ELSEVIER

Aquaculture 190 (2000) 11–25

Aquaculture

www.elsevier.nl/locate/aqua-online

# Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*

A.I. Olsen [a,*], Y. Olsen [b], Y. Attramadal [c,1], K. Christie [d], T.H. Birkbeck [d], J. Skjermo [a], O. Vadstein [b]

[a] *SINTEF Fisheries and Aquaculture, N-7465 Trondheim, Norway*
[b] *Trondhjem Biological Station, Norwegian University of Science and Technology, N-7491 Trondheim, Norway*
[c] *Stolt Sea Farm A.S. Øye, N-4484 Øyestranda, Norway*
[d] *Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Joseph Black Building, Glasgow G12 8QQ, UK*

Received 8 November 1999; received in revised form 10 April 2000; accepted 12 April 2000

**Abstract**

*Artemia franciscana* cultivated for 2 days for use as feed for Atlantic halibut larvae (*Hippoglossus hippoglossus* L.) had high numbers of associated bacteria with high counts of presumptive vibrios (TCBS-agar) and haemolytic bacteria (blood agar). The food used for the cultivation of *A. franciscana*, fish meal and fish oil, is a rich substrate for proliferation of opportunistic bacteria. A procedure for incubation of 2-day-old *A. franciscana* with the microalga *Tetraselmis* sp. was used to test the hypothesis that a change in the gut bacterial flora of *A. franciscana* could be induced by incorporating algal cells into the diet. It was expected that this would reduce bacterial proliferation in the *Artemia*, and introduce new bacterial species from the algal culture resulting in a more stable and diverse flora associated with the animals. The standard 2-day-old *A. franciscana* had 24,000 ± 10,700 colony forming units (CFU, mean ± STD) per animal with presumptive vibrios and haemolytic bacteria constituting 58% and 10% of the total, respectively. The flora was dominated by *Vibrio alginolyticus*-like bacteria that constituted 34 out of 40 isolates tested. During 4-h incubation the numbers of associated bacteria were reduced, on average by 75%, with the flora less dominated by *V. alginolyticus*. The relative diversity ($J'$) of the associated bacterial

* Corresponding author. Tel.: +47-73596387; fax: +47-73596363.
*E-mail address:* atle.i.olsen@fish.sintef.no (A.I. Olsen).
[1] Present address: Risørfisk, P.O. Box 283, Kranveien 60C, 4951 Risør, Norway.


PII: S0044-8486(00)00396-3

flora increased from 0.17 to 0.40. A 24-h incubation to simulate first feeding conditions for halibut larvae gave further decreases in bacterial numbers associated with both treated and untreated *A. franciscana*. The prevalence of *Vibrio* was lower in these two samples, and the flora was now dominated by isolates of other genera. The relative diversity of the flora of *A. franciscana* increased to 0.82 and 0.63 for samples previously incubated with *Tetraselmis* sp. and those which were not, respectively. Data from a first feeding experiment showed that the bacterial flora of the live feed was directly transferred to the larvae. The algal component was shown to influence the numbers and composition of the associated flora of the live feed, giving *A. franciscana* with lower numbers of bacteria as well as a more diverse bacterial community.


*Keywords:* *Artemia franciscana*; *Tetraselmis* sp.; *Vibrio*; Haemolytic bacteria; Relative diversity; *Hippoglossus hippoglossus*; Atlantic halibut; Larval rearing

## 1. Introduction

During first feeding of marine fish species high mortality rates have been reported, and both nutritional and bacteriological explanations have been given for this. Microbiological problems seem to be attributed to proliferation of opportunistic pathogenic bacteria rather than specific obligate pathogenic bacteria (Skjermo and Vadstein, 1999). An important source of bacteria is the live feed (Muroga et al., 1987; Perez Benavente and Gatesoupe, 1988; Nicolas et al., 1989; Keskin et al., 1994; Olsen et al., 1999) and a correlation between bacterial counts on the live feed and larval mortality has been reported (Perez Benavente and Gatesoupe, 1988; Munro et al., 1999). Often, the bacteria associated with live feed show haemolytic activity (Olsen et al., 1999), and in larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.) (Olsen et al., 1999) and turbot (*Scophtalmus maximus* L.) (Nicolas et al., 1989) these bacteria originate from the live feed.

