Using inorganic clay as compared to marine microalgae for environmental shading during first feeding of Atlantic halibut *Hippoglossus hippoglossus* larvae: effects on the cultivable bacterial community and larval survival.

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1. Abstract

High bacterial numbers and the establishment of an unfavourable bacterial community has been identified as a possible cause of high mortalities commonly observed during the early life stages of intensively reared Atlantic halibut (*Hippoglossus hippoglossus* L.). The study describes larval survival and numbers of cultivable bacteria in the tank water environment and in the gastrointestinal tract of first feeding larvae with environmental shading provided by either marine microalgae or inorganic clay. Using inorganic clay instead of marine microalgae was not found to affect larval survival. Higher bacterial numbers were observed in the tank water with environmental shading provided by marine algae as compared with inorganic clay, primarily during the first days of exogenous feeding. Gram negative, fermentative bacteria dominated the cultivable community of the gastrointestinal tract of larvae during the first weeks in feeding, with the numbers of non-fermentative groups increasing as first feeding proceeded. Using inorganic clay has been found economically advantageous compared with the microalgae and the commercial producer Fiskey Ltd. has used the product exclusively for environmental shading during first feeding of halibut larvae since 2003.

2. Introduction

Atlantic halibut is one of the most valued flatfish species and is produced in aquaculture in a number of countries of the Northern Hemisphere. It is a cold water species, characterised by a very long larval yolk-sac stage following hatching, and a long period with offering live feed (Olsen *et al.*, 1999; Shields, 2001). An overall poor survival is commonly experienced during the early life stages and occasionally resulting in a total collapse in the production of individual batches, with mean values of ~20% when calculated from hatching of yolk sac larvae (Fiskey Ltd., Hjalteyri, IS-601 Akureyri, Iceland). Marine microalgae are commonly added to the culture water environment during production of marine larvae in order to provide the appropriate shading effects needed for normal larval behaviour including grazing on the live feed offered during the first weeks of exogenous feeding (Olsen *et al.*, 1999; Fiskey Ltd.). The algae also serve as a feeding source and have been reported to be beneficial through the first stages in the rearing of marine larvae (Muller-Feuga, 2000; Shields, 2001; van der Meeren *et al.*, 2007). Marine microalgae have furthermore been suggested to positively affect the composition of the intestinal bacterial community of fish larvae as bacterial selecting factor but may, however, at the same time be carriers of opportunistic pathogens (Makridis *et al.*, 2006; Olsen *et al.*, 2000; Liao *et al.*, 2003). Organic debris from dead larva and offering live feed with the addition of microalgae represents a reservoir of organic material that has been reported to support and stimulate bacterial growth (Ritar *et al.*, 2004; Skjermo and Vadstein, 1999; Qian *et al.*, 2007). The association of *Vibrio* bacteria with organisms and organic matter in general is well documented and *Vibrio* sp. have been identified as a part of the natural bacterial community of fish, with beneficial effects suggested (Olafsen, 2001; Hansen and Olafsen, 1999; Hjelm *et al.*,

2004; Austin *et al.*, 1995). A number of *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; Hjelm *et al.*, 2004). A general understanding of the bacterial community associated with rearing of marine larvae and continuous improvements in production efficiency are therefore required for an improvement in rearing performances (Olafsen, 2001).

The present work describes a study of the cultivable bacterial community and larval survival in a large number of production units of first feeding halibut larvae with environmental shading provided by either marine microalgae or inorganic clay. Presented are 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.

3. Materials and Methods

Production methods and evaluation of larval success

A continuous production with manipulation of the photoperiod results in three distinct groups spawning at advanced and delayed periods compared to the normal group at Fiskey Ltd. The treatments were carried out in three different spawning groups with environmental shading provided by marine microalgae (1999-2000) and three different spawning groups with environmental shading provided by inorganic clay (2001-2002). 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) with enriched 24 h *Artemia franciscana* nauplii (Great Salt Lake, Utah, USA) offered 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) then cultured semi-continuously, with renewal rates between 10-50% and a mixture of ~1/3 *T. suecica* and ~2/3 *I. galbana* was added to the tank water environment prior to offering enriched 24 h and 32 h *Artemia* to larvae in two daily feedings. 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 in higher concentrations during the first weeks in feeding, with reduced quantities needed as the first feeding of larvae proceeds (algal densities ranging between 100 – 200 M mL^{-1} of tank water). The concentration of the inorganic clay (particle size ≤ 5 µm) was visually adjusted in order to achieve similar shading effects throughout the first feeding period (0.0015 – 0.0030 g L^{-1}). In cases of contamination or collapse of the algal cultures, a commercial algal paste (*Nanochloropsis* spp.; Reed Mariculture, California, USA) was applied.

