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# Survival and quality of halibut larvae (*Hippoglossus hippoglossus* L.) in intensive farming: Possible impact of the intestinal bacterial community

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ABSTRACT

The high mortality commonly observed during the early life stages of intensively reared halibut (*Hippoglossus hippoglossus* L) is believed to be caused by e.g. opportunistic bacteria. However, the impact of particular bacterial species is poorly defined and still remains disputable. The study describes the bacterial diversity in the gastrointestinal tract of halibut larvae in a large number of incubators at a commercial production site. The overall success of larvae was found to be highly variable and analysis of the gut microbiota revealed high variation of the cultivable part as well as the bacterial community of surface sterilised larvae analysed by denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16s rDNA products. Analysis of the bacterial community of unfed yolk sac larvae revealed higher diversity than previously reported, with *Marinomonas*, *Marinobacter*, *Aeromonas* and *Shewanella* dominating the community structure. There are indications that *Marinomonas* is found only in the overall most successful first feeding larvae of the period where the *Vibrio* group dominated the bacterial community together with *Shewanella*. *Vibrio wodanis* was identified as a part of the bacterial community of feeding larvae that yielded the poorest overall success of the period. α-*Proteobacteria*, not previously reported in halibut, were also found as a part of the bacterial community of first feeding larvae. The diverse bacterial community was only partly reflected in the cultivable part which, however, may reflect the dominating bacterial groups of the highly heterogeneous bacterial community of larvae in the production system as a whole. The bacterial community of the *Artemia* was found to be highly variable in different samples collected through the period. Only a small part of the different groups observed in the bacterial community of surface sterilised larvae was reflected in the cultivable part which was dominated by highly variable groups in different samples of *Artemia*. Also, the numbers of cultivable bacteria were found to positively correlate with jaw deformation of unfed yolk sac larvae as well as incomplete metamorphosis of feeding larvae.



## 1. Introduction

The production of larvae has been the main obstacle for the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) farming throughout the world (Olsen et al., 1999; Jensen et al., 2004; Kvale et al., 2007; Le Vay et al., 2007). High mortalities due to constraints in morphology and physiology may result in a total collapse in the production of individual batches, with an overall general survival reported to be in the range of 0–10% (Olsen et al., 1999). The bacterial load and contamination of the production system with opportunistic bacteria may be an important reason for an overall poor survival of larvae (Olafsen, 2001). Environmental bacterial concentrations of $10^6$ CFU $ml^{-1}$ have commonly been reported, of which only a small fraction being found to be cultivable on non-selective agar media (Hugenholtz and Pace, 1996; Jensen et al., 2004; Hovda et al., 2007). However, the impact of any specific bacterial species or composition of species is poorly defined and still remains disputable. The intestinal environment of the yolk sac larvae of halibut has been found to possess a distinct and specific normal flora regardless of geographical origin (Jensen et al., 2004). The microbiota of feeding larvae, on the other hand, has been found to reflect the microbial composition of the live feed offered during the first weeks (Verner-Jeffreys et al., 2003b; Jensen et al., 2004; Korsnes et al., 2006). The microbiota has by numerous authors been found to affect the quality and survival of unfed as well as feeding larvae and an unpredictable development of the bacterial community has been suggested to follow actions carried out in order to keep the numbers of bacteria low by various forms of disinfection (Olafsen, 2001).

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Organic debris from dead larva and feeding live feed with the addition of live micro-algae to provide the environmental conditions considered needed for normal larval growth, thus provides a reservoir of organic material that has been reported to support and stimulate growth of opportunistic bacteria (Korsnes et al., 2006).

*Vibrio* bacteria have been found to dominate the bacterial community as a normal flora of fish and seawater species in brackish and costal waters and a number of *Vibrio* species have been recognised as opportunistic pathogens in many marine fish species (Colwell and Grimes, 1984; Egidius, 1987; Hjelm et al., 2004). Furthermore, *Vibrio* spp. have commonly been found to represent the main flora of the *Artemia* used during first feeding of halibut larvae (Verner-Jeffreys et al., 2003b). The natural filtering activity of *Artemia* nauplii will result in large amounts of bacteria in addition to the risk of live feed being contaminated with undesirable and pathogenic species (Makridis et al., 2000; Savas et al., 2005).

The specific immune system of halibut is not mature at the first stage of feeding and the larvae therefore have to rely on the innate, unspecific response of the immune system for their protection against environmental bacteria (Magnadottir et al., 2005; Lange et al., 2006). The microbial community of fish feeds has been found to quantitatively and qualitatively affect the intestinal microflora of larvae (Savas et al., 2005). Bacteria present in the live feed and larval environment may thus represent a major stress on the larvae at this stage when the larvae are found to be extremely vulnerable against effects of environmental parameters (Olsen et al., 1999). A proper balance of the bacterial community and a general understanding of the microbial community associated with rearing of marine larvae is therefore required for an improvement in rearing performances (Skjermo and Vadstein, 1999; Verner-Jeffreys et al., 2003b; Ritar et al., 2004).

The objective of the present study was to get a better understanding of the intestinal bacterial community of halibut larvae in intensive farming and how this may affect survival and larval quality measured as success of metamorphosis.

## 2. Materials and methods

### 2.1. Production methods and evaluation of larval success

The study was carried out in a hatchery in Iceland, Fiskey Ltd., producing annually between 300 000 and 750 000 halibut juveniles, representing 35–55% of the annual global production of halibut larvae. A continuous production with manipulation of the photoperiod results in three distinct groups spawning at advanced and delayed periods compared to the normal group. The overall success of individual spawning groups has been shown to vary considerably between groups and years. The normal spawning group (N) represents the largest group, hence supplying a large part of the annual production of larvae at Fiskey Ltd. Samples were collected from all production units of one spawning group (N) during August 2006 through October 2006, with the first eggs brought in during May 2006 and the last larvae transferred to weaning at approximately 115 days post hatch (dph) in October 2006.

The fertilized eggs were kept in 0.25 $m^3$ tanks at 5.0–5.3 °C for 14 days before hatching. The eggs were then transferred to 10 $m^3$ silos where the eggs hatched and the yolk sac larvae were held at 5.0–5.3 °C for ~50 days prior to transferring to first feeding tanks (3.5 or 7.0 $m^3$) with the addition of enriched 24 h *Artemia franciscana* nauplii (Great Salt Lake, Utah, USA) two times a day for a period of ~65 days at 11 °C when weaning onto formulated feed was started.

Survival and quality of unfed yolk sac larvae was estimated at transfer to first feeding tanks at 50 dph (48–52 dph). Larval survival was calculated as the percentage of surviving larvae at the end of the period in relation to L of eggs transferred to each silo (one L is estimated to hold ~40 000 eggs). The percentage of jaw deformation of yolk sac larvae (% gaping) was estimated by counting the number of gaping larvae from a pool of ~150 larvae from each incubator (silo) at the end of the period. Survival through first feeding was calculated after all juveniles had been transferred to weaning tanks. This was estimated as the percentage of normal larvae originally transferred to the first feeding tanks. The total number of larvae was the sum of juveniles transferred to weaning tanks plus the number of dead larvae, which were counted on a daily basis from each individual tank throughout the first feeding period. The success of metamorphosis was estimated at the onset of weaning and calculated as the percentage of juveniles with incomplete eye migration or poor pigmentation.

Growth of larvae was estimated by measuring the weight of ~150 (50 dph) to ~15 (100 dph) larvae from individual tanks at approximately weekly intervals throughout the first feeding period. The larvae were dried for 4–5 days at 70–75 °C before weighing (dry weight) and the dry weight was found to correlate with the wet weight of the larvae which 87–89% water content of larvae up to 15 g and 83–85% water content of over 40 g larvae.

### 2.2. Sampling and treatment of samples

Samples of unfed yolk sac larvae were collected immediately before the onset of feeding (~50 dph) and after 7, 17 and 36 days of feeding (57, 67 and 87 dph, respectively). Sampling at 57 dph was selected in relation to elevated numbers of dead larvae observed in all tanks after 6–12 days in feeding (56–62 dph). Sampling at 67 dph was carried out in relation to a variable percentage of non-feeding larvae occasionally observed in individual production units at ~70 dph (results not shown). The sampling at 87 dph was expected to reflect the intestinal bacterial community of larvae at a period in the development when further death of larvae was in general not to be expected. Larval samples were collected from the incubators and at the same dph in every production unit. Unfed yolk sac larvae were sampled from a total of 9 production units and first feeding larvae from a total of 14 production units in addition to numerous samples of enriched *Artemia* collected approximately weekly throughout the period.

Samples were transported to the laboratory and processed within 4 h post collection.