Several techniques have been tested to improve the microbiological quality of the live feed, and freshwater baths (Rodríguez et al., 1991), chemical disinfection (GomezGil-RS et al., 1994) and UV treatment (Munro et al., 1999) have been reported effective in reducing the number of live feed-associated bacteria. The bacterial load on both rotifers and *Artemia* has been reduced with antibiotics (Perez Benavente and Gatesoupe, 1988; Rodríguez et al., 1991), and the use of rotifers with reduced bacterial load improved the survival of turbot larvae during first feeding (Perez Benavente and Gatesoupe, 1988). Good agreement between the bacterial flora of the rotifers and the cultivation water indicates that the flora of rotifers can be manipulated experimentally (Skjermo and Vadstein, 1993a; Makridis et al., 2000).

Microalgae have been shown to have positive effects on fish larvae during first feeding, and this has been attributed to improvement in the nutritional quality of the live feed (Reitan et al., 1993), and to microbial factors (Skjermo and Vadstein, 1993b; Støttrup et al., 1995). Addition of algae to the water in fish tanks alters the composition of the bacterial flora associated with larvae (Skjermo and Vadstein, 1993b; Bergh et al., 1994), and bacterial growth and composition of the flora in the water depends on both the algal species and state of growth of the algae (Salvesen et al., 2000).

How microalgae affect the bacterial flora of live feed animals has so far not been studied. One hypothesis is that microalgae contribute to a change in the bacterial composition of live feed organisms, and also reduce numbers, by expelling the gut contents which act as a substrate for bacterial proliferation. Algae can possibly also affect the bacterial community in the live feed by production of antibacterial substances, as reported for *Tetraselmis* (Duff and Bruce, 1966; Kellam and Walker, 1989; Austin and Day, 1990; Austin et al., 1992).

Here we report a technique for reduction of bacterial numbers and a change in the bacterial composition in the gut of juvenile *A. franciscana* by use of *Tetraselmis* sp. It was important to develop a simple procedure that could be incorporated into the daily routines at first-feeding facilities. In preliminary experiments, 4-h incubation of 2-day-old *A. franciscana* with *Tetraselmis* sp. resulted in reduced numbers of associated bacteria, and reduced the percentage of haemolytic bacteria and counts on TCBS agar. Subsequent experiments were done to further characterise and quantify the reproducibility of the changes in the microflora of the live feed after the rinsing procedure, and during simulated first feeding conditions. This was done to investigate changes occurring during the time after addition of the live feed to the fish tanks. In addition, a small first feeding experiment to investigate the possible impact of this treatment on the microflora of Atlantic halibut was conducted.

## 2. Materials and methods

The experiments were performed at SINTEF Fisheries and Aquaculture in Trondheim and at Stolt Sea Farm (SSF) Øye.

### 2.1. Cultivation of algae and A. franciscana

At SINTEF the microalga *Tetraselmis* sp., our own isolate, was cultivated in 300-l volumes with f/2 medium (Guillard and Ryther, 1962) at 28°C, cultures being aerated with air plus added $CO_2$ ($< 0.1\%$). The algae were cultivated semi-continuously at $\mu = 0.5\mu_{max}$ as described by Reitan et al. (1994). At SSF Øye the same *Tetraselmis* sp. was produced at 28°C with aeration in 300-l cultures in plastic bags for 4–7 days, with 3/4 of the volume harvested and seawater and fertiliser added every 2 days (Walne, 1966).

The 2-day-old *Artemia franciscana* (EG quality, INVE Aquaculture, Belgium) produced at SINTEF were decapsulated and hatched according to standard procedures (Sorgeloos et al., 1986). The incubation temperature was 25–26°C, and the juveniles were fed micronised fish meal (Norwegian Herring Oil and Meal Industry Research Institute, Bergen, Norway), and fish oil concentrate (EPAX 1040 TG, Pronova Biocare, Sandefjord, Norway) with added emulsifier (Tween 20, 10%, Sigma-Aldrich, St Louis, MO, USA) and α-tocopherol (1%, Sigma-Aldrich), as described by Olsen et al. (1999).