Larval survival was estimated at the onset of weaning unto formulated feed and is presented as the ratio of surviving larvae 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 were kept chilled on transport to the laboratory where they were processed within 3-5 h post collection. Samples were collected prior to the first of two daily feedings with *Artemia*, following the first of two daily additions of marine microalgae (n=12) or inorganic clay (n=10). Samples

were collected from individual tank units at approximately weekly intervals for a period of 51 days through each period and processed as previously described (Bjornsdottir *et al.*, 2009). Briefly, a group of ~10 (51 days post onset of first feeding, dpff) to ~100 (0 dpff) larvae were anaesthetized using Hypnodil (51µg mL^{-1}), followed by surface sterilization in a solution of 0.1% benzalkonium chloride for 30 sec and rinsing three times in sterile 2% NaCl prior to enumeration and homogenization in a tenfold dilution of peptone-seawater containing 0.1% w/v Bacto peptone dissolved in aged seawater (seawater stored in the dark at room temperature for a minimum of three weeks prior to filtration through 1.22 µm and subsequent dilution to 70% using distilled H_2O) and the pH then adjusted to 8.6.

Analysis of the cultivable bacterial community.

Colony forming units (CFU) in the tank water and in surface sterilized larvae 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 (TCBS; Oxoid). Results are expressed as the mean numbers of CFU mL^{-1} and $larvae^{-1}$ from a minimum of seven production units at individual sampling dates. From each sample, 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 inoculated and then incubated with and without a paraffin seal at 15 °C with results recorded after 7, 14 and 28 days.

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. and the mean values from a minimum of seven production units were used to analyse the difference in bacterial numbers between the groups at various sampling dates using a *t*-test. A *t*-test and was also used to compare mean values of larval survival in production units with environmental shading provided by marine microalgae and inorganic clay. Larval survival in individual production units containing larvae of common silo origin was compared using the Chi-square test. Differences were considered statistically significant when $p<0.05$. A regression analysis was used to analyse the relationship between larval survival and bacterial numbers at selected sampling points.

4. Results

Cultivable bacteria in tank water

A rapid increase in bacterial numbers was observed during the first two weeks of offering live *Artemia* to larvae (Fig.1A). The highest numbers of CFU on MA were observed 15 days post the onset of first feeding (dpff) with the addition of 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 after one ($p=0.009$) as well as thirteen ($p\leq0.001$), fifteen ($p=0.006$) and twenty eight ($p=0.014$) days in feeding with the addition of inorganic clay as compared to marine microalgae.

Figure 1

The CFU on TCBS were generally 2–3 log-units lower than the observed CFU on MA in tank water samples (Fig. 1A and 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}) and 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 one (p=0.01) as well as thirteen (p=0.025), fifteen (p=0.024), twenty one (p=0.02) and thirty six (p=0.03) days in feeding with addition of inorganic clay as compared to marine microalgae. A positive relationship was furthermore observed between the CFU on MA and TCBS in tank water after one day (R^2= 0.81) and three weeks (R^2= 0.99) in feeding with the addition of marine algae and after one week in feeding with the addition of inorganic clay (R^2=0.78).

Grouping of the isolates to fermentative and non-fermentative Gram negative rods revealed a relative dominance of fermentative Gram negative bacteria in the tank water from both groups (Fig. 1C and 1D). The numbers of fermentative groups tended to be higher with the addition of marine algae compared with inorganic clay, but the difference between the two groups was not found to be significant at any of the sampling dates (p=0.07-0.2). No significant difference in the numbers of non-fermentative Gram negative bacteria in the culture water was observed when the two groups were compared (p=0.06-0.8).

Cultivable bacteria of surface sterilized larvae

Statistical analysis revealed significantly lower CFU on MA in larvae after one (p=0.001) and thirty six (p=0.017) days, but significantly higher after thirteen (p=0.008) days in feeding with environmental shading provided by inorganic clay as compared to marine

microalgae (Fig. 2A). The highest numbers of CFU on MA were reached after two weeks in feeding with the addition of inorganic clay ($6.8*10^5 \pm 10^5$ CFU larvae^{-1}), 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}). The highest numbers of CFU on TCBS in surface sterilized larvae were reached towards the end of the first feeding period with the addition of marine microalgae ($1.4*10^5 \pm 10^5$ CFU larvae^{-1}), but already after two weeks in feeding with the addition of inorganic clay ($2.5*10^4 \pm 10^4$ CFU larvae^{-1}) (Fig. 2B). No significant differences were observed between the two groups at any of the sampling points (p=0.06-0.9). A positive relationship was, however, observed between the numbers of CFU on MA and TCBS in larvae after one and three weeks in feeding as well as at the end of the first feeding period with the addition of inorganic clay (R^2=0.74, 0.82 and 0.92, respectively), but only after the first day in feeding with the addition of marine algae (R^2=0.97).

