Effects of different environmental shading on the cultivable bacterial community and survival of first feeding Atlantic halibut larvae

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ABSTRACT

High mortality rates commonly observed during the early life stages of intensively reared Atlantic halibut have among other things been related to high bacterial numbers and an unfavourable bacterial community. The study describes the effects of two different methods for environmental shading on larval survival and numbers of cultivable bacteria in the culture water and the gastrointestinal tract of first feeding larvae at Fiskey Ltd. Larval survival was not affected by the method used for environmental shading. Lower bacterial numbers were observed in the tank water with environmental shading provided by inorganic clay as compared with marine algae, primarily during the first days of exogenous feeding. Gram negative, fermentative bacteria dominated the cultivable community in the gastrointestinal tract of larvae during the first weeks in feeding. The use of inorganic clay has clear economic advantages as compared to the use of marine microalgae, and the commercial producer has used the product exclusively for environmental shading during first feeding of halibut larvae since 2003.

Keywords: Aquaculture, halibut, environmental shading, bacteria, survival

YFIRLIT

Skygging eldisumhverfis - áhrif á ræktanlega bakteríuflóru og afkomu lúðulirfa í startfóðrun

Mikil afföll sem verða á fyrstu stigum lúðueldis hafa meðal annars verið tengd fjölda baktería og óæskilegri samsetningu bakteríuflóru. Rannsóknin fjallar um áhrif tveggja mismunandi aðferða við skyggingu eldisumhverfis á afkomu lúðulirfa og fjölda ræktanlegra baktería í eldisvökva og meltingarvegi lirfa hjá Fiskey hf. en fyrirtækið hefur um árabil verið stærsti framleiðandi lúðuseiða á heimsvísu. Ekki var munur á afkomu lirfa í meðferðarhópunum. Fjöldi baktería í eldisvökva lirfa var minni þegar leir var notaður til skyggingar samanborið við notkun þörunga, sér í lagi fyrstu dagana í fóðrun. Gram neikvæðar, gerjandi bakteríur voru ríkjandi hluti ræktanlegrar flóru í meltingarvegi lirfa fyrstu vikurnar í fóðrun. Sparnaður er af notkun leirs samanborið við þörunga og hefur lúðuseiðaframleiðandinn alfarið notað ólífrænan leir til skyggingar eldisvökva við frumfóðrun lirfa frá árinu 2003.

INTRODUCTION

Atlantic halibut (*Hippoglossus hippoglossus* L.) is one of the highest valued flatfish species. It is a cold water species, characterised by a long larval yolk-sac stage following hatching, and a long period of live feed requirements under culture conditions (Shields 2001).

An overall poor survival rate is experienced during early life stages, occasionally resulting in total collapse in the production of individual batches and with only ~20% survival observed from hatching at Fiskey Ltd. in Iceland, the world's largest commercial producer. Larvae are given live feed during the first weeks of exogenous feeding and the appropriate concentration of marine microalgae is commonly added to the culture water in order to provide the appropriate shading effects needed for normal larval grazing behaviour (Mueller-Feuga 2000). The algae also serve as a feeding source and have been reported to be beneficial during early developmental stages of marine larvae (Muller-Feuga 2000, van der Meeren et al. 2007). It has furthermore been suggested that the algae have positive effects on the intestinal bacterial community of fish larvae but may at the same time be carriers of opportunistic pathogens (Liao et al. 2003, Makridis et al. 2006, Olsen et al. 2000). The association of *Vibrio* bacteria with marine organisms is well documented and beneficial effects have been suggested for various *Vibrio* sp. that have been identified as a part of the natural bacterial community of fish (Austin et al. 1995, Olafsen 2001). Many *Vibrio* species have furthermore been recognised as opportunistic pathogens in marine fish species, reflecting the vast diversity of species belonging to this group (Egidius 1987). Organic debris from dead larvae, the live feed and algal supplements are a reservoir of organic material that supports bacterial growth (Ritar et al. 2004). A general understanding of the bacterial community associated with rearing of marine larvae is therefore required for improved rearing performances (Olafsen 2001).