Isolation of bacteria from the larval intestines was carried out using a modification of the method of (Muroga et al., 1987) where a group of ~120 (50 dph) to ~25 (87 dph) larvae were killed with an overdose of Hypnodil (51 μg $ml^{-1}$) prior to immersion in 0.1% benzalkonium chloride solution for 30 s to remove surface bacteria (Grisez et al., 1997). The larvae were then rinsed three times in sterile NaCl solutions (2%) before enumeration and subsequent homogenization in a tenfold dilution of peptone-seawater (PS; 0.1% w/v Bacto peptone dissolved in 1.22 μm filtered 70% aged seawater and pH adjusted to 8.6). Homogenization was carried out at 133 rotations per sec for 60 s with 10 s bursts at 10 s intervals (Ultra Turrax T25; IKA). The live feed (*Artemia*) was harvested into 150 μm mesh net and rinsed under running tap water for two min before homogenization in a tenfold dilution of PS as described above.

### 2.3. Analysis of the bacterial community of surface sterilised larvae

The bacterial community of the larval intestines was analysed by cultivation on non-selective nutrient media (colony forming units, CFU) as well as by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR–DGGE). Serial dilutions were prepared in PS and 100 μl aliquots plated in duplicate unto MA (Marine Agar; Difco) and TCBS (Thiosulphate Citrate Bile salts Sucrose; Difco) agar plates for the measurement of total marine heterotrophic bacteria and numbers of presumptive *Vibrio* bacteria, respectively (duplicate samples). The plates were incubated at 15 °C for 5–7 days prior to enumeration of the colonies and a random selection of isolates for further characterization. Results are expressed as number of CFU $larvae^{-1}$. From each sample, twelve randomly selected colonies were picked from MA plates containing 25–250 CFU $plate^{-1}$ and sub-cultured to ensure purity of the isolates before presumptive identification through morphological and biochemical

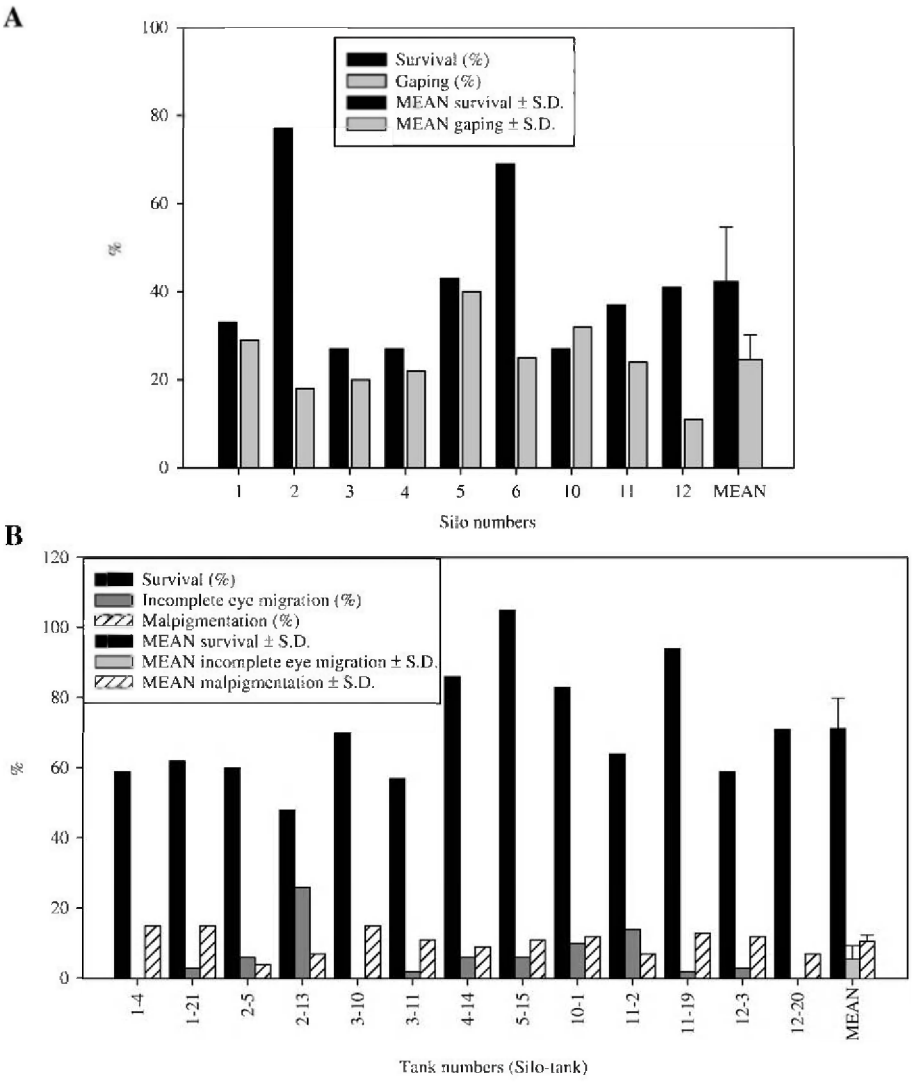


**Fig. 1.** Survival and quality of unfed yolk sac larvae at 50 dph (A) and first feeding larvae at ~115 dph (B), with tank labelling indicating the silo-origin of feeding larvae, dark bars represent % survival and grey bars representing either % gaping (A) or % larvae with incomplete eye migration (B). Striped bars represent % larvae with deviation in pigmentation (B). Mean values ± S.D. in all tanks during the period are also shown. A total collapse of larvae occurred early after the onset of feeding in tanks 6–12, which is excluded in B. Statistical analysis revealed a significant difference in the success of larvae in various tanks ($P < 0.05$).

characterization using eight distinct tests: Gram reaction; motility; cytochrome-oxidase and catalase reactions; oxidative or fermentative dissimilation of glucose; growth in the presence or absence of NaCl (2% w/v) as well as sensitivity against Novobiosin (0.2% w/v) and the O/129 *Vibrio* static compound (2,4-Diamino-6,7-diisopropyl pteridine) using the disc diffusion method (150 μg disc$^{-1}$). A variable number of isolates from individual samples grew poorly and expressed indistinct response in the test applied. These isolates were grouped as unidentified.

The subsequent grouping of a total of 540 randomly selected bacterial isolates resulted in 24 main groups. Representative isolates (8–20% of the total number of isolates within each group, depending on the size of individual groups) were selected from each group and characterized further using the API 20E system (BioMérieux). The isolates were furthermore identified by partial 16S rDNA sequencing using *Archaea* sequential primer, 805.R (Barns et al., 1994) with ABI 377 DNA sequencer by using a BigDye terminator cycle sequencing ready reaction kit according to the instructions of the manufacturer (PE Applied Biosystems) and grouped with 98% limits in the Sequencer programme (work carried out by Matis-Prokaria Ltd.). The BLAST alignment program was then used to match these sequences with known entries in the GenBank and linked sequence databases, in order to further identify the strains.

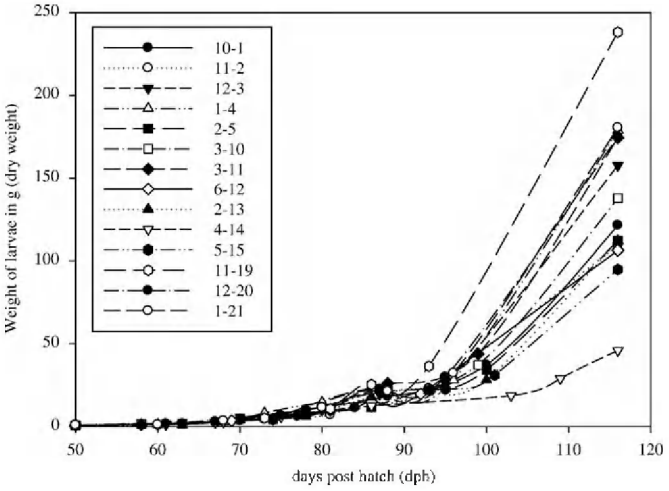


**Fig. 2.** Dry weight of larvae (g) in individual production units throughout the first feeding period studied. The lines show the weight of larvae in individual tanks at various samplings throughout the first feeding period, with each line extrapolated to 115 dph from the last sampling carried out in individual tank units (90–108 dpf). Labelling indicates the silo-tank origin of larvae.