For the experiments at SSF Øye, the nauplii (EG quality, INVE Aquaculture) were hatched, separated from empty cyst shells and rinsed with seawater at 25°C. The 2-day-old animals were produced in 3000-l volumes at a density of approximately 100

animals $ml^{-1}$. The tanks were fed about 0.27 g $l^{-1}$ SSF fishmeal with added 8% cod liver oil each day. After harvesting, the animals were carefully washed in seawater (25°C) before incubation with *Tetraselmis*.

### 2.2. Experimental conditions

The procedure was designed to allow the 2-day-old *A. franciscana* to ingest enough microalgae to change the gut contents several times to eliminate faeces and remains of the fish meal and oil, and entail a moderate starvation period at the end of the incubation. The gut filling time of 1.1 mm long *A. franciscana* is about 30 min (Nimura, 1989), and as a 4-h period gave promising results in preliminary experiments this time was used, but algal and animal density were optimised. The following conditions were chosen as standard:

| | |
|---|---|
| Algal species | *Tetraselmis* sp. |
| Algal concentration | 7 mg C $l^{-1}$ at start |
| Animal density | $100 \pm 10$ $ml^{-1}$ |
| Temperature | $20 \pm 1$°C |
| Time | 4 h |

The animals were washed thoroughly in UV-treated seawater before and after incubation, until all coloration of the water from algae and faeces was removed. Algal concentration was adjusted based on spectrophotometer (Shimadzu Double Beam Spectrophotometer, UV-150-3) readings at 750 nm, and using an empirical relationship between absorption and carbon content (Kjell Inge Reitan, unpublished results). The cultures were aerated strongly to achieve good oxygenation. The temperature was reduced for partial acclimation to the first feeding conditions of Atlantic halibut larvae (11–12°C) and to preserve the nutritional value of the live feed (Danielsen et al., 1995).

For simulation of first feeding conditions, *A. franciscana* were transferred to 10-l buckets and kept under first feeding conditions with the same light intensity, temperature ($12 \pm 1$°C), slight aeration, algal density (1–2 mg C $l^{-1}$ *Tetraselmis* sp.) and a density of *A. franciscana* of approximately 1 $ml^{-1}$. As controls, untreated 2-day-old *A. franciscana* were transferred to the same conditions.

Samples for bacteriological studies were taken from the algal culture (*Tsp*), of *A. franciscana* before (*A*) and after the 4-h incubation (*TA*), and after the 24-h simulation experiment (*A24* and *TA24*). The experiment was repeated twice at the two different laboratories, Stolt Sea Farm Øye, and SINTEF Fisheries and Aquaculture. From the final experiment at SINTEF, pure cultures were obtained from M65 agar plates and characterised phenotypically.

### 2.3. Bacteriological methods

Bacteria associated with algae, *A. franciscana* and Atlantic halibut larvae were quantified as colony forming units (CFU) using three different types of agar. The total number of culturable bacteria was estimated by plating on M65 seawater agar (0.5 g peptone, 0.5 g tryptone, 0.5 g yeast extract, 15 g agar, 200 ml distilled water, and 800

ml sterile seawater, sterilised at 121°C for 20 min, Slaatebræk, 1975). To estimate the number of presumptive *Vibrio* spp., TCBS Cholera Agar (Oxoid) was used, and the number of bacteria with haemolytic activity was estimated using blood agar base (Merck) with added calf blood (50 ml $l^{-1}$ medium) and NaCl (15 g $l^{-1}$). *A. franciscana* were washed in 5 ml sterile seawater (SSW) on a plankton net (100 μm) for 1 min and a known number (20–30 individuals) were picked with a sterile 100-μl pipette and transferred to a Potter–Elevhjem tissue grinder. The samples were homogenised, the volume adjusted to 10 ml, and thereafter serially diluted. All dilutions were plated out in triplicate and the plates were incubated in darkness at 20°C and counted after 2 and 7 days.

Eighteen plates were used for the isolation to establish pure bacterial cultures for phenotypic characterisation (two dilutions from *A24*, one dilution from the rest). From each agar plate circa 12 colonies per plate were picked at random using a grid, plated out on marine agar and examined by microscopy. The plates for sample *TA24* contained only 17 colonies so all were used.