The aim of the present work was to study the cultivable bacterial community in relation to survival of first feeding halibut larvae when two different methods were used for providing environmental shading. The work describes the bacterial numbers in the culture water and in the gastrointestinal tract of larvae from a large number of production units at a commercial production site during 1999-2002, with shading provided by either marine microalgae or inorganic clay.

MATERIALS AND METHODS

The effects of different environmental shading were studied in larvae originating from three distinct spawning groups of halibut broodfish using marine microalgae during 1999-2000 and from other three spawning groups using inorganic clay during 2001-2002. Samples of larvae and their culture water were collected from all production units (tanks) of each spawning group studied (~20 tanks group^{-1}).

Samples were collected from each tank after one-five days of feeding and then at approximately weekly intervals for a period of 51 days. Only time points with sampling from a minimum of seven tanks for each treatment (7-12 tanks) were selected for calculation of the average numbers of cultivable bacteria in surface sterilized larvae and their tank water.

Production methods and evaluation of larval success

Fertilized eggs were kept in 0.25 m^3 tanks at 5.0–5.3 °C for 14 days prior to surface disinfection using 400 ppm glutaraldehyde and transferring to 10 m^3 silos where the eggs hatched. 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), fed with 24 h cultures of fatty acid enriched *Artemia franciscana* nauplii originating from the Great Salt Lake in Utah, USA. The larvae were fed twice a day for ~60 days at 11 °C, when weaning onto formulated feed was started. The microalgae, *Tetraselmis suecica* (diameter 7-10 μm) and *Isochrysis galbana* (diameter 5-6 μm) were cultured semi-continuously, with renewal rates between 10-50%. A mixture of *T. suecica* and *I. galbana* (~1/3 and 2/3, respectively) was

added to the tank water of larvae prior to the first of two daily rations of Artemia fed to the tanks. For algal culturing, fluorescent lamps provided an illumination of 15,000-20,000 lux with a photoperiod of 16:8 hours of light:dark.

The algae were added to the tank water in progressive amounts from the onset of first feeding (~200 million algae mL^{-1}) until the end of the first feeding period (~100 million algae mL^{-1}). The concentration of the inorganic clay (particle size ≤ 5 µm) was visually adjusted in order to achieve similar shading effects at different periods during first feeding of larvae (0.0030 – 0.0015 g L^{-1}). Larval survival was evaluated at the onset of weaning unto formulated feed, calculated from the estimated number of yolk sac larvae transferred to each tank at the onset of exogenous feeding.

Sampling and sample preparation

Tank water samples were collected in sterile 250 mL glass bottles that were opened 5-10 cm below the water surface. Larvae were collected using a mesh net and then transferred to sterile 100 mL glass bottles along with seawater from the respective tank. Samples from daily produced cultures of enriched *Artemia* were collected at approximately weekly intervals throughout both periods. Samples were kept chilled on transport to the laboratory where they were processed within 3-5 h post collection. All samples were collected prior to the first of two daily additions of marine microalgae or inorganic clay to the tanks and processed as previously described (Bjornsdottir et al. 2009). Briefly, a group of ~10 (51 days after onset of first feeding, dpff) to ~100 (0 dpff) larvae were anaesthetized using Hypnodil (51µg mL^{-1}). The larvae were then surface sterilized in a solution of 0.1% benzalkonium chloride for 30 sec followed by rinsing three times in a solution of sterile 2% NaCl. Enumeration was followed by homogenization in a tenfold w/w dilution of peptone-seawater containing 0.1% w/v Bacto peptone.

Analysis of the cultivable bacterial community

Colony forming units (CFU) were determined by cultivation at 15 °C for 5–7 days on marine agar plates (MA; Difco) and CFU of presumptive *Vibrio* bacteria on thiosulphate citrate bile salt sucrose agar plates (TCBS; Oxoid).