**Table 1A**
Numbers of cultivable bacteria (A) in unfed yolk sac larvae sampled immediately before the onset of feeding (50 dph) and at selected time points through the first weeks of feeding (57–87 dph)

| Tank | 50 dph | 57 dph | 67 dph | 87 dph |
|---|---|---|---|---|
| 10-1 | n.d. | $2.3*10^5$ (b) | $1.2*10^5$ (c) | $1.2*10^5$ (a) |
| 11-19 | $1.5*10^4$ (b) | n.d. | **$2.0*10^6$**(b) | $2.0*10^5$ (a) |
| 11-2 | $1.5*10^4$ (b) | **$3.2*10^7$**(a) | **$1.7*10^6$**(b) | $9.0*10^5$ (a) |
| 12-3 | $2.0*10^3$ (b) | $3.3*10^4$ (b) | **$1.1*10^6$**(b) | **$3.0*10^6$**(a) |
| 12-20 | $2.0*10^3$ (b) | $5.0*10^4$ (b) | **$4.2*10^6$**(a) | **$1.2*10^6$**(a) |
| 1-21 | $1.1*10^3$ (b) | $7.7*10^5$ (b) | $2.2*10^5$ (c) | $3.1*10^5$ (a) |
| 1-4 | $1.1*10^3$ (b) | $4.0*10^5$ (b) | $2.2*10^5$ (c) | $2.8*10^5$ (a) |
| 2-13 | $3.9*10^5$ (a) | $3.8*10^5$ (b) | $1.1*10^5$ (c) | $5.3*10^5$ (a) |
| 2-5 | $3.9*10^5$ (a) | $4.4*10^5$ (b) | $1.8*10^5$ (c) | $1.1*10^5$ (a) |
| 3-10 | $2.1*10^3$ (b) | $4.1*10^5$ (b) | **$1.0*10^6$**(b) | **$4.1*10^6$**(a) |
| 3-11 | $2.1*10^3$ (b) | $1.7*10^4$ (b) | **$2.0*10^6$**(b) | **$1.7*10^6$**(a) |
| 4-14 | $3.0*10^1$ (b) | $1.4*10^5$ (b) | $4.2*10^5$ (c) | $2.4*10^5$ (a) |
| 5-15 | $1.8*10^4$ (b) | **$1.2*10^6$**(b) | $2.1*10^5$ (c) | $1.4*10^5$ (a) |
| 6-12 | $6.1*10^1$ (b) | **$2.9*10^6$**(b) | $3.8*10^5$ (c) | n.d. |
| **Mean** | **$6.5*10^4$** | **$3.0*10^6$** | **$1,0*10^6$** | **$9.8*10^5$** |
| **±S.D.** | **±$1.5*10^5$** | **±$8.7*10^6$** | **±$1.2*10^6$** | **±$1.2*10^6$** |

n.d., not detected.
Different superscript letters within the same column indicate significant difference in bacterial counts in larvae from individual tanks at each sampling date ($P<0.05$).
The tables show the numbers of CFU larvae$^{-1}$ with mean values±S.D. Bacterial counts above $10^6$ are marked in bold.

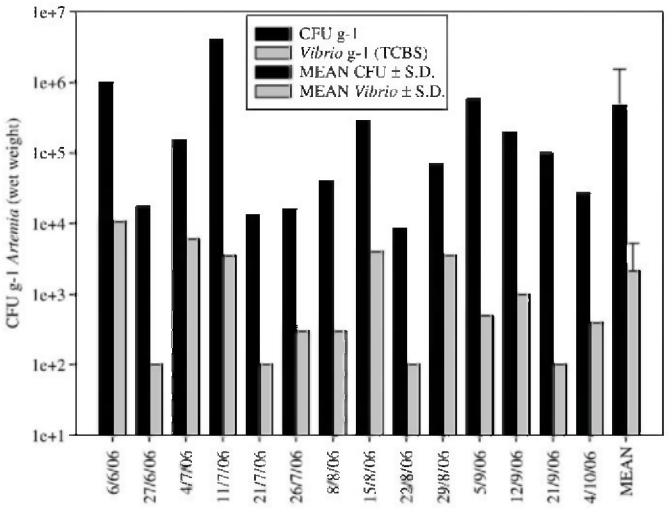


**Fig. 3.** Numbers of cultivable bacteria (CFU) and presumptive *Vibrio* bacteria in *Artemia* sampled throughout first feeding period of the group of larvae studied. The figure shows bacterial numbers in each g of *Artemia* (wet weight).

The bacterial 16S rDNA profiles of surface sterilised larvae were analysed using PCR–DGGE. DNA from 300 µl of homogenized samples was extracted using a PureGene DNA Extraction Kit (Gentra) with some modifications. Samples were centrifuged at 16000 ×*g* for 3 min and 300 µl of cell lysis solution then added to the precipitate, vortexed and incubated at 80 °C for 5 min. 1.5 µl of RNase A solution (4 mg ml$^{-1}$) were then added to each tube, mixed by inversion and the samples incubated at 37 °C for 15 min. After cooling on ice for 1 min, 100 µl of protein precipitation solution were added to each tube, mixed thoroughly for 20 s and the samples were then centrifuged for 3 min (13000 ×*g*). The supernatant was then carefully transferred into a clean tube, 300 µl of 2-propanol added and the solution mixed by inverting the tube 50 times prior to centrifugation at 13000 ×*g* for 3 min. The supernatant was then poured off and the precipitate dried on paper for 20 min before washing in 300 µl of 70% ethanol by

**Table 1B**
Numbers of *Vibrio* bacteria in unfed yolk sac larvae sampled immediately before the onset of feeding (50 dph) and at selected time points through the first weeks of feeding (57–87 dph)

| Tank | 50 dph | 57 dph | 67 dph | 87 dph |
|---|---|---|---|---|
| 10-1 | n.d. | $1.5*10^5$ (a) | $8.4*10^3$ (a) | $1.2*10^4$ (a) |
| 11-19 | $3.0*10^0$ (a) | $9.2*10^3$ (b) | $3.8*10^4$ (a) | $2.3*10^4$ (a) |
| 11-2 | $3.0*10^0$ (a) | $9.9*10^4$ (a) | $1.3*10^5$ (a) | $1.4*10^5$ (a) |
| 12-3 | $3.0*10^0$ (a) | $6.2*10^2$ (b) | $1.9*10^5$ (a) | $8.2*10^4$ (a) |
| 12-20 | $3.0*10^0$ (a) | $1.8*10^3$ (b) | $2.1*10^5$ (a) | $1.6*10^4$ (a) |
| 1-4 | $3.0*10^0$ (a) | $2.3*10^3$ (b) | $1.5*10^4$ (a) | $4.3*10^5$ (b) |
| 1-21 | $3.0*10^0$ (a) | $7.7*10^3$ (b) | $8.8*10^3$ (a) | $1.2*10^5$ (a) |
| 2-13 | $1.8*10^3$ (b) | $3.1*10^4$ (b) | $3.0*10^3$ (a) | $6.1*10^3$ (a) |
| 2-5 | $1.8*10^3$ (b) | $2.8*10^3$ (b) | $2.1*10^3$ (a) | $3.3*10^3$ (a) |
| 3-10 | $3.0*10^0$ (a) | $1.6*10^4$ (b) | **$2.3*10^6$**(b) | $1.1*10^5$ (a) |
| 3-11 | $3.0*10^0$ (a) | $5.7*10^2$ (b) | $4.2*10^4$ (a) | $9.2*10^4$ (a) |
| 4-14 | $3.0*10^0$ (a) | $3.1*10^3$ (b) | n.d. | $2.4*10^4$ (a) |
| 5-15 | $1.2*10^3$ (b) | $1.1*10^4$ (b) | $1.4*10^4$ (a) | $6.1*10^3$ (a) |
| 6-12 | $3.0*10^0$ (a) | $2.0*10^5$ (a) | $1.5*10^4$ (a) | n.d. |
| **Mean** | **$3.7*10^2$** | **$3.9*10^4$** | **$2.3*10^5$** | **$8.2*10^4$** |
| **±S.D.** | **±$7.0*10^2$** | **±$6.5*10^4$** | **±$6.3*10^5$** | **±$1.1*10^5$** |

n.d., not detected.
Different superscript letters within the same column indicate significant difference in bacterial counts in larvae from individual tanks at each sampling date ($P<0.05$).
The tables show the numbers of CFU larvae$^{-1}$ with mean values±S.D. Bacterial counts above $10^6$ are marked in bold.