Two isolates were Gram-positive (1 Gram-positive coccus in *A24* and 1 Gram-positive bacillus in *A*), and the remaining 183 isolates were Gram-negative. These were analysed by the following criteria: morphology (rod or coccus), pigmentation (marine agar), luminescence, swarming, motility, growth on TCBS agar, sucrose fermentation (TCBS), growth on CLED agar, oxidase, Hugh and Leifson O/F test, *o*-nitrophenyl-β-D-galactopyranoside hydrolysis (ONPG, measuring β-galactosidase activity), Voges-Proskauer and Indole (Cowan, 1974; Furniss et al., 1979). All were non-luminescent Gram-negative rods, and these criteria were omitted in analysis of the populations. In addition, 156 of the isolates were tested for their antibiotic sensitivity pattern using Mastring diagnostic discs M11, M14 and M46 (Mast Diagnostics, Merseyside, UK) with chloramphenicol (25 μg), erythromycin (5 μg), fusidic acid (10 μg), methicillin (10 μg), novobiocin (5 μg), penicillin G (1 unit), streptomycin (10 μg), tetracycline (25 and 100 μg), ampicillin (10 and 25 μg), cephalothin (5 μg), colistin sulphate (25 μg), gentamicin (10 μg), sulphatriad (200 μg), cotrioxazole (25 μg), nitrofurantoin (50 μg), ticarcillin (75 μg), nalidixic acid (30 μg), trimethoprim (2.5 μg) and sulphamethoxazole (50 μg), providing 21 further characteristics for each strain, and the dendrogram analysis to compare properties of the bacteria from each source relied upon a total of 33 criteria.

### 2.4. Data analysis

The data were recorded in binary form and analysed using the simple matching coefficient of Sokal and Michener (1958). Dendrograms were obtained using unweighted average kinage clustering (Sneath and Sokal, 1973).

The relative diversity among the isolates (at 85% similarity; *J'*) was calculated using Shannon's index (Zar, 1996). For 26 isolates, representatives of the phena from dendrogram analysis, a further 27 biochemical tests were applied to aid identification of the organisms: amylase, gelatinase, aesculin hydrolysis, arginine decarboxylase, lysine decarboxylase, ornithine decarboxylase, nitrate reduction, acid from the carbohydrates: arbutin, salicin and sucrose, utilisation of the single carbon sources: L-arabinose,

Table 1
Summary of number of isolates used in the different tests

| Group | Total isolates | Number tested for antibiotic sensitivity | Number used in further biochemical tests |
|---|---|---|---|
| *A* | 40 | 36 | 7 |
| *TA* | 41 | 30 | 10 |
| *TA24* | 16 | 16 | 3 |
| *A24* | 47 | 36 | 4 |
| *Tsp* | 39 | 38 | 2 |
| Total | 183 | 156 | 26 |

D-cellobiose, D-galactose, D-melibiose, D-gluconate, D-glucuronate, L-citrulline, L-leucine, D-glucosamine, DL-3-hydroxybutyrate and succinate, growth at 4°C and 37°C, resistance to 10 and 150 μg O/129 (Oxoid), penicillin G and polymyxin B (Furniss et al., 1979). The number of isolates tested is summarised in Table 1. The identification keys of Furniss et al. (1979), Oliver (1982) and Muroga et al. (1987) were used to assign isolates to genus level.

### 2.5. First feeding experiment

Larvae of Atlantic halibut were cultivated at Stolt Sea Farm Øye, as described by Olsen et al. (2000). The only exception was a different feeding regime with Short Term enriched (ST) nauplii (Olsen et al., 2000) for the first 6 days, then 2-day-old *A. franciscana* either incubated with *Tetraselmis* sp. for 4 h (*T2A*, two tanks), or untreated 2-day-old *A. franciscana* (*2A*; one tank). *A. franciscana* and larvae were homogenised and plated on M65 seawater agar, blood agar and TCBS as described in Olsen et al. (1999), except that the larvae were not surface disinfected. Due to problems with the M65 plates, no estimates for total bacterial numbers were obtained in this experiment.

## 3. Results

The density of *A. franciscana* in the incubation experiments was $101 \pm 9$ (mean $\pm$ STD, range 90–111) individuals $ml^{-1}$, and algal density $7.2 \pm 0.1$ mg C $l^{-1}$ at the start and $3.1 \pm 0.5$ mg C $l^{-1}$ at the end of the experiment ($n = 4$). The temperature was stable at $20 \pm 1$°C in all four experiments. The *A. franciscana* length, $1.07 \pm 0.03$ mm (four experiments) was similar in the four experiments, and did not change measurably during the 4-h treatment (checked in two of the experiments). The dry weight was $2.99 \pm 0.34$ μg individual$^{-1}$ when measured in one experiment. Total lipids and fatty acid composition did not change during 4-h incubation with the *Tetraselmis* sp. (results not shown here).