Results are expressed as the mean numbers of CFU mL^{-1} and $larvae^{-1}$ from a minimum of seven production units at each sampling date. Twelve randomly selected colonies were picked from MA plates containing 25 - 250 CFU $plate^{-1}$ and sub-cultured at 15 °C to ensure purity before grouping a total of 3,500 isolates to Gram negative fermentative and non-fermentative rods. The fermentative activity was determined as the ability to dissimilate glucose according to the modified method of Lemos and his co-workers (1985). Duplicate tubes containing 5 mL of oxygen depleted medium were then inoculated and results recorded after incubation with and without a paraffin seal for 7, 14 and 28 days at 15 °C.

Statistical analysis

Data were analysed using SigmaStat® release 3.5 (Systat Software, Inc. CA 94804-2028 USA). The normality of the data distribution was analysed using the Kolmogorov-Smirnov test. Bacterial numbers are expressed as mean ± S.D. in the tank water and larvae from a minimum of seven tanks at the sampling points selected. A *t*-test was used to analyse the difference in bacterial numbers between the groups at each sampling point. Differences were considered statistically significant when $p<0.05$. Mean values of larval survival in tanks with environmental shading provided by marine microalgae or inorganic clay were compared using a *t*-test. A regression analysis was used to analyse the relationship between larval survival and bacterial numbers at selected sampling points.

RESULTS

Tank water environment

A rapid increase in bacterial numbers in the tank water was observed during the first days