Figure 2

No significant relationship was observed between larval survival in individual production units at the end of the first feeding period and the numbers of CFU on MA found in larvae from the respective production units after two weeks in feeding (Table 1). No relationship was furthermore observed between the numbers of CFU on TCBS and MA at this sampling point (p>0.05). 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).

Table 1

Grouping of the isolates to fermentative and non-fermentative heterotrophic Gram negative bacteria revealed significantly higher numbers of fermentative bacteria in larvae already after one day in feeding (p=0.012) as well as after five weeks in feeding (p=0.032), but significantly lower numbers after two weeks in feeding (p=0.019) with environmental shading provided by marine algae as compared with inorganic clay (Fig. 2C). The numbers of non-fermentative bacteria were not found to differ significantly between the two groups at any of the sampling points (Fig. 2D).

An analysis of the cultivable bacterial community of the live feed revealed no significant differences during the two periods studied, with mean numbers of $2.1*10^7$ $\pm10^7$ CFU on MA g^{-1} and $2.4*10^6$ $\pm10^6$ CFU on TCBS g^{-1} observed during the period with environmental shading provided by marine microalgae (n=28) and $0.4*10^7$ $\pm10^7$ CFU on MA g^{-1} and $1.1*10^6$ $\pm10^5$ CFU on TCBS g^{-1} observed during the period with environmental shading provided by inorganic clay (n=20).

Larvae of common silo origin

An analysis of the culture water in tanks containing larvae of common silo origin (sibling tank units) revealed similar numbers of CFU on MA throughout the period (Fig. 3A). Relatively lower numbers of CFU on TCBS were, however, observed in the culture water during the first three weeks in feeding with environmental shading provided by inorganic clay as compared to marine algae (Fig. 3B). Furthermore, lower numbers of CFU on both MA and TCBS were observed in larvae after the first days in feeding with environmental shading provided by inorganic clay as compared to marine algae (Fig. 3C and 3D).

Figure 3

Survival of first feeding larvae

The survival of first feeding larvae in individual tank units varied considerably, with values ranging between 0-75% in individual production units during both periods (Table 1). The mean values of larval survival were found to be 40.3% ±30 and 45.2% ±24 with environmental shading provided by marine algae and inorganic clay, respectively. The difference between the two groups was not found to be significant (p=0.7). Furthermore, the use of inorganic clay as compared with marine microalgae for environmental shading in tanks containing larvae of common silo origin was not found to affect larval survival (Fig. 3).

5. Discussion

The study summarizes the results from a three year examination of the cultivable bacterial community and survival of first feeding halibut larvae and their tank water environment at a commercial hatchery, with environmental shading provided by either marine algae or inorganic clay. The use of inorganic clay for environmental shading was not found to affect larval survival. The results furthermore indicate elevated bacterial numbers following introduction of algae into the system that may be explained by an increase in organic nutrient availability that has been found to support the multiplication of opportunistic bacteria (Nakase and Eguchi, 2007; Olafsen, 2001; Pinhassi *et al.*, 2004). The highest bacterial numbers in tank water were observed after ~two weeks in feeding with the addition of marine algae and coinciding with the period of a sudden and extensive death of larvae commonly experienced in the production of halibut larvae at Fiskey Ltd. A stable

environment has been suggested to prevent an unpredictable development of bacterial communities (Olsen *et al.*, 2000). No relationship was, however, observed between larval survival and the highly variable bacterial numbers observed especially in the culture water environment during the first two weeks in feeding. 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 has been suggested to impact the survival and overall quality of larvae (Nakase *et al.*, 2007; Olafsen, 2001). The microalgae are, however, believed to improve the nutritional value of the live feed used in fish larvae culture 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; Olsen *et al.*, 2000). A positive relationship was observed between the numbers of CFU on MA and TCBS in tank water after one and three weeks in feeding with environmental shading provided by inorganic clay, but only after the first day in feeding with the addition of marine algae. The lack of relationship between the numbers of CFU on MA and TCBS in larvae after two weeks in feeding may indicate a shift in the groups dominating the bacterial community of larvae during this period as has previously been suggested (Bergh *et al.*, 1994). Following this period, a further death of larvae is rarely to be expected (Olsen *et al.*, 1999) and the similar bacterial numbers found in the tank water throughout the period may indicate improved stability when the first feeding proceeds as previously observed by other authors (Eddy and 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; Olafsen, 2001). 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; Verner-Jeffreys *et al.*, 2006; Nakase and Eguchi, 2007). 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). The 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 (Olsen *et al.*, 2000; Verner-Jeffreys *et al.*, 2003). Hence, enumeration of *Vibrio* spp. is highly important when studying the bacterial community during the early production stages of marine larvae. Presumptive *Vibrio* bacteria dominated the cultivable bacterial community of larvae in the present study, but the addition of inorganic clay was found to result in reduced numbers of presumptive *Vibrio* in tank water as compared with the addition of marine microalgae, however, without affecting larval survival. The lower initial numbers of bacteria and the sudden increase in the numbers of cultivable bacteria observed in surface sterilised larvae during the first days in feeding with addition of inorganic clay may, however, indicate an immediate colonization of the bacterial community carried to the larvae through the live feed as previously 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; Shields, 2001).