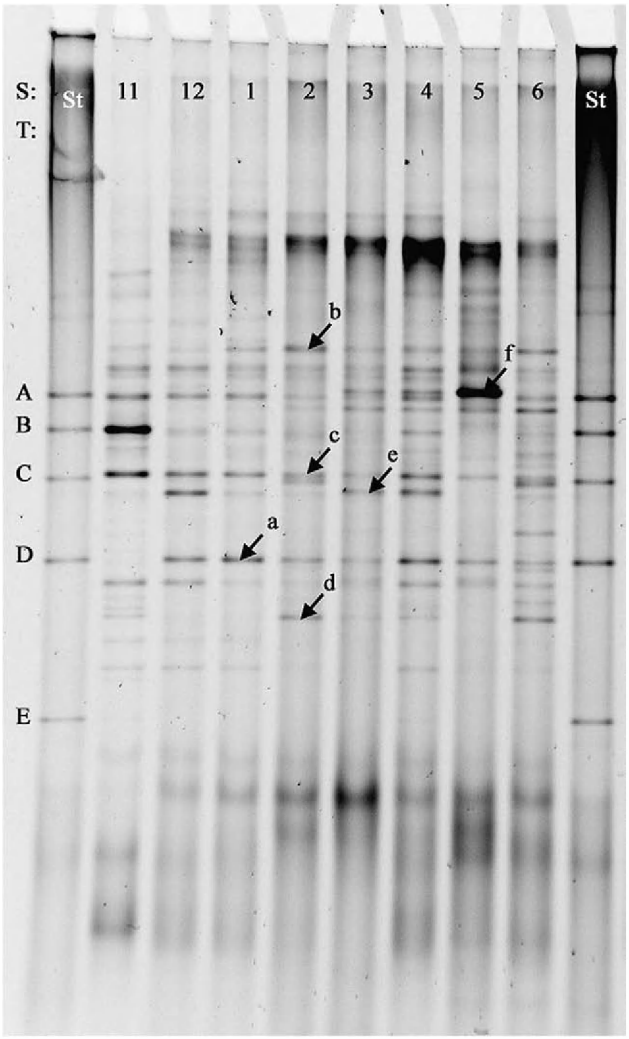


**Fig. 4.** DGGE profiles of the intestinal bacterial community in a pool of ~120 unfed yolk sac larvae from individual incubators (S) at 50 dph. Relative mobility standards A–E (St) are also shown. Labelled arrows indicate bands that were excised from the gel and identified by sequence analysis. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787).

inverting the tube a few times. After centrifugation at 13 000 ×g for 2 min, the supernatant was carefully poured off and the open tubes then dried on clean paper for 10 min before adding 50 µl of DNA hydration solution to each tube. Samples were then incubated at 65 °C for 1 h and left overnight at room temperature with gentle shaking. The V4 part of the bacterial 16S ribosomal RNA gene was amplified in PCR with primers 515F-GC and 806R (TAC Copenhagen) as described by (Griffiths et al., 2001) with slight modifications. The PCR reaction mixture consisted of 10 mM Tris pH 8.8, 50 mM KCl, 1,5 mM $MgCl_2$, 0,1% Triton X-100, 0.1 µM dNTP, 0.8 µM of each primer and 2.4 units of TEG polymerase (Matis-Prokaria Ltd.) per 50 µl reaction volume. The PCR amplification was carried out in a PTC-200 thermal cycler (MJ Research Inc. Watertown, USA) with an initial denaturation step of 94 °C for 5 min followed by 30 cycles of alternating denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension for 10 min at 72 °C. Reaction products were routinely examined on 2% agarose gels, visualised by staining with 1.0 mg l-1 ethidium bromide. The primer combination amplifies a ~291 bp fragment suitable for DGGE analysis performed essentially as described by (Muyzer et al., 1993). The amplified DNA was then loaded onto a 40–70% denaturing gradient of urea-formamide in a 6% acrylamide-bis gel and electrophoresed on a Dcode DGGE system (Dcode, BioRad) at 60 °C under 60 V and 20 mA for 14 h. A relative mobility standard was used, consisting of PCR products from laboratory subcultures and type strains with various GC content and hence resolving in different regions of the gel. The standard was represented by A: *Pseudoalteromonas elyakovii* (99% similar to GenBank accession number AB000389), B: *Vibrio splendidus* (100% similar to GenBank accession number AJ874364), C: *Marinovum algicola* (DSM 10251), D: *Shewanella baltica* (99% similar to GenBank accession number CP000891) and E: *Streptomyces* sp. (99% similar to GenBank accession number EU 257269), which was included because of high GC content. The PCR-primers were selected in order to specifically amplify the 16S rDNA gene, and at the same time to avoid the co-amplification of 18S DNA (Jensen et al., 2004) from the larvae or the live feed. DGGE bacterial community profiles were highly reproducible. Gels were stained for 15 min in SYBR Gold nucleic acid stain (Invitrogen), resolving a clear view of bacterial isolates present in lower relative quantities that were not visible by staining with ethidium bromide. The gels were then imaged under UV light using an InGenius LHR gel imaging system (Syngene). Images were recorded with GeneSnap software (Syngene) and analysed with Gene Tools software (Syngene).

For characterization of amplified products, a sterile pipette tip was used to excise a small core, approximately 0.5 mm in diameter, from the centre of bands of interest while under the UV trans-illuminator. These gel slices were then placed into 150 µl sterile milliQ water, crushed with a sterile pipette tip, shaken for 10 min and then centrifuged at 10 000 ×g for 2 min to remove gel fragments. The supernatant was used as template DNA for another PCR reaction, the quality of the re-amplified bands was then checked by DGGE and the 16S rDNA fragment was sequenced. The products were identified by sequence analyses, alignment with the BLAST program to closest matches in GenBank and related databases, and further phylogenetic identification using the Ribosomal Database Project II programme (Wang et al., 2007).

### 2.4. Statistical analysis

Data were analysed using SigmaStat® release 3.5 (Systat Software, Inc. CA 94804-2028 USA). Normality of the data distribution was

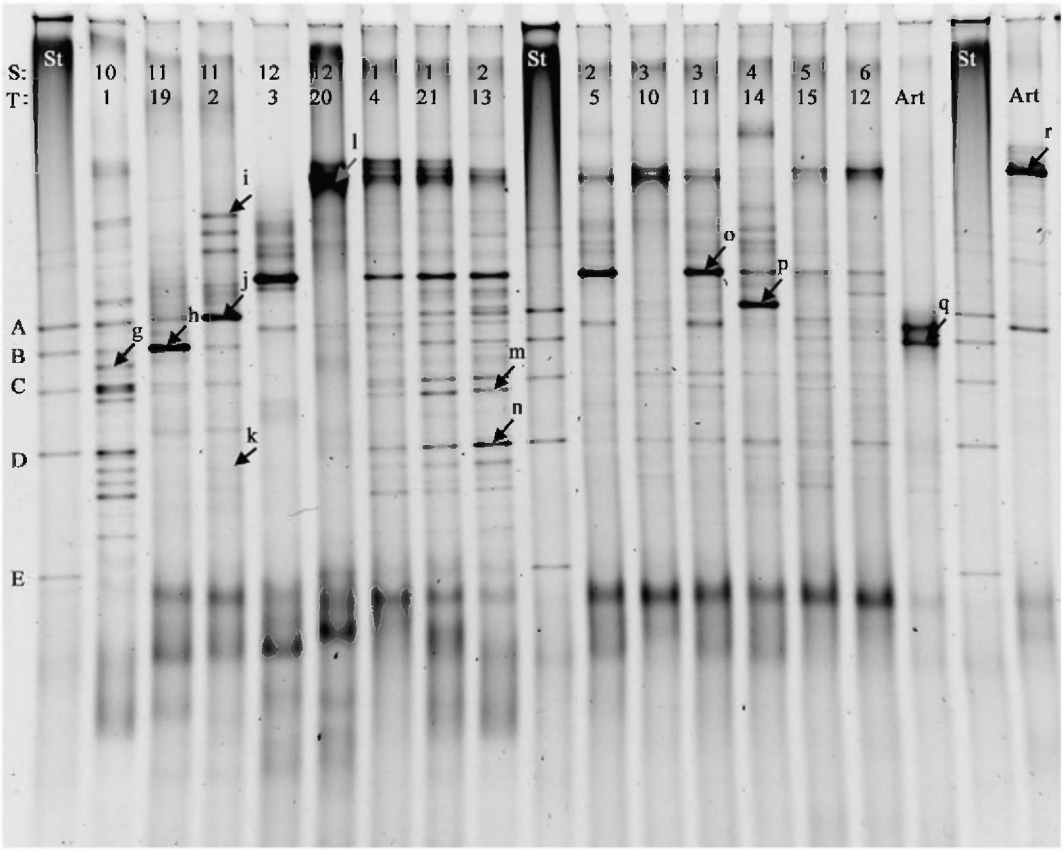


**Fig. 5.** DGGE profiles of the intestinal bacterial community in a pool of 80–100 larvae from individual tanks at 57dph. Also shown are profiles of the live feed (Art) and relative mobility standards A–E (St). Tank labelling (T) indicates the silo-origin (S) of larvae. Labelled arrows indicate bands that were excised from the gel and identified by sequence analysis. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787).

analysed by Normality Test (Kolmogorov–Smirnov). Survival of larvae was analysed by comparing the number of surviving, dead and total number of larvae in individual production units using the Chi-square test. Growth of larvae in individual production units was analysed by comparing the confidence interval of the extrapolated weight of larvae at 115 dph, using the mean values and standard error of the mean. A Pearson correlation was used to analyse the relationship between bacterial numbers and larval survival as well as metamorphoses characteristics used to evaluate the overall success of larvae. A regression analysis was used to analyse the relationship between parameters found to be linearly related.