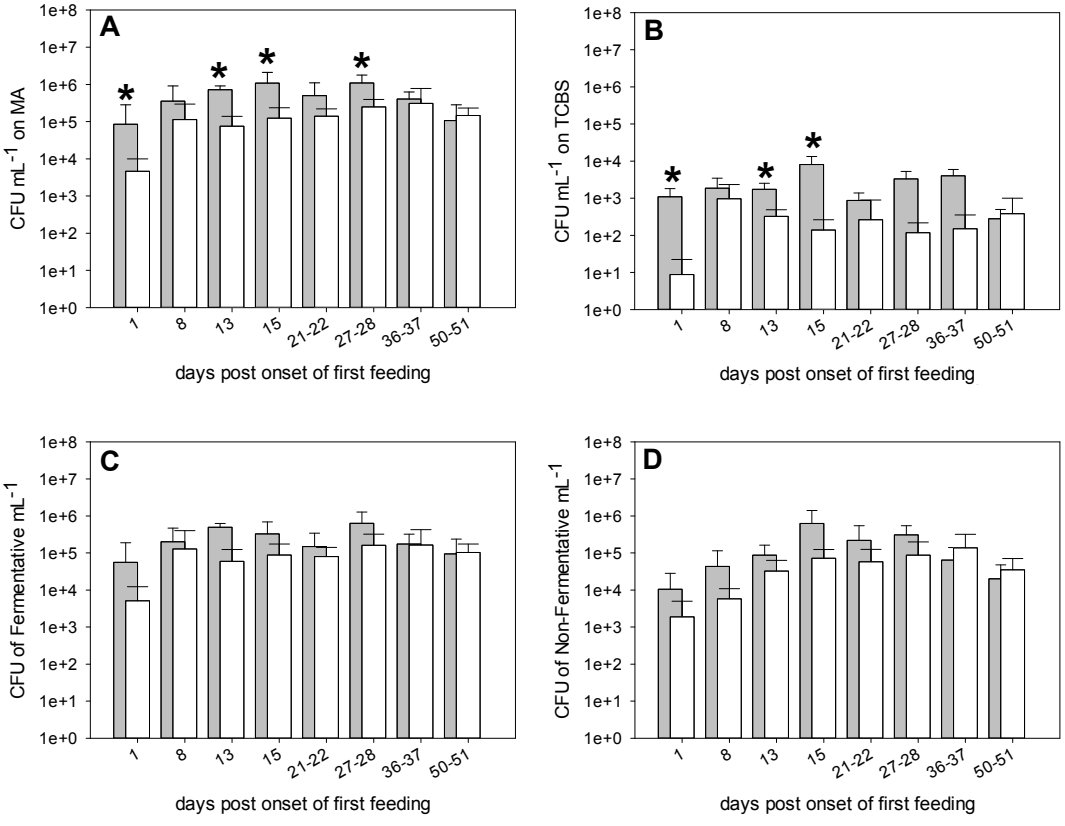

Figure 1. Number of colony forming units (CFU) in tank water of first feeding larvae, with environmental shading provided by marine algae (▓) or inorganic clay (□): (A) CFU on marine agar (MA); (B) CFU on thiosulphate citrate bile salt sucrose (TCBS) agar (presumptive *Vibrio* bacteria); (C) CFU of fermentative Gram negative bacteria; (D) CFU of non-fermentative Gram negative bacteria. Shown are mean values ±S.D. from a minimum of seven tanks from each group at each sampling date. Statistically significant differences in bacterial numbers between the two groups are denoted with an asterisk (*).

in feeding larvae with *Artemia* (Figure 1A). The highest numbers of CFU on MA were observed 15 dpff with environmental shading provided by marine algae ($1.1*10^6 \pm 10^6$ CFU mL^{-1}), but relatively late during first feeding (36–37 dpff) with the addition of inorganic clay ($2.51*10^5 \pm 10^5$ CFU mL^{-1}). Statistical analysis revealed significantly lower CFU on MA in the tank water with shading provided by inorganic clay as compared to marine microalgae, after 1 (p=0.009) as well as 13 ($p \leq 0.001$), 15 (p=0.006) and 28 (p=0.014) days in feeding.

The CFU on TCBS were generally 100 to 1000 times lower than the observed CFU on MA in tank water samples (Figures 1A & 1B). The highest numbers were observed at 15 dpff with the addition of marine algae ($0.8*10^4 \pm 10^4$ CFU mL^{-1}), compared to a week earlier with the addition of inorganic clay ($1.0*10^3 \pm 10^3$ CFU mL^{-1}). Significantly lower numbers of CFU on TCBS were observed in tank water after 1 (p=0.01) as well as 13 (p=0.025) and 15 (p=0.024) days in feeding with the addition of inorganic clay as compared to marine micro-algae. Grouping of the isolates to fermentative and non-fermentative Gram negative rods revealed a relative dominance of fermentative

Table 1. Number of colony forming units (CFU) in surface sterilized larvae sampled from individual production units 13-15 days after onset of exogenous feeding, with environmental shading provided either by marine microalgae (A) or inorganic clay (C). Also shown are the spawning group origin (1999-2002), tank numbers and larval survival (%) in the corresponding production units, calculated at the end of the first feeding period and based on the total number of yolk sac larvae transferred to each tank at the onset of feeding.

	Marine microalgae				Inorganic clay		
Tank No.	**CFU (MA)†**	**CFU (TCBS)††**	**Survival (%)**	**Tank No.**	**CFU (MA)†**	**CFU (TCBS)††**	**Survival (%)**
1999-A16	$1.1*10^4$	$0.4*10^3$	45	2001-C12	$7.4*10^4$	$1.4*10^3$	25
1999-A5	$4.7*10^4$	$35.0*10^3$	75	2001-C16	$20.1*10^4$	$4.2*10^3$	23
1999-A12	$6.0*10^4$	$7.9*10^3$	70	2001-C18	$15.6*10^4$	$8.5*10^3$	0
1999-A2	$5.6*10^4$	$4.5*10^3$	64	2001-C15	$2.2*10^4$	$1.4*10^3$	52
1999-A9	$4.7*10^4$	$2.1*10^3$	30	2001-C7	$7.1*10^4$	$2.6*10^3$	47
1999-A18	$14.9*10^4$	$4.0*10^3$	7	2001-C14	$5.3*10^4$	$1.3*10^3$	66
1999-A6	$16.6*10^4$	$43.3*10^3$	71	2002-C15	$39.7*10^4$	$5.0*10^3$	36
1999-A11	$17.2*10^4$	$3.7*10^3$	10	2002-C4	$20.1*10^4$	$0.9*10^3$	70
2000-A1	$13.3*10^4$	$7.1*10^3$	73	2002-C13	$3.6*10^4$	$1.3*10^3$	75
2000-A2	$7.6*10^4$	$59.6*10^3$	33	2001-C8	$3.0*10^4$	$2.0*10^3$	54
2000-A10	$0.5*10^4$	$0.5*10^3$	5				
2000-A12	$11.0*10^4$	$0.7*10^3$	0				
Mean	***$8.6*10^4$***	***$14.1*10^3$***	***40.3***	***Mean***	***$12.4*10^4$***	***$2.8*10^3$***	***44.8***
S.D.	***$6*10^4$***	***$20*10^3$***	***30***	***S.D.***	***$12*10^4$***	***$2*10^3$***	***24***