Using marine microalgae for the necessary environmental shading during the first weeks of offering exogenous feed to marine larvae and keeping the continuous algal cultures going represents a considerable addition to the production costs, with the main cost items

represented by manpower and housing in addition to the necessary device and electricity costs. While the inorganic clay may be sterilized prior to use, the use of marine microalgae furthermore represents a pending risk of contamination of the algal cultures. The periodic collapses of the algal cultures commonly experienced and adversely affecting the overall production safety may therefore be bypassed with only minimal costs represented by the purchase of inorganic clay. Hence, there has been a significant pressure to replace the algae and the annual production costs have been reduced by 50-100.000 Euro by the use of inorganic clay as compared with marine microalgae at Fiskey Ltd. Marine microalgae are, however, still considered an essential nutritional supplement and are commonly applied in intensive production of marine fish larvae (Palmer *et al.*, 2007; Rocha *et al.*, 2008; van der Meeren *et al.*, 2007).

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References

Austin, B., Baudet, E. & Stobie, M. (2006) Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. *J. Fish Dis.,* **15,** 55-61.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I. & Griffith, D.R.W. (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *J. Fish Dis.,* **18,** 93-96.

Bergh, O., Naas, K.E. & Harboe, T. (1994) Shift in the intestinal microflora of Atlantic halibut (*Hippoglossus hippoglossus*) larvae during first feeding. *Can. J. Fish Aquat. Sci.,* **51,** 1899-1903.

Bjornsdottir, R., Johannsdottir, J., Coe, J., Smaradottir, H., Agustsson, T., Sigurgisladottir, S. & Gudmundsdottir, B.K. (2009) Survival and quality of halibut larvae (*Hippoglossus hippoglossus* L.) in intensive farming: possible impact of the intestinal bacterial community. *Aquaculture,* **286,** 53-63.

Eddy, S.D. & Jones, S.H. (2002) Microbiology of summer flounder *Paralichthys dentatus* fingerling production at a marine fish hatchery. *Aquaculture,* **211,** 9-28.

Egidius, E. (1987) Vibriosis: Pathogenicity and pathology. A review. *Aquaculture,* **67,** 15-28.

Hansen, G.H. & Olafsen, J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. *Microb. Ecol.,* **38,** 1-26.

Hjelm, M., Riaza, A., Formoso, F., Melchiorsen, J. & Gram, L. (2004) Seasonal incidence of autochthonous antagonistic *Roseobacter* spp. and Vibrionaceae strains in a turbot larva (*Scophthalmus maximus*) rearing system. *Appl. Environ. Microbiol.,* **70,** 7288-7294.

Hongoh, Y., Yuzawa, H., Ohkuma, M. & Kudo, T. (2003) Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. *FEMS Microbiol. Letters,* **221,** 299-304.

Lemos, M.L., Toranzo, A.E. & Barja, J.L. (1985) Modified medium for the oxidation-fermentation test in the identification of marine-bacteria. *Appl. Environ. Microbiol.,* **49,** 1541-1543.

Liao, W.R., Lin, J.Y., Shieh, W.Y., Jeng, W.L. & Huang, R. (2003) Antibiotic activity of lectins from marine algae against marine vibrios. *Journal of Industrial Microbiology and Biotechnology,* **30,** 433-439.

Makridis, P., Costa, R.A. & Dinis, M.T. (2006) Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched *Artemia* metanauplii. *Aquaculture,* **255,** 76-81.

Marques, A., Thanh, T.H., Sorgeloos, P. & Bossier, P. (2006) Use of microalgae and bacteria to enhance protection of gnotobiotic *Artemia* against different pathogens. *Aquaculture,* **258,** 116-126.

Muller-Feuga, A. (2000) The role of microalgae in aquaculture: situation and trends. *Journal of Applied Phycology,* **12,** 527-534.