## 3. Results

### 3.1. Survival, quality and growth of larvae

The success of the spawning group examined turned out to be below the average success commonly experienced at Fiskey Ltd., with ~200000 larvae weaned and only 160000 individuals developing to fingerlings. The survival (%) and gaping (%) of yolk sac larvae in individual incubators immediately before transfer to first feeding (50 dph) is shown in Fig. 1A and B. It shows the survival (%) and success of metamorphosis (%) of first feeding larvae in individual tanks, evaluated at the end of the period (~115 dph). Daily registration of dead larvae in individual production units revealed elevated numbers of dead larvae in all tanks between 4 and 12 days after the onset of feeding (results not shown). Two distinct peaks in the mortality curve are commonly observed at Fiskey Ltd., at 55–59 dph and 60–63 dph. Up to 5000 larvae were commonly found dead $day^{-1}$ in the least successful production units and up to 2000 larvae $day^{-1}$ in the most successful production units. The relative numbers of surviving larvae were found to vary significantly when individual production units were compared ($P<0.05$).

Growth of larvae in individual tanks throughout the first feeding period is shown in Fig. 2. The growth curves shown are extrapolated to 115 dph that is the approximate time of transferring the larvae to weaning. Larval growth was found to differ significantly in individual first feeding tanks ($P<0.05$). However, correlation was neither found between growth and survival of feeding larvae ($R^2<0.1$) nor the quality of the larvae measured as incomplete eye migration ($R^2=0.16$) or poor pigmentation ($R^2=0.20$).

### 3.2. The cultivable part of the bacterial community

A considerable variation in bacterial counts was found in unfed yolk sac larvae from various production units, with numbers ranging from $6.1*10^1$–$3.9*10^5$ CFU $larvae^{-1}$ (Table 1A). The number of bacteria growing on TCBS agar (presumptive *Vibrio* bacteria) was also found to vary considerably, ranging from $3.0*10^0$–$1.8*10^3$ *Vibrio* $larvae^{-1}$ (Table 1B). The highest numbers of CFU and *Vibrio* were found in larvae from the most successful production unit (Silo 2), with CFU numbers significantly higher than in larvae from other production units of the period ($P<0.05$). The survival of unfed yolk sac larvae was found to positively correlate with the numbers CFU $larvae^{-1}$ ($R^2=0.32$) as well as the numbers of *Vibrio* $larvae^{-1}$ ($R^2=0.49$). Bacterial numbers were not found to correlate with gaping of unfed yolk sac larvae ($R^2<0.01$). However, excluding the most successful production unit of the period revealed a positive correlation between jaw deformation and the numbers of *Vibrio* bacteria ($R^2=0.60$) as well as numbers of CFU $larvae^{-1}$ ($R^2=0.36$) in the unfed yolk sac larvae.

The same overall variation was observed during the first week in feeding, with numbers ranging from $1.7*10^4$–$3.2*10^7$ CFU $larvae^{-1}$ and $5.7*10^2$–$2.0*10^5$ *Vibrio* $larvae^{-1}$ at 57 dph. The numbers of cultivable bacteria in larvae at later samplings were found to be less variable, with $1.1*10^5$–$4.1*10^6$ CFU $larvae^{-1}$ found in larvae from various production units at 87 dph.

Higher counts in unfed yolk sac larvae were found to positively correlate with poor quality of larvae after the onset of feeding, measured as incomplete eye migration ($R^2=0.74$). However, the correlation was negative with respect to poor pigmentation of first feeding larvae from the corresponding incubators ($R^2=0.52$).

**Table 2**
Groups identified as a part of the bacterial community of halibut larvae

| Band | | BLAST identification | Division (similarity) | GenBank accession numbers |
|---|---|---|---|---|
| a | ■ | *Pelomonas aquatica* | β-Proteobacteria (98%) | AM501435 |
| | | *Shewanella* spp. | γ-Proteobacteria (99%) | EU075118 |
| b | ■ | *Marinomonas* spp. | γ-Proteobacteria (100%) | EU052766 |
| c | ■ | Uncultured *Aeromonas* spp. | γ-Proteobacteria (100%) | EU887532 |
| | | Uncultured *Citrobacter* spp. | γ-Proteobacteria (99%) | EU704221 |
| d | ■ | Uncultured beta proteobacterium | β-Proteobacteria (100%) | AY225604 |
| | | *Citrobacter* spp. | γ-Proteobacteria (99%) | EU073953 |
| e | ■ | Uncultured *Ralstonia* spp | β-Proteobacteria (100%) | EU706276 |
| | | *Ralstonia* spp. | β-Proteobacteria (93%) | AB441670 |
| f | ■ | *Marinobacter* spp. | γ-Proteobacteria (99%) | EU052740 |
| g | □ | *Stenotrophomonas* spp. | γ-Proteobacteria (96%) | EF620472 |
| h | □ | *Vibrio* spp. | γ-Proteobacteria (100%) | EU655423 |
| i | □ | *Acinetobacter* spp. | γ-Proteobacteria (100%) | EU848481 |
| j | □ | *Hippoglossus hippoglossus* 12S | 12S rRNA (97%) | AM749125 |
| | | *Pseudoalteromonas* spp. | γ-Proteobacteria (100%) | EU489558 |
| k | □ | Uncultured *Aquicella* spp. | γ-Proteobacteria (96%) | EU012271 |
| | | *Vibrio* spp. | γ-Proteobacteria (99%) | EU655427 |
| l | □ | *Mesonia* spp. | Flavobacteria (99%) | DQ873771 |
| m | □ | *Ralstonia* spp. | β-Proteobacteria (98%) | AB441670 |
| n | □ | *Shewanella* spp. | γ-Proteobacteria (100%) | AB334772 |
| o | □ | *Pseudomonas* spp. | γ-Proteobacteria (100%) | AY091598 |
| p | □ | *Marinobacter* spp. | γ-Proteobacteria (100%) | EU052754 |
| q | □ | *Artemia* spp. | 18S rRNA (96%) | X69067 |
| r | □ | *Tenacibaculum* spp. | Flavobacteria (100%) | AF530136 |
| s | □ | *Blastomonas* spp. | α-*Proteobacteria* (99%) | AB242676 |
| t | □ | Rhodocyclaceae | β-Proteobacteria (96%) | EF636156 |
| u | □ | Uncultured *Janthinobacterium* | β-Proteobacteria (100%) | EU300542 |
| v | □ | Uncultured *Citrobacter* spp. | γ-Proteobacteria (99%) | EU704221 |
| w | □ | *Psychrobacter* spp. | γ-Proteobacteria (100%) | EU836736 |
| x | □ | *Acinetobacter* spp. | γ-Proteobacteria (99%) | EU848481 |
| y | □ | *Marinomonas* spp. | γ-Proteobacteria (99%) | EU052766 |
| z | □ | *Shewanella* spp. | γ-Proteobacteria (100%) | EU075118 |
| aa | □* | Uncultured *Acidovorax* spp. | β-Proteobacteria (100%) | EU341283 |
| bb | □* | *Vibrio* spp. | γ-Proteobacteria (99%) | EU177084 |

The marks indicate bands excised from gels of unfed yolk sac larvae (■) and first feeding larvae (□). Also shown are selected products excised from samples of first feeding larvae in gels not shown (□*). The products identified are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787).

Highly variable numbers of CFU and presumptive *Vibrio* bacteria growing on TCBS were isolated from samples of *Artemia* collected throughout the period (Fig. 3). The numbers of CFU ranged from $8.4*10^3$ to $4.1*10^6$ $g^{-1}$ *Artemia* (wet weight). Presumptive *Vibrio* bacteria were found to dominate the cultivable bacterial community at all samplings, with counts ranging from $1.0*10^2$ to $1.1*10^4$ $g^{-1}$ *Artemia* (wet weight).