### 3.1. Quantitative and semi-qualitative changes in bacterial numbers

The 4-h incubation reduced the total CFU on all three agar media (Table 2), although for haemolytic bacteria this was not statistically significant in all experiments. After 24 h

Table 2
Summary of bacteriological data from the four different experiments. CFU individual$^{-1}$ in 2-day-old *A. franciscana* before (*A*) and after (*TA*) 4-h incubation with *Tetraselmis* sp. (*Tsp*), after 24 h at simulated first feeding conditions (*A24* and *TA24*), and in *Tsp* culture, and data from the experiment used for isolation of bacterial strains ($n = 3–6$)

| | CFU individual$^{-1}$ ± STD | Isolation exp. |
|---|---|---|
| *M65* | | |
| *A* | 24,000 ± 10,700[a] | 26,500 ± 5200[a] |
| *TA* | 5900 ± 4400[b] | 2750 ± 1400[c] |
| *A24* | 9000 ± 1550[b] | 7700 ± 2200[b] |
| *TA24* | 660 ± 460[c] | 280 ± 70[d] |
| *Haemolytic counts (blood agar, CFU individual$^{-1}$ ± STD)* | | |
| *A* | 2500 ± 1400[a,b] | 960 ± 920[a] |
| *TA* | 1750 ± 1900[b] | 170 ± 110[b] |
| *A24* | 2600 ± 2700[a] | 480 ± 260[a] |
| *TA24* | 170 ± 140[c] | 130 ± 70[b] |
| *TCBS agar (CFU individual$^{-1}$ ± STD)* | | |
| *A* | 14,000 ± 9800[a] | 18,200 ± 1200[a] |
| *TA* | 4100 ± 5200[b] | 1300 ± 390[b] |
| *A24* | 2500 ± 3600[b] | 470 ± 40[c] |
| *TA24* | 240 ± 105[c] | 120 ± 30[d] |
| *Tetraselmis sp. (CFU ml$^{-1}$ ± STD)* | | |
| M65 ($\times 10^5$) | 8.9 ± 5.7 | 13.2 ± 2.1 |
| Blood agar | 1000 ± 1100 | 500 ± 440 |
| TCBS | 840 ± 1400 | 3000 ± 300 |

Different superscript letters in columns indicate significantly different numbers ($P < 0.05$) for total, haemolytic and vibrio counts, respectively, from *A. franciscana*.

in simulated first feeding conditions, the numbers were reduced significantly for all three agars. The *TA24* contained less than 3% of the total CFU of *A*. After 24 h in first feeding conditions, the untreated *A. franciscana* (*A24*) had approximately the same total bacterial levels as *TA* ($P > 0.05$), but significantly higher numbers of haemolytic bacteria. The percentage of haemolytic bacteria increased in all samples after transfer to algae, from about 10% to almost 30% of total numbers. For CFU on TCBS, the percentage of total CFU increased from about 60% to 70% after the 4 h incubation, but then decreased after 24 h to about 36% of the total CFU. For *A*, the percentage of vibrios in the total numbers declined from 60% to 28% during the 24-h incubation. The bacterial concentration in the *Tetraselmis* sp. cultures used in the different experiments varied widely, but the percentages of TCBS and haemolytic counts were always low, i.e. approximately 1% of total CFU for both.

### 3.2. Qualitative changes and identification of isolates

At a level of 85% similarity, 10 groups of four or more bacteria were recognised in the dendrogram for 156 isolates × 33 tests but comparison at the level of 80% produced

seven major phena containing 141 of the isolates (Fig. 1). The remaining 15 isolates comprised four groups of two isolates and seven individual strains. The principal feature of the dendrogram was that the 156 isolates were divided into two roughly equal groups. The first contained all except 7 of the 66 *A* and *TA* isolates, and the second contained all 38 of the *Tetraselmis* sp. (*Tsp*) isolates and the majority (24/36) of the *A24* isolates. The *TA24* isolates were more evenly distributed with 9 of 16 isolates in the second group.