Nakase, G. & Eguchi, M. (2007) Analysis of bacterial communities in *Nannochloropsis* sp. cultures used for larval fish production. *Fish. Sci.,* **73,** 543-549.

Nakase, G., Nakagawa, Y., Miyashita, S., Nasu, T., Senoo, S., Matsubara, H. & Eguchi, M. (2007) Association between bacterial community structures and mortality of fish larvae in intensive rearing systems. *Fish. Schi.,* **73,** 784-791.

Olafsen, J.A. (2001) Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture,* **200,** 223-247.

Olsen, A.I., Olsen, Y., Attramadal, Y., Christie, K., Birkbeck, T.H., Skjermo, J. & Vadstein, O. (2000) Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. *Aquaculture,* **190,** 11-25.

Olsen, Y., Evjemo, J.O. & Olsen, A. (1999) Status of the cultivation technology for production of Atlantic halibut (*Hippoglossus hippoglossus*) juveniles in Norway/Europe. *Aquaculture,* **176,** 3-13.

Palmer, P.J., Burke, M.J., Palmer, C.J. & Burke, J.B. (2007) Developments in controlled green-water larval culture technologies for estuarine fishes in Queensland, Australia and elsewhere. *Aquaculture,* **272,** 1-21.

Pinhassi, J., Sala, M.M., Havskum, H., Peters, F., Guadayol, O., Malits, A. & Marrase, C. (2004) Changes in bacterioplankton composition under different phytoplankton regimens. *Appl. Environ. Microbiol.,* **70,** 6753-6766.

Qian, P.-Y., Lau, S., Dahms, H.-U., Dobretsov, S. & Harder, T. (2007) Marine biofilms as mediators of colonization by marine macroorganisms: implications for antifouling and aquaculture. *Mar. Biotechnol.,* **9,** 399-410.

Ritar, A.J., Dunstan, G.A., Nelson, M.M., Brown, M.R., Nichols, P.D., Thomas, C.W., Smith, E.G., Crear, B.J. & Kolkovski, S. (2004) Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation. *Aquaculture,* **239,** 351-373.

Rocha, R.J., Ribeiro, L., Costa, R. & Dinis, M.T. (2008) Does the presence of microalgae influence fish larvae prey capture? *Aquacul. Res.,* **39,** 362-369.

Shields, R.J. (2001) Larviculture of marine finfish in Europe. *Aquaculture,* **200,** 55-88.

Skjermo, J. & Vadstein, O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture,* **177,** 333-343.

Tolomei, A., Burke, C., Crear, B. & Carson, J. (2004) Bacterial decontamination of on-grown *Artemia*. *Aquaculture,* **232,** 357-371.

van der Meeren, T., Mangor-Jensen, A. & Pickova, J. (2007) The effect of green water and light intensity on survival, growth and lipid composition in Atlantic cod (*Gadus morhua*) during intensive larval rearing. *Aquaculture,* **265,** 206-217.

Verner-Jeffreys, D.W., Nakamura, I. & Shields, R.J. (2006) Egg-associated microflora of Pacific threadfin, *Polydactylus sexfilis* and amberjack, *Seriola rivoliana*, eggs. Characterisation and properties. *Aquaculture,* **253,** 184-196.

Verner-Jeffreys, D.W., Shields, R.J., Bricknell, I.R. & Birkbeck, T.H. (2003) Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. *Aquaculture,* **219,** 21-42.

Table 1

Numbers of colony forming units (CFU) in larvae sampled from individual production units thirteen to fifteen days post onset of first feeding, with environmental shading provided by either marine microalgae or inorganic clay. Also shown is larval survival in the corresponding production units presented as the ratio (%) of surviving larvae calculated from the total number of yolk sac larvae transferred to each tank at the onset of feeding.

	Marine microalgae		
	CFU (MA)[†]	**CFU (TCBS)[††]**	**survival (%)**
	$1.1*10^4$	$0.4*10^3$	45
	$4.7*10^4$	$35.0*10^3$	75
	$13.3*10^4$	$7.1*10^3$	73
	$6.0*10^4$	$7.9*10^3$	70
	$5.6*10^4$	$4.5*10^3$	64
	$7.6*10^4$	$59.6*10^3$	33
	$4.7*10^4$	$2.1*10^3$	30
	$14.9*10^4$	$4.0*10^3$	7
	$0.5*10^4$	$0.5*10^3$	5
	$17.2*10^4$	$3.7*10^3$	10
	$11.0*10^4$	$0.7*10^3$	0
	$16.6*10^4$	$43.3*10^3$	71
Mean	***$8.6*10^4$***	***$14.1*10^3$***	***40.3***
S.D.	***$6*10^4$***	***$20*10^3$***	***30***