Using selected morpho-physiological tests, a total of 540 randomly selected isolates from the cultivable part of the bacterial community (12 isolates from each sample) were grouped into 8 main groups of bacteria that were found in >15% abundance in larvae sampled from individual production units at various dph. Representative isolates from the groups (8–20% of the isolates within each group) were sequenced and identified. Subsequent analysis of the DGGE profiles of the selected isolates revealed a distinct product for each of the isolates (results not shown). Bacterial isolates belonging to 5 of the groups were each found in larvae from three or more of the 23 production units examined and are therefore considered to represent the dominating groups of the cultivable part of the production system's total bacterial community. These groups were found to be represented by *Vibrio* sp., *Shewanella* sp. and *Photobacterium* sp. in first feeding larvae and *Pseudoalteromonas* sp. and *Marinomonas* sp. in addition to *Shewanella* sp. in unfed yolk sac larvae. Bacteria belonging to the other groups identified were only found in limited numbers (<9%) or sporadically in individual production units, and are therefore not considered to be a dominating part of the cultivable fraction of the bacterial community. A considerable variation in the composition of the cultivable bacterial community was observed in unfed yolk sac larvae from various production units (results not shown). The highest survival was found with co-domination of the *Pseudoalteromonas* and *Marinomonas* groups but the overall success of yolk sac larvae in individual units could not be related to the presence or absence of bacterial groups identified as a part of the cultivable bacterial community. *Vibrio* were found to dominate the bacterial community of first feeding larvae (results not shown), but this group was not identified as a part of the cultivable bacterial community of unfed yolk sac larvae. *Vibrio wodanis* was detected only in larvae from the least successful production unit at 87 dph (results not shown). Other species found to dominate the cultivable bacterial community of first feeding larvae included *Pseudoalteromonas* and *Shewanella* early during first feeding and *Photobacterium* in addition to *Shewanella* later during first feeding of larvae (results not shown). *Photobacterium* was only detected in larvae from two tanks and in one of the tanks at all samplings throughout the first feeding period (tanks 2–13). The overall success of larvae from this tank was found to be the poorest observed during the period.

A variable percentage of the isolates did not grow upon sub-culturing in the laboratory or responded poorly in the morpho-physiological tests used. These isolates were characterized as "uni-dentified". This was more commonly observed for colonies isolated from unfed yolk sac larvae (8–100% of isolates in individual samples)

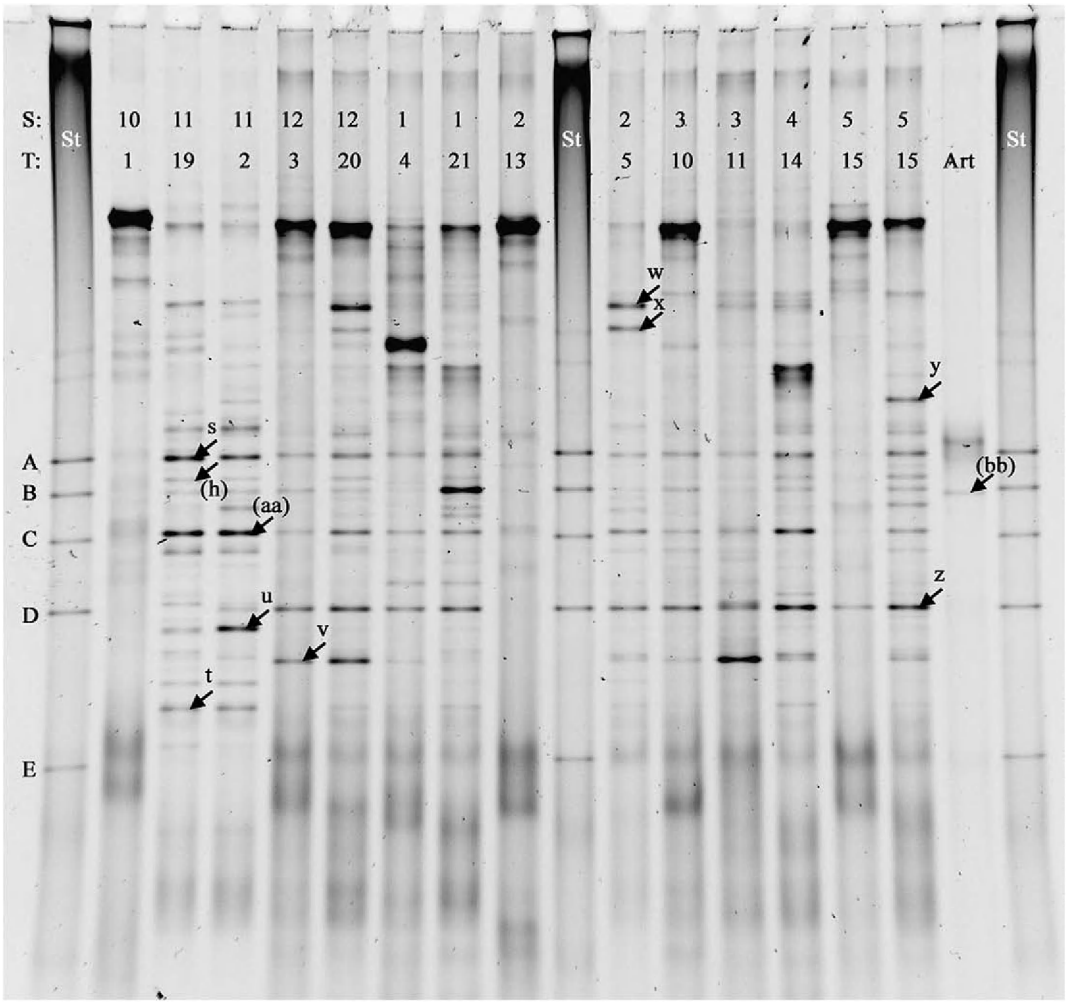


**Fig. 6.** DGGE profiles of the intestinal bacterial community in a pool of ~50 larvae from individual tanks 87 dph with tank numbers (T) indicating the silo-origin of larvae (S). Also shown is the profile of a sample of the live feed (Art) and relative mobility standards A–E (St). Labelled arrows indicate bands that were excised from the gel and identified by sequence analysis. Arrows labelled in parentheses indicate presumptively identical bands excised from other gels and identified by sequence analysis. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787).

compared to first feeding larvae (8–34% of isolates from individual samples). It is important to note that the grouping of bacteria was based on only 11 morpho-physiological tests and subsequent identification of 8–20% of the isolates within each group. The results presented therefore do not provide a confirmed identification of all isolates within each group.

### 3.3. The total bacterial community

PCR–DGGE profiles of the bacterial community of surface sterilised larvae were found to be highly diverse with a large number of amplified products visible in the gels. Three products appear in all samples of unfed yolk sac larvae (Fig. 4 — arrows labelled a, c and f) as well as in most samples of first feeding larvae at 87 dph (Fig. 6), but are not visible in larvae from all tanks at 57 dph (Fig. 5). Excising and subsequent sequence analysis identified *Marinobacter*, *Aeromonas* and *Pelomonas/Shewanella* as the closest relatives with 98–100% similarity (Table 2). An additional product identified as mitochondrial 12S rRNA from the larvae was found in larvae sampled at 57 dph (Fig. 5 — arrow labelled j) and may also be represented in bands resolving at a similar location in the gels at 87 dph. However, excising and subsequent identification of excised bands at 87 dph revealed α-*Proteobacterium* only (Table 2) and the product may be present in larvae from most tanks at 87 dph.

Excising and subsequent identification of other products from the gels revealed a variety of bacterial groups including *Pseudoalteromonas* and *Marionomonas* in unfed yolk sac larvae and a number of groups belonging to the γ and β-*Proteobacteria* as well as α-*Proteobacteria* in feeding larvae (Table 2). The bacterial community of surface sterilised first feeding larvae at 67 dph was also analysed. Identification of only one product is shown in Table 2, selected due to the apparently common appearance of this products in larvae sampled from all production units at 87 dph and possibly at 57 dph as well.

The quality of *Artemia* was found to vary considerably with respect to the total bacterial community of surface sterilised larvae reflected in the DGGE gels (Figs. 5–7). *Flavobacterium* and *Vibrio* were the only groups identified in the samples analysed. However, products visible in the DGGE gels were only excised from a restricted number of the samples of live feed collected during the period. The diversity of products in various samples that were not analysed (Fig. 7A) reveal a considerably higher diversity of the bacterial community of *Artemia* compared to the samples that were analysed (Figs. 5, 6). The different bacterial profiles of first feeding larvae may be partly explained by the highly heterogeneous bacterial community observed in various samples of the live feed collected through the period.