† *Marine Agar*
†† *Thiosulphate Citrate Bile Salt Sucrose Agar*

Gram negative bacteria in the tank water from both groups at most sampling dates (Figure 1C & 1D). Similar numbers of fermentative bacteria were found in both groups at all sampling dates (p=0.07-0.2).

Halibut larvae

Statistical analysis revealed significantly lower CFU on MA in larvae after 1 (p=0.001) and 36 (p=0.017) days in feeding with environmental shading provided by clay as compared with algae, but significantly higher numbers were observed with clay after 13 (p=0.008) days in feeding (Figures 2A). No significant differences in CFU numbers on TCBS were observed between the two groups at any of the sampling points (p=0.06-0.9). The highest numbers of CFU were reached after two weeks in feeding with the addition of inorganic clay ($6.8*10^5 \pm 10^5$ CFU larvae^{-1} on MA and $2.5*10^4 \pm 10^4$ CFU larvae^{-1} on TCBS), but only towards the end of the first feeding period with the addition of marine algae ($1.1*10^6 \pm 10^6$ CFU larvae^{-1} on MA and $1.4*10^5 \pm 10^5$ CFU larvae^{-1} on TCBS) (Figure 2A & 2B).

No significant relationship was observed between larval survival in individual production units, calculated at the end of the first feeding period, and the numbers of CFU on MA in larvae from the respective production units after two weeks in feeding (Table 1). Larval survival was, however, found to be positively correlated to the numbers of CFU on TCBS observed in larvae after two weeks in feeding with environmental shading provided by inorganic clay (p=0.005).

Grouping of the isolates to fermentative and non-fermentative heterotrophic Gram negative bacteria revealed significantly higher numbers of fermentative bacteria in larvae already after the first day in feeding (p=0.012), but the numbers were significantly lower after two weeks in feeding (p=0.019) with environmental shading provided by marine algae as compared with inorganic clay (Figure 2C). The numbers of non-fermentative bacteria were also found

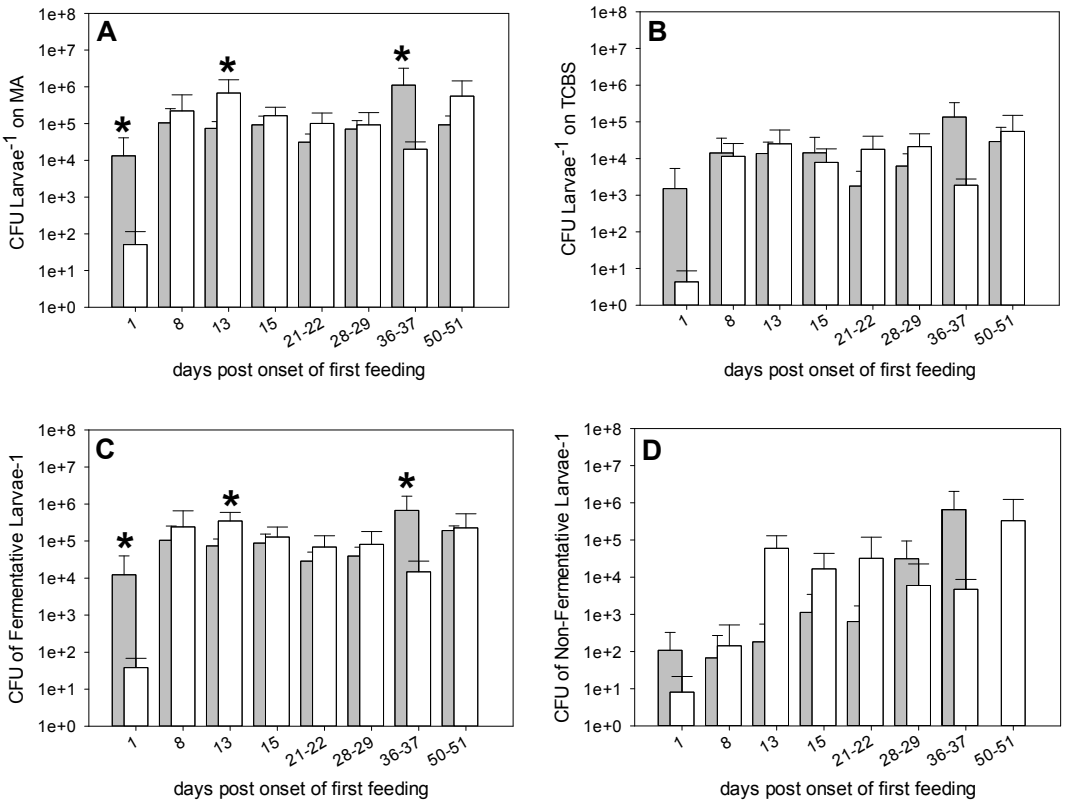

Figure 2. Number of colony forming units (CFU) in surface sterilized larvae from tanks with environmental shading using marine algae (▓) or inorganic clay (□): (A) CFU on marine agar (MA); (B) CFU on thiosulphate citrate bile salt sucrose (TCBS) agar (presumptive *Vibrio* bacteria); (C) CFU of fermentative Gram negative bacteria; (D) CFU of non-fermentative Gram negative bacteria. Shown are mean values ±S.D. from a minimum of seven tanks from each group at each sampling date. Statistically significant differences in bacterial numbers between the two groups are denoted with an asterisk (*).

to accumulate during the first 13-15 days of feeding, but no significant difference in numbers was observed between the two groups at any of the sampling points (Figure 2D).

An analysis of the cultivable bacterial community of the live feed revealed no significant differences during the two periods studied. Mean numbers of $2.1*10^7 \pm 10^7$ CFU on MA g^{-1} and $2.4*10^6 \pm 10^6$ CFU on TCBS g^{-1} wet weight of *Artemia* were observed during the period with environmental shading provided by marine microalgae (n=28) and $0.4*10^7 \pm 10^7$ CFU on MA g^{-1} and $1.1*10^6 \pm 10^5$ CFU on TCBS g^{-1} during the period with environmental shading provided by inorganic clay (n=20).

The survival of first feeding larvae in individual tank units varied considerably, with values ranging between 0-75% during both periods (Table 1). Larval survival in the two groups was not found to differ significantly (p=0.7).

DISCUSSION

The work summarizes the results from a three year study of the cultivable bacterial community of first feeding halibut larvae and their tank water related to larval survival at a commercial hatchery, with environmental shading provided by either marine algae or inorganic clay. The use of inorganic clay for environ-

mental shading was not found to affect larval survival. The elevated bacterial numbers observed following introduction of algae into the system may be the result of increased organic nutrient availability that has been found to support the multiplication of opportunistic bacteria (Nakase & Eguchi 2007, Olafsen 2001). With the addition of marine algae, the highest bacterial numbers were observed after approximately two weeks of feeding, while similar numbers were observed between 8-50 days in feeding with the addition of inorganic clay. A sudden and often extensive death of larvae is commonly experienced after about two weeks in feeding in the production of halibut larvae at Fiskey Ltd. Highly variable bacterial numbers were observed in the culture water during the first two weeks of feeding live *Artemia* to larvae, however, without any relationship with larval survival observed.

The intestinal bacterial community of marine larvae is established by the ingestion of bacteria by drinking long before the larvae actually start feeding, and elevated numbers of bacteria and poor water quality have been suggested as factors affecting the survival and overall quality of larvae (Olafsen 2001). The microalgae are, however, believed to improve the nutritional value of the live feed and have furthermore been suggested to positively affect the composition of the intestinal flora of fish larvae as bacterial selecting factor (Austin et al. 2006, Liao et al. 2003, Marques et al. 2006, Nakase et al. 2007, Olsen et al. 2000). A positive relationship was observed between the numbers of cultivable bacteria (CFU on MA) and presumptive *Vibrio* bacteria (CFU on TCBS) in the tank water after one and three weeks in feeding and with environmental shading provided only by inorganic clay. As previously suggested (Bergh et al. 1994), the lack of a significant relationship between the numbers of CFU on MA and TCBS in larvae after two weeks of feeding may indicate a shift in the groups dominating the bacterial community of larvae during this period. Following this period, further larval mortalities are rarely to be expected. The similar bacterial numbers found in the tank water when the first feeding proceeded may furthermore indicate improved stability, as previously suggested (Eddy & Jones 2002).

Recovery of bacteria from the marine environment has proven inconclusive by culturing methods and the cultivable part of the bacterial community has been found to represent only a small part of the microbial diversity in environmental samples (Hongoh et al. 2003). A community dominated by fast-growing bacteria may, however, to a large extent be recoverable on traditional nutrient media (Bjornsdottir et al. 2009, Tolomei et al. 2004). The TCBS medium is commonly used for isolation of presumptive *Vibrio* bacteria and a satisfactory recovery of *Vibrio* bacteria from fish as well as environmental samples has been obtained (Olsen et al. 2000, Tolomei et al. 2004). A relationship between *Vibrio* spp. and marine algae has been reported and *Vibrio* spp. have commonly been found to dominate the bacterial community of the live feed of marine fish larvae (Olsen et al. 2000, Verner-Jeffreys et al. 2003). Presumptive *Vibrio* bacteria dominated the cultivable bacterial community of larvae in the present study, emphasizing the importance of the enumeration of *Vibrio* spp. when studying the bacterial community during early developmental stages of marine larvae. The addition of inorganic clay was found to result in reduced numbers of presumptive *Vibrio* in tank water, however, without affecting overall larval survival at the end of the first feeding period. An analysis of the tank water containing larvae of a common silo origin furthermore revealed lower numbers of CFU on TCBS during the first three weeks in feeding with environmental shading provided by inorganic clay as compared to marine algae (Bjornsdottir et al. unpublished data). Also, lower numbers of CFU on both MA and TCBS were observed in surface sterilized larvae from the respective tank units during the first days in feeding with environmental shading provided by inorganic clay as compared to marine algae. The lower initial numbers of bacteria and the sudden increase in the numbers of cultivable bacteria

observed in larvae during the first days in feeding when inorganic clay was used as compared with marine algae may, however, indicate an immediate colonization of the bacterial community carried to the larvae through the live feed, as has previously been suggested (Verner-Jeffreys et al. 2003). A diverse bacterial community established by non-opportunists has been suggested to inhibit the proliferation of opportunistic pathogenic bacteria in the culture water as well as in the gastrointestinal tract of larvae. Thus, maintaining good water quality by reducing the overall substrate for bacterial growth may prevent the establishment of unfavourable opportunistic bacteria (Olafsen 2001). Considerable production costs are associated with maintaining the algal cultures, but only minimal costs are associated with purchasing inorganic clay. The clay may furthermore be sterilized prior to use, while there is a pending risk of contamination of the algal cultures, with periodic collapses commonly experienced and adversely affecting the overall production safety. Hence, there has been a significant pressure to replace the algae and the annual production costs have been reduced by 50,000-100,000 euros by the use of inorganic clay as compared with marine microalgae at Fiskey Ltd. Marine microalgae are, however, generally considered an essential nutritional supplement and are consequently commonly applied in intensive production of marine fish larvae (Rocha et al. 2008, van der Meeren et al. 2007).

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