	Inorganic clay		
	CFU (MA)[†]	**CFU (TCBS)[††]**	**survival (%)**
	$7.4*10^4$	$1.4*10^3$	25
	$20.1*10^4$	$4.2*10^3$	23
	$20.1*10^4$	$0.9*10^3$	70
	$15.6*10^4$	$8.5*10^3$	0
	$2.2*10^4$	$1.4*10^3$	52
	$3.6*10^4$	$1.3*10^3$	75
	$7.1*10^4$	$2.6*10^3$	47
	$5.3*10^4$	$1.3*10^3$	66
	$3.0*10^4$	$2.0*10^3$	54
	$39.7*10^4$	$5.0*10^3$	36
Mean	***$12.4*10^4$***	***$2.8*10^3$***	***44.8***
S.D.	***$12*10^4$***	***$2*10^3$***	***24***

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

Figure legends

Figure 1. Numbers of colony forming units (CFU) in tank water with environmental shading provided by marine algae (grey bars) or inorganic clay (white bars): (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. ($n \geq 7$) at selected days post onset of first feeding. Statistically significant differences in bacterial numbers between the two groups are denoted with an asterisk (*).

Figure 2. Numbers of colony forming units (CFU) in surface sterilized larvae in tanks with environmental shading provided by marine algae (grey bars) or inorganic clay (white bars): (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. ($n \geq 7$) at selected days post onset of first feeding. Statistically significant differences in bacterial numbers between the two groups are denoted with an asterisk (*).

Figure 3. Numbers of colony forming units (CFU) on marine agar (MA) (A, C) and thiosulphate citrate bile salt sucrose (TCBS) agar (presumptive *Vibrio* bacteria) (B, D). Shown are CFU numbers in tank water (A, B) and surface sterilized larvae (C, D) from sibling tank units containing larvae of common silo origin. Shown are mean numbers of CFU ± S.D. in two tanks with environmental shading provided by marine algae and one tank with environmental shading provided by inorganic clay.