For the individual *A. franciscana* and *Tetraselmis* sp. samples analysed it was evident that the strains isolated from *A* dominated Phenon 1 (Fig. 1), comprising 32 of

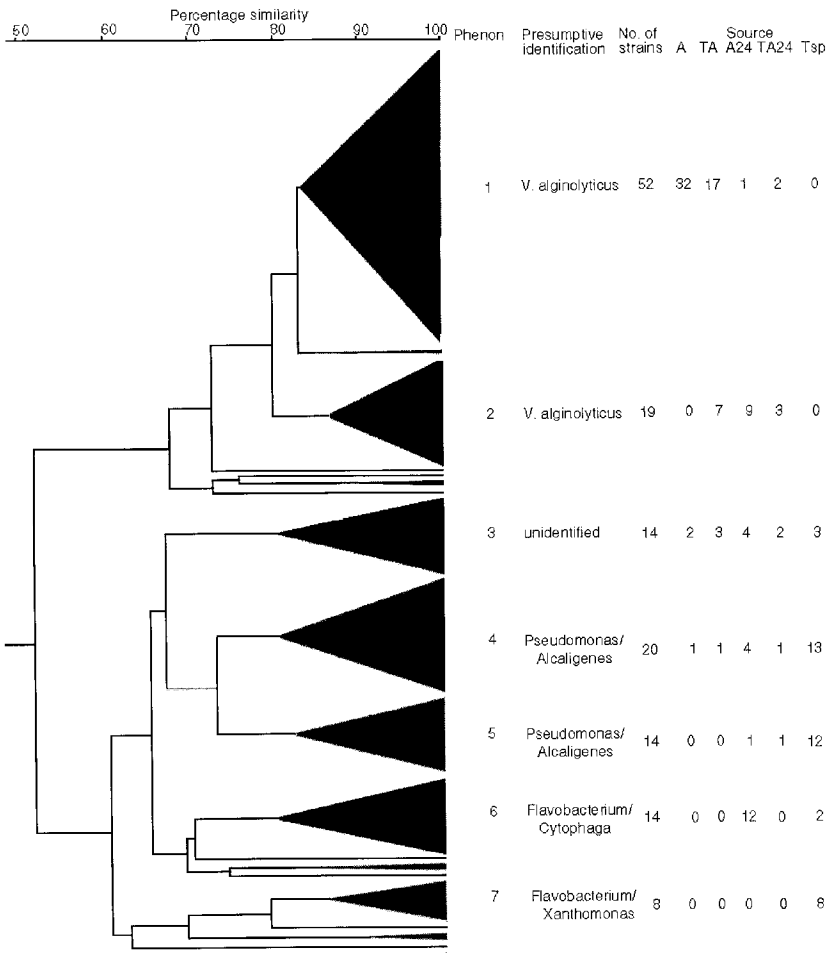


Fig. 1. Simplified dendrogram using the simplified matching coefficient and unweighted average linkage method of clustering showing the relationship between 156 isolates from *A. franciscana*, with 33 biochemical and morphological and antibiotic sensitivity tests. Seven phena containing 141 of the 156 isolates are recognised at a level of 80%. The number of strains in each phenon and their source (*A*, *TA*, *A24*, *TA24* and *Tsp*) is also shown.

the 52 isolates. They formed a fairly homogeneous group of Gram-negative rods which were non-pigmented, motile, oxidase positive, fermentative (Hugh and Leifson), swarming, and forming yellow colonies on TCBS (sucrose positive). All 18 isolates tested further reduced nitrate and were resistant to the vibriostat O/129 (10 μg). These properties are characteristic of *Vibrio alginolyticus*, and this was the dominant organism in the initial *A. franciscana* culture. There was little diversity ($J' = 0.17$) in the *A* population since this organism (or group of closely related organisms) constituted 88% (32/36) of the *A* bacteria. The prevalence of this organism, however, declined during the incubation with *Tetraselmis* sp. at high concentration. This led to a shift in the microbial population of the *A. franciscana* as the population diversity increased with the introduction of three isolates (Phenon 3) in common with *Tsp,* and seven more of Phenon 2 not detected in either *A* or *Tsp.* In the *TA* group 17 of 30 isolates belonