61 %
Novosphingobium	2,261	2.57 %
Sphingobacterium	2,191	2.49 %

Total Genus-level Taxonomic Categories Identified: 354. This table show

Comparison of water and larval samples:
As the results given in **Table 2** are from samples taken at the same unit at the same time, we can also compare the bacterial content of the water (a, b and c) with the bacterial content of the larvae (d, e, and f). Interestingly, the bacterial flora is quite different between the water and the larvae. As these samples were taken from the silo, with no feed supply, the origin of the microbes must mainly be the water, which again indicates the that the larvae constitute a microenvironment selecting for growth of certain bacteria. Similar tendencies were observed for other sampling times and units, even after the start-feeding. **Table 3** shows representative data of water and larvae samples from a RAS-tank at day 20 after first feeding (22th May).

Table 3. Results from the water and larval samples taken from the RAS tank 22th May.

Water

Top Genus Classification Results V37

Classification	Number of Reads	% Total Reads
Polaribacter	22,491	14.68 %
Tenacibaculum	20,269	13.23 %
Thalassobius	17,870	11.66 %
Unclassified at Genus level	15,782	10.30 %
Glaciecola	11,938	7.79 %
Sediminicola	7,508	4.90 %
Aliivibrio	6,885	4.49 %
Microbulbifer	5,178	3.38 %

Total Genus-level Taxonomic Categories Identified: 400. This table show

Larvae

Top Genus Classification Results L80

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	33,716	35.18 %
Aliivibrio	10,139	10.58 %
Vibrio	8,661	9.04 %
Thalassomonas	3,399	3.55 %
Leucothrix	2,513	2.62 %
Polaribacter	2,367	2.47 %
Legionella	1,982	2.07 %
Planctomyces	1,945	2.03 %

Total Genus-level Taxonomic Categories Identified: 378. This table shows

Comparison of water samples of RAS- and FT-silos:
Table 4 shows representative results of water samples taken from RAS- and FT-silos at 23 and 42 days after hatching, respectively. The results reveal a significant difference of the microflora between the RAS- and the FT-systems. The genus *Polaribacter* is dominating in the RAS system, while this geneus was not found between the eight most abundant genera of the FT system. Furthermore, it shows the ratio between the different bacteria changes with time. For example, are bacteria of the *Microbulifer* genus increasing in the FT system with time.

Comparison of larval samples from RAS- and FT-silos:
Table 5 reveals that there are significant differences between the bacterial content of the larvae of the two different systems. *Colwellia* is the most abundant genus in the RAS system, while *Marinomonas* is dominating in the FT system. *Hyphomicrobiom* is the only genus found among the eight most abundant genera in both systems.

Comparison of larval samples of RAS and FT first feeding tanks:
Table 6 shows the ratio between the bacterial genera in the larval samples in the RAS and FT tanks at day 20 after first feeding (22th May). At this stage the microbiota composition is more similar between the two systems; the *Aliivibrio* genus is the most abundant in both systems. On explanation may be that the microbial content is to a larger extent determined by the feed.

The effect of antibiotic treatment on the bacterial composition:
The larvae were treated with antibiotics after transfer from the silos to the first feeding tanks by feeding the larvae *Artemia* enriched with florfenicol. **Table 7** shows the bacterial composition of the water before (a and c) and after treatment (b and d) of the RAS tanks R2 and R3). The great effect of the antibiotic can be seen for example on the *Colwellia* which was dominating before the treatment. For other genera, like the *Polaribacter*, the effect is less.

Table 8 shows the bacterial composition of the larvae before (day 2 after first feeding (3th May), the parallels a, b and c) and after the treatment (day 20 after first feeding (22th May), the parallels d, e, and f). The antibiotic treatment here also had a great effect, for example on the *Marinomonas* content which was dominating before the treatment.

Table 4. Classification results from water samples taken from RAS- and FT-silos at 23 and 42 days after hatching.

RAS

3.april

Top Genus Classification Results V11

Classification	Number of Reads	% Total Reads
Polaribacter	16,882	15.75 %
Unclassified at Genus level	16,165	15.08 %
Psychroflexus	15,061	14.05 %
Microbulbifer	13,038	12.16 %
Thalassobius	10,914	10.18 %
Tenacibaculum	3,531	3.29 %
Sediminicola	3,465	3.23 %
Flavobacterium	2,996	2.79 %

Total Genus-level Taxonomic Categories Identified: 336. This table show

25.april

Top Genus Classification Results V18

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	42,712	12.49 %
Polaribacter	37,951	11.10 %
Thalassobius	35,291	10.32 %
Pedobacter	26,291	7.69 %
Psychrilyobacter	23,985	7.01 %
Arcobacter	14,057	4.11 %
Clostridium	12,231	3.58 %
Tenacibaculum	11,797	3.45 %

Total Genus-level Taxonomic Categories Identified: 479. This table show

FT

Top Genus Classification Results V9

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	39,645	33.07 %
Flavobacterium	10,145	8.46 %
Acidiphilium	8,087	6.75 %
Peptoniphilus	5,689	4.75 %
Psychroflexus	4,157	3.47 %
Legionella	3,647	3.04 %
Microbulbifer	2,814	2.35 %
Nitrospina	2,496	2.08 %

Total Genus-level Taxonomic Categories Identified: 448. This table show

Top Genus Classification Results V13

Classification	Number of Reads	% Total Reads
Microbulbifer	23,950	28.77 %
Unclassified at Genus level	18,497	22.22 %
Flavobacterium	6,642	7.98 %
Acidiphilium	2,969	3.57 %
Marinomonas	2,924	3.51 %
Peptoniphilus	1,862	2.24 %
Colwellia	1,722	2.07 %
Sandarakinotalea	1,594	1.91 %

Total Genus-level Taxonomic Categories Identified: 390. This table show

Table 5. Comparison of larval samples in RAS and FT silos

RAS

Top Genus Classification Results L22

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	40,045	35.03 %
Colwellia	8,518	7.45 %
Oceanospirillum	4,902	4.29 %
Hyphomicrobium	4,552	3.98 %
Tenacibaculum	4,154	3.63 %
Fluviicola	3,363	2.94 %
Oleispira	2,910	2.55 %
Shewanella	2,629	2.30 %

Total Genus-level Taxonomic Categories Identified: 392. This table shows

FT

Top Genus Classification Results L47

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	21,986	35.28 %
Marinomonas	14,439	23.17 %
Pseudoalteromonas	2,925	4.69 %
Shewanella	1,925	3.09 %
Microbulbifer	825	1.32 %
Flavobacterium	824	1.32 %
Hyphomicrobium	824	1.32 %
Sandarakinotalea	778	1.25 %

Total Genus-level Taxonomic Categories Identified: 335. This table shov

Table 4 .Comparison of larval samples of RAS and FT tanks.

RAS

Top Genus Classification Results L80

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	33,716	35.18 %
Aliivibrio	10,139	10.58 %
Vibrio	8,661	9.04 %
Thalassomonas	3,399	3.55 %
Leucothrix	2,513	2.62 %
Polaribacter	2,367	2.47 %
Legionella	1,982	2.07 %
Planctomyces	1,945	2.03 %

Total Genus-level Taxonomic Categories Identified: 378. This table show

FT

L94

Top Genus Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	31,406	23.22 %
Aliivibrio	28,745	21.26 %
Aureispira	20,483	15.15 %
Thalassomonas	6,391	4.73 %
Cobetia	6,357	4.70 %
Vibrio	4,951	3.66 %
Pseudoalteromonas	3,321	2.46 %
Sandarakinotalea	2,558	1.89 %

Total Genus-level Taxonomic Categories Identified: 394. This table shows t

Table 5. The effect of antibiotic treatment on the microbiota of the water (RAS tanks R2 and R3).

3th May

Top Genus Classification Results V22

a

Classification	Number of Reads	% Total Reads
Colwellia	25,640	14.86 %
Unclassified at Genus level	19,125	11.08 %
Thalassomonas	17,707	10.26 %
Marinomonas	17,701	10.26 %
Sandarakinotalea	12,322	7.14 %
Polaribacter	10,989	6.37 %
Flavobacterium	9,139	5.30 %
Pseudoalteromonas	8,141	4.72 %

R2

Total Genus-level Taxonomic Categories Identified: 440. This table shows t

22th May

Top Genus Classification Results V40

b

Classification	Number of Reads	% Total Reads
Thalassobius	18,546	14.10 %
Polaribacter	18,292	13.91 %
Tenacibaculum	17,553	13.35 %
Unclassified at Genus level	13,910	10.58 %
Glaciecola	9,976	7.58 %
Sediminicola	7,085	5.39 %
Microbulbifer	4,362	3.32 %
Aliivibrio	4,205	3.20 %

Total Genus-level Taxonomic Categories Identified: 354. This table show

Top Genus Classification Results

c

Classification	Number of Reads	% Total Reads
Colwellia	32,687	19.92 %
Unclassified at Genus level	21,980	13.40 %
Thalassomonas	18,707	11.40 %
Flavobacterium	12,657	7.72 %
Marinomonas	10,803	6.59 %
Polaribacter	8,211	5.01 %
Sandarakinotalea	5,919	3.61 %
Pseudoalteromonas	5,570	3.40 %

R3

Total Genus-level Taxonomic Categories Identified: 411. This table shows

Top Genus Classification Results V43

d

Classification	Number of Reads	% Total Reads
Polaribacter	19,124	14.39 %
Tenacibaculum	18,280	13.76 %
Thalassobius	17,511	13.18 %
Unclassified at Genus level	14,727	11.08 %
Glaciecola	10,080	7.59 %
Sediminicola	7,092	5.34 %
Microbulbifer	5,120	3.85 %
Methylotenera	3,537	2.66 %

Total Genus-level Taxonomic Categories Identified: 377. This table show

Table 6 The effect of antibiotic treatment on the larval microbiota (tank 5)

3th May

a

Classification	Number of Reads	% Total Reads
Marinomonas	30,850	59.97 %
Unclassified at Genus level	6,562	12.76 %
Rubritalea	2,068	4.02 %
Sandarakinotalea	1,383	2.69 %
Thalassomonas	1,008	1.96 %
Caulobacter	803	1.56 %
Vibrio	755	1.47 %
Pseudoalteromonas	589	1.14 %

b

Classification	Number of Reads	% Total Reads
Marinomonas	26,388	45.99 %
Unclassified at Genus level	8,178	14.25 %
Rubritalea	4,208	7.33 %
Vibrio	2,920	5.09 %
Tenacibaculum	1,730	3.02 %
Pseudoalteromonas	1,260	2.20 %
Thalassomonas	1,241	2.16 %
Aliivibrio	1,159	2.02 %

c

Classification	Number of Reads	% Total Reads
Marinomonas	9,215	31.99 %
Unclassified at Genus level	5,235	18.17 %
Vibrio	2,167	7.52 %
Tenacibaculum	1,322	4.59 %
Pseudoalteromonas	1,101	3.82 %
Thalassomonas	1,077	3.74 %
Aliivibrio	558	1.94 %
Shewanella	537	1.86 %

22th May

d

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	37,421	28.93 %
Cobetia	11,273	8.71 %
Vibrio	11,245	8.69 %
Thalassomonas	9,043	6.99 %
Pseudoalteromonas	6,986	5.40 %
Aliivibrio	6,606	5.11 %
Aureispira	5,289	4.09 %
Oleispira	3,768	2.91 %

e

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	31,520	29.90 %
Vibrio	13,854	13.14 %
Aliivibrio	12,711	12.06 %
Pseudoalteromonas	5,254	4.98 %
Thalassomonas	5,026	4.77 %
Cobetia	3,846	3.65 %
Aureispira	3,277	3.11 %
Marinomonas	2,931	2.78 %

f

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	21,148	17.07 %
Vibrio	18,228	14.71 %
Thalassomonas	11,609	9.37 %
Cobetia	9,560	7.72 %
Aureispira	8,734	7.05 %
Aliivibrio	6,107	4.93 %
Oleispira	4,595	3.71 %
Pseudoalteromonas	3,482	2.81 %

Summary and conclusions:

- 300-400 different bacterial genera were detected in the rearing systems
- Significant differences were detected in the microbiota composition of the RAS and FT systems: both in silos and tanks, and in the water and the larvae.
- No obvious correlation was seen between the microbiota in the water and the microbiota of the larvae.
- Antibiotic treatment had a big influence on the composition of the microbiota.

Deviations:

The original plan for this deliverable was to perform *in vitro* challenge trials with probiotic candidates for use in larval rearing systems. However, addition of probiotics has proven to be problematic in cold-water systems and an alternative strategy for finding candidates was chosen, based on new and more specific molecular methods (metagenomics) that have recently become available for characterization of bacteriological environments both within ecosystems, water and individual larvae. Further, as interest for using RAS in marine aquaculture is increasing, we tested how the microbiome in these systems develops in yolk sac and first feeding larvae, as an alternative way of establishing a probiotic effect.

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