Figures

Figure 1

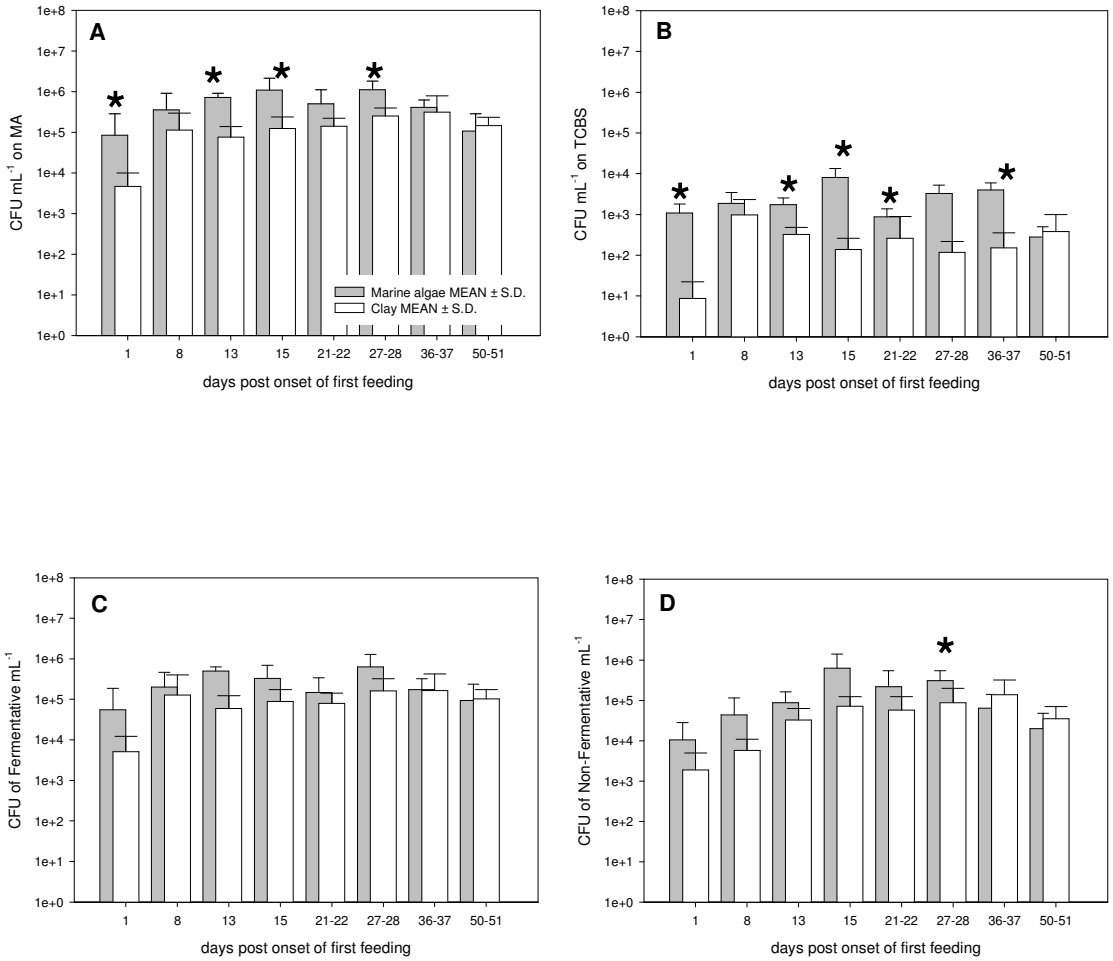


Figure 2

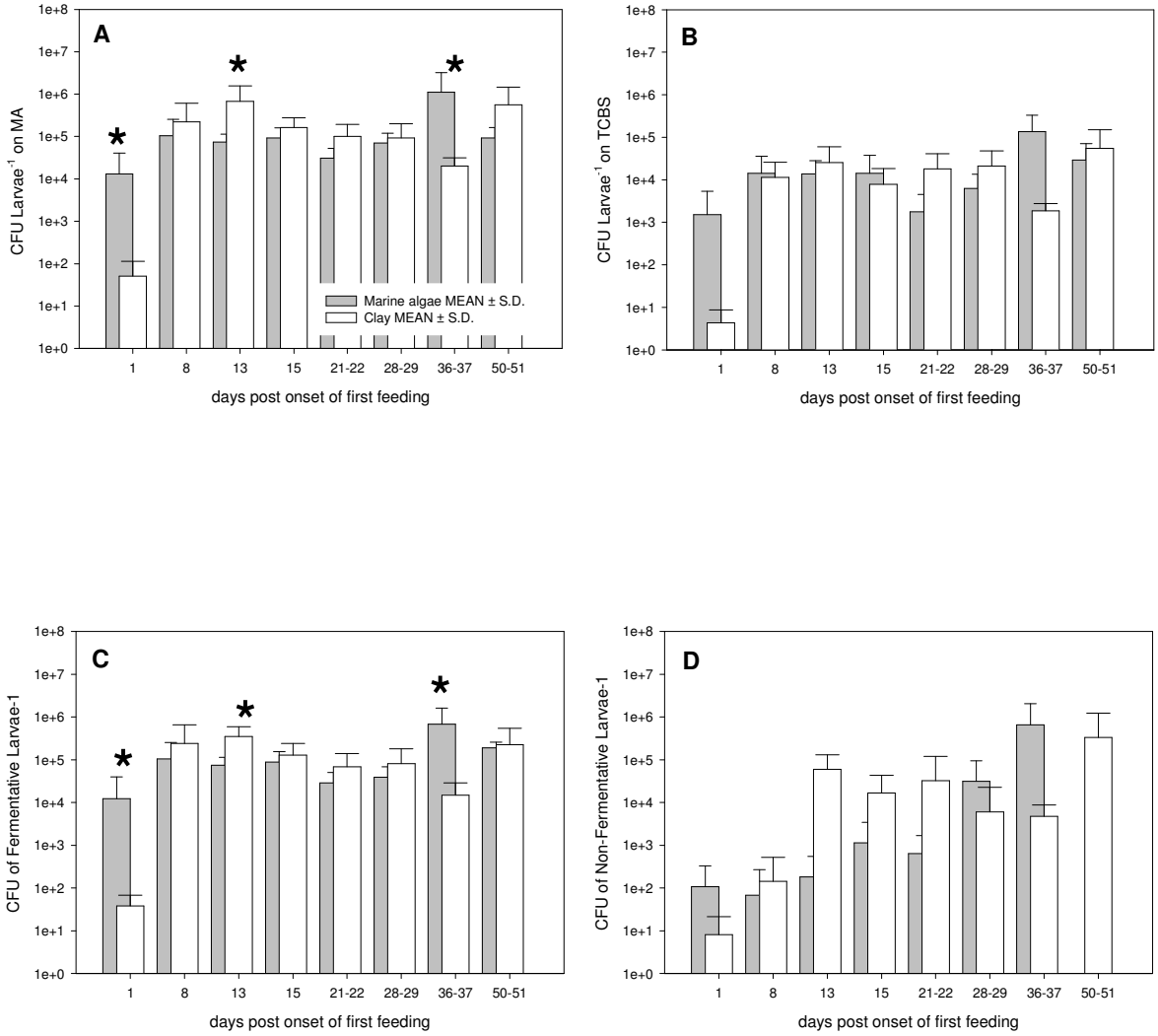
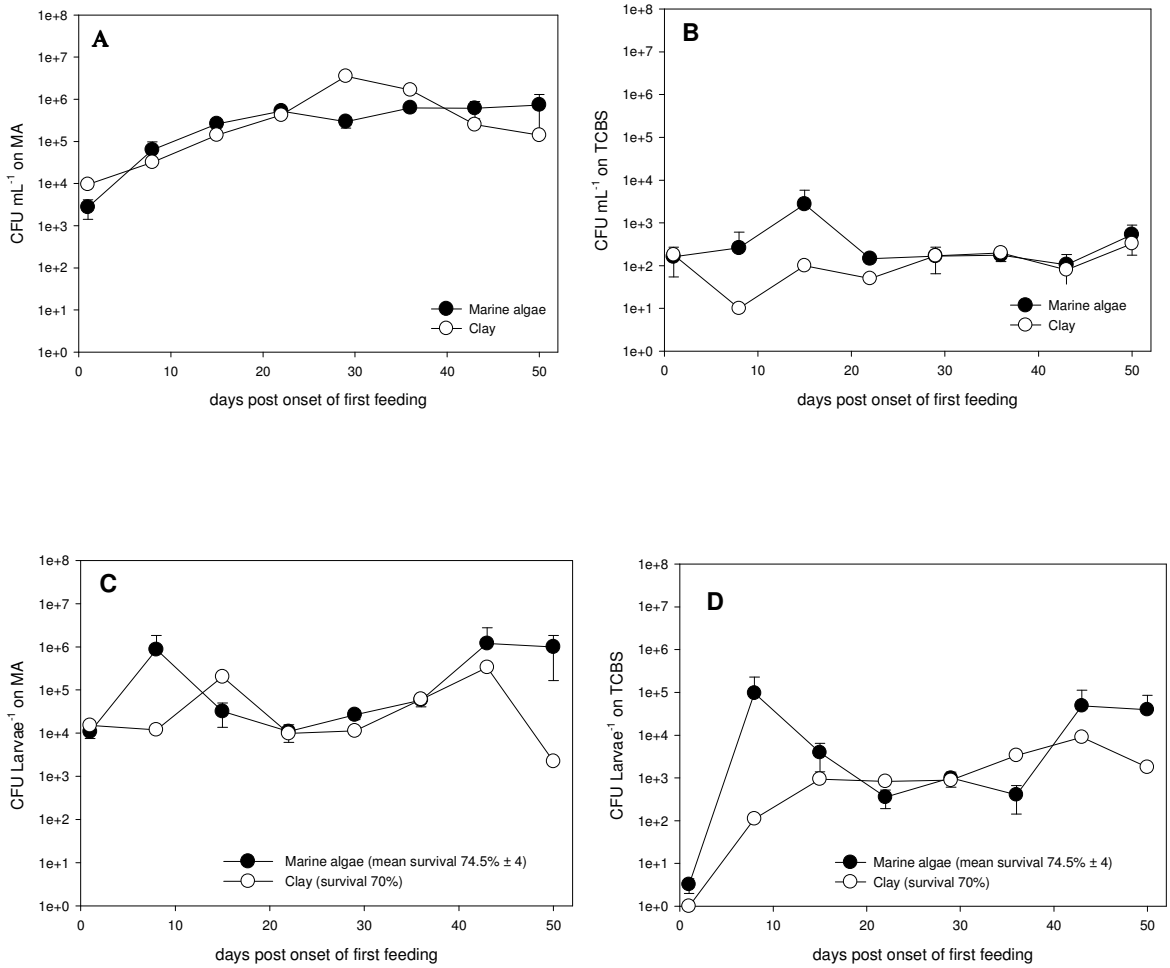

A
B
C
D
CFU Larvae-1 on MA
CFU Larvae-1 on TCBS
CFU of Fermentative Larvae-1
CFU of Non-Fermentative Larvae-1
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
Marine algae MEAN ± S.D.
Clay MEAN ± S.D.
1
8
13
15
21-22
28-29
36-37
50-51
days post onset of first feeding

Figure 3


A
CFU mL-1 on MA
Marine algae
Clay
days post onset of first feeding
B
CFU mL-1 on TCBS
Marine algae
Clay
days post onset of first feeding
C
CFU Larvae-1 on MA
Marine algae (mean survival 74.5% ± 4)
Clay (survival 70%)
days post onset of first feeding
D
CFU Larvae-1 on TCBS
Marine algae (mean survival 74.5% ± 4)
Clay (survival 70%)
days post onset of first feeding