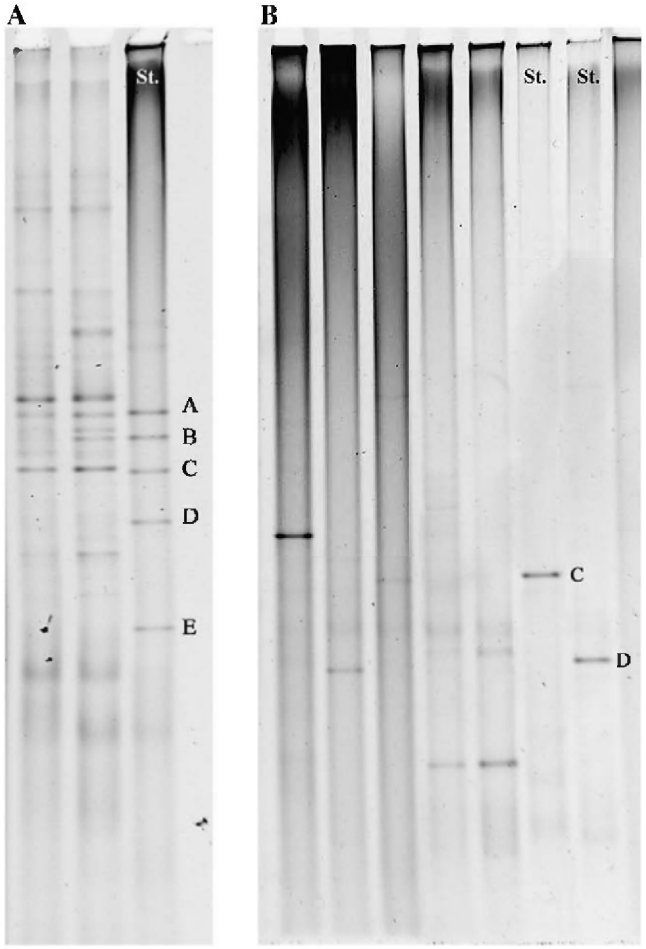


**Fig. 7.** DGGE profiles of the total (A) and cultivable (B) part of selected samples of the live feed collected during the period. Relative mobility standards A–E (Fig. 7A) and C–D (Fig. 7B) are included in the figures. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787).

## 4. Discussion

The study describes an analysis of the bacterial community of surface sterilised halibut larvae at the onset of feeding and through the first weeks of offering live feed.

The survival and overall success of larval development was found to be highly variable, as has previously been reported in larvae of halibut (Shields et al., 1999; Ottesen and Olafsen, 2000; Imsland et al., 2002; Verner-Jeffreys et al., 2003a), as well as other marine fish species (Skjermo and Vadstein, 1999; Griffiths et al., 2001; Brunvold et al., 2007; Chiu et al., 2007; Le Vay et al., 2007; Nakase et al., 2007). The survival of yolk sac larvae in various tank units was from 27% up to 77% and during first feeding from 48% to 100%. This is within the range of the overall survival of up to 50–70% that has previously been reported in intensive production of halibut larvae (Olsen et al., 1999). The mean weight of larvae at the end of the first feeding period was also found to vary significantly but larval growth was not found to correlate with survival of first feeding larvae ($R^2$<0.1). The results furthermore indicate that high survival of yolk sac larvae may lead to poor survival amongst first feeding larvae. The negative relationship may be explained by the death of already moribund larvae during the yolk sac stage. Similarly, higher survival through the yolk sac stage could result in higher percentage of poorish larvae collapsing already during the first two weeks in feeding. The relative numbers of dead larvae in individual first feeding tank units were found to be highly variable. The increased numbers of dead larvae observed at 55–59 dph have been found to represent yolk sac larvae with jaw deformation that represents one of the main problems in the production of halibut larvae (Morrison and Macdonald, 1995). This deformation has been associated with abrasion of the surface layers and subsequent invasion by microorganisms as explained by Ottesen and Olafsen (2000) who found that bacteria in the seawater environment affect the proliferation of mucus cells in halibut larvae. The death of larvae observed at 60–63 dph may be expected to reflect the overall quality of the yolk sac larvae, as an indication of how the larvae manage in the variable environmental conditions in individual first feeding units where the dominating bacterial community is suspected to play an important role (Olafsen, 2001; Schulze et al., 2006; Nakase et al., 2007). A recess in the feeding activity of larvae in individual production units is furthermore commonly observed at Fiskey Ltd. after 3–4 weeks of feeding, occasionally resulting in elevated numbers of dead larvae and even a total collapse of all the larvae within a unit as observed in one of the first feeding tanks of the period. This may be the result of the continuing development of larvae (metamorphosis) presenting new challenges in relation to differential colonization of opportunistic pathogens. Also, poor pigmentation of the fry is frequently observed

and may be caused by various environmental parameters (Olsen et al., 1999; Le Vay et al., 2007). However, incomplete pigmentation was not found to correlate with the bacterial colonization of feeding larvae in the present study.

The numbers of cultivable bacteria in larvae from individual production units were found to vary considerably. In unfed yolk sac larvae the numbers were from $6.1*10^1$ to $3.9*10^5$ CFU larvae$^{-1}$, which is in agreement with the findings of Verner-Jeffreys et al. (2003b) and Jensen et al. (2004) who estimated the numbers of cultivable bacteria in yolk sac larvae to be $<5*10^5$ CFU larvae$^{-1}$. We studied the bacterial community in a pool of many larvae, while others have examined individual larvae obtaining similar results (Jensen et al., 2004). Interestingly, the highest numbers of CFU were found in yolk sac larvae from the most successful production unit of the period. This may indicate the colonization of a favourable bacterial community despite the overall results indicating a positive relationship between the numbers of CFU larvae$^{-1}$ and the prevalence of jaw deformation amongst unfed yolk sac larvae. The cultivable part of the bacterial community of larvae from this tank was found to be co-dominated by *Pseudoalteromonas* and *Marinomonas*. However, the co-dominance of *Marinomonas* and *Shewanella* was observed in relation to poor overall success of unfed yolk sac larvae. *Pseudoalteromonas* spp. produce a range of biologically active compounds and have been suggested to counter protection to unfed yolk sac larvae (Verner-Jeffreys et al., 2004). This group was found to dominate the cultivable bacterial community of yolk sac larvae in most production units in the present study. This is in agreement with previous findings of Verner-Jeffreys et al. (2003b) who found this group as well as *Vibrio* frequently dominating the intestines of unfed yolk sac larvae and occasionally also in first feeding larvae. *Pseudoalteromonas* were also detected in larvae early in first feeding (57 dph), indicating that this group represents an inherent part of the bacterial community of yolk sac larvae as previously observed by Verner-Jeffreys et al. (2003b). Our results, however, indicate that this group may recede in the variable environment established in larvae during first feeding. *Photobacterium* was only detected in larvae from one tank at all samplings throughout the period and the survival of larvae from this tank was the poorest observed during the period. This group was, however, occasionally detected in larvae from another tank, yielding the best overall outcome of the period and *Photobacterium* therefore highly unlikely to control the overall success of first feeding larvae.

The numbers of cultivable bacteria in unfed yolk sac larvae were found to be positively related to survival as well as jaw deformation of larvae, reinforcing a previously observed correlation of jaw deformation and bacterial counts of unfed yolk sac larvae (unpublished results). Elevated numbers of cultivable bacteria in unfed yolk sac larvae were furthermore found to be positively correlated with poor quality of feeding larvae from the corresponding tanks, measured as incomplete eye migration. The relationship was, however, found to be negative with respect to quality measured as incomplete pigmentation of first feeding larvae, indicating a general and highly complicated impact of the intestinal bacterial community of larvae.

*Vibrio* bacteria dominated the cultivable bacterial community of first feeding larvae, representing from 33% to 70% of the isolates from larvae in individual production units at various samplings. However, *Vibrio* bacteria were not identified as a part of the bacterial community of unfed yolk sac larvae. This is in disagreement with the observations of Verner-Jeffreys et al. (2003b) who commonly found *Vibrio* spp. colonizing the intestines of unfed yolk sac larvae. The relative dominance of *Vibrio* bacteria in the cultivable bacterial community of larvae after the onset of first feeding may be explained by the commonly observed dominance of *Vibrio* in samples of the live feed collected through the period (Olsen et al., 2000; Ritar et al., 2004; Savas et al., 2005; Korsnes et al., 2006). A member of the *Vibrio* group, *V. wodanis*, was detected in larvae from the least successful unit but at 87 dph only. This is a species that along with *Vibrio viscosus* has been isolated in association with winter ulcer disease affecting salmonid fish reared in saline water (Benediktsdottir et al., 2000; Lunder et al., 2000). The sporadic identification of *V. wodanis* spp. may therefore be related to survival of first feeding larvae, but further attempts to relate the structure of the cultivable bacterial community to the overall success of unfed yolk sac larvae proved unsuccessful.

A variable percentage of the strains were classified as "unidentified" due to their lack of response the morpho-physiological tests used or the inability to grow up on sub-culturing. This was more commonly observed when analysing the gut community of yolk sac larvae and may be caused by a relatively low overall organic load of the larval environment before the onset of feeding, known to contribute to the success of cultivation of bacteria from the marine environment (Kemp and Aller, 2004). Furthermore, the API 20E system was found to be insufficient for grouping of the cultivable part of the microbial community as recently reviewed by (Popovic et al., 2007).

Only a small part of the microbial communities of aquatic environments has been found to be cultivable on non-selective nutrient media (Kemp and Aller, 2004), and culture-independent methods therefore more commonly applied in order to study the diversity of these communities. In the present study we used the PCR–DGGE method described by Muyzer et al. (1993). The method has commonly been applied when studying the bacterial diversity of species in intensive aquaculture and often combined with the traditional culturing of bacteria on non-selective nutrient media (Griffiths et al., 2001; Huber et al., 2004; Jensen et al., 2004; Dorigo et al., 2005; Brunvold et al., 2007; Hovda et al., 2007). More than one group of bacteria were occasionally identified in products excised from the gels and may be explained by a similar arrangement of base-pairs within the V4 region of the 16S rDNA gen of the respective groups. The denaturing gradient of the gel will furthermore affect the general separation of individual bacterial groups found in the samples.

The bacterial community of yolk sac larvae was found to be highly diverse, but relatively similar in larvae from various production units. This is in disagreement with the observations of Verner-Jeffreys et al. (2003b) who found the bacterial community of surface sterilised larvae from different incubators to be highly variable. Our results are therefore more in agreement with the findings of Jensen et al. (2004), who suggest that a distinct and specific community of bacteria may have adopted the strategy to proliferate in the yolk sac halibut larvae. However, the DGGE profiles of unfed yolk sac larvae of the present study were found to reveal considerably higher diversity compared to the results of Jensen et al. (2004), who studied the bacterial community of unfed yolk sac larvae from the same hatchery and sampled immediately before the onset of feeding as in the present study. The authors found the *Pseudomonas* and *Marinomonas* groups as the primary colonizers of yolk sac halibut larvae in addition to *Janthinobacterium*, which was not identified as a part of the bacterial community of yolk sac larvae in the present study. This group may, however, be present as an "uncultured β-*Proteobacterium*" that was identified as a dominating product of the larval intestines. Identification of the excised bands in the present study furthermore revealed an overall dominance of *Marinomonas, Marinobacter, Aeromonas* and *Shewanella* in unfed yolk sac larvae. The different profiles of larvae observed in the two studies may be explained by the different set of primers, denaturing gradient and % acrylamide used in the DGGE gels in the studies. Interestingly, the identification of excised bands in the study of Jensen et al. (2004) revealed in unfed yolk sac larvae the presence of 18S rDNA from the live feed (*Artemia franciscana* AJ238061). Our previous observations indicate that the co-amplification of 18S rDNA, due to the overall dominance of 18S rDNA in all samples, may be expected to exclude bacterial groups present in lower relative quantities (results not shown). The various results of the two studies may furthermore be explained by the addition of phytoplankton for the appropriate shading of the environment of first feeding larvae commonly practised in the production of halibut larvae. Live algae were applied for shading during the period of sampling of Jensen et al.

(2004) at Fiskey Ltd., while inorganic clay was used during the present study. Live algae represents an addition to the total biologic load of the production system as a whole and high amounts of organic wastes present in the environment may cause fluctuations in bacterial growth and support differential colonization of opportunistic pathogens as hypothesized by Hjelm et al. (2004).

The bacterial community of first feeding larvae was also found to be highly diverse. Two main products seem to be present in larvae from all tanks and at all samplings. Excising and subsequent sequencing of the products identified *Vibrio* and *Shewanella*. The *Pseudomonas* and *Marinobacter* groups were also found in larvae from most production units at 57 dph, but not at later sampling of first feeding larvae at 87 dph. Interestingly, α-*Proteobacterium* were identified in first feeding larvae and the DGGE pattern indicates the presence of this group in larvae from most tanks at 87 dph, and possibly also at other sampling dates. As pointed out by Jensen et al. (2004), the α-*Proteobacterium* group has not previously been reported in halibut larvae, but has been identified in the marine environment (Schafer et al., 2002; Selje et al., 2004). The *Roseobacter* clade belong to the α-*Proteobacterium* group and have been found in high densities in a larval rearing environment (Hjelm et al., 2004). The absence of this product in larvae from some of the tanks may indicate that α-*Proteobacteria* are only present in lower proportions or with seasonal incidence as has been suggested (Hjelm et al., 2004). The predominance of α-*Proteobacteria* and the *Cytophaga–Flavobacterium* cluster has been found to be a good indicator for successful production of e.g. sea bream larvae (Nakase et al., 2007). Both groups were identified as a part of the community of halibut larvae in the present study, but *Flavobacterium* only sporadically in samples of the live feed collected through the period as well as in some of the larval samples. The *Marinomonas* group may be present only in larvae from the most successful production unit of the period, but the pattern profile of larvae in other overall successful tank units of the period was found to be highly variable and no obvious relation to larval success could be established. This may indicate the importance of the co-dominance of various bacterial species rather than the presence of any particular species.

The quality of the live feed was shown to vary considerably, with respect to the numbers of cultivable bacteria as well as the total bacterial pattern reflected in the DGGE gels. The numbers of cultivable bacteria in *Artemia* ranged from $8.4*10^3$ to $4.1*10^6$CFU $g^{-1}$, which is considerably lower than the 1.7 to $3.5*10^7$ CFU $g^{-1}$ that Savas et al. (2005) found, but within the range observed by Verner-Jeffreys et al. (2003b). The various numbers of cultivable bacteria may be explained by different methods used for enrichment and that have been found to affect the bacterial community of the live feed both quantitatively and qualitatively (Olsen et al., 2000; Eddy and Jones, 2002; Korsnes et al., 2006). *Vibrio* sp. dominated the bacterial community as has previously been reported by others (Olsen et al., 2000; Eddy and Jones, 2002; Tolomei et al., 2004; Savas et al., 2005). Flavobacteraceae was the only group identified in addition to *Vibrio* and the occasional identification of 18S rDNA originating from the live feed. Bacteria identified as *Pseudomonas*, *Alcaligenes*, *Pseudoalteromonas*, *Acinetobacter* and *Aeromonas* have previously been observed in enriched *Artemia* (Olsen et al., 2000; Verner-Jeffreys et al., 2003b; Tolomei et al., 2004). A number of products were observed in the gels from various samples of *Artemia* in the present study, but only a part of the products was excised and subsequently identified. The different bacterial profiles of first feeding larvae in the present study may therefore be partly explained by the highly diverse bacterial composition of the live feed. However, not all of the bacterial products observed in various samples of *Artemia* can be found in first feeding larvae. This is in agreement with the previous observations of Verner-Jeffreys et al. (2003b), who hypothesised that only a selected proportion of the *Artemia*-associated bacteria are able to establish themselves in the gut of larvae. Our results may furthermore indicate that a certain period of feeding live feed is needed for the species composition stability within the gut, as has been pointed out by Possemiers et al. (2004).

The overall results indicate that only a small part of the bacterial groups associated with halibut larvae in intensive aquaculture may be cultivable. However, excising and subsequent identification of the DGGE products indicates that the dominating bacterial groups of surface sterilised larvae at various developmental stages may be cultivable.

## 5. Conclusion

In conclusion, we found the bacterial community structure of surface sterilised halibut larvae to be more heterogeneous than previously reported. The PCR–DGGE method applied was found to reveal an acceptable overview of the highly diverse bacterial community structure of larvae. The numbers of cultivable bacteria in the intestines of larvae were found to positively correlate with the quality of larvae. Furthermore, our results indicate that individual bacterial groups may affect the overall success of halibut larvae in intensive farming. The high degree of variability observed in the bacterial community of various samples of the live feed could therefore affect larvae at different stages of the development to a various degree. Elevated numbers of bacteria and less favourable bacterial community structure of the live feed may thus represent an additional challenge if fed to larvae immediately at the onset of feeding, when larvae are found to be highly vulnerable. The dominating bacterial community of feeding larvae was furthermore reflected in the cultivable part, indicating that cultivation on non-selective nutrient media may prove useful for surveillance of the bacterial composition and overall quality of feeding larvae. Periodic surveillance of the live feed may also be of value in promoting stability and hence, effective management strategies in the production of marine larvae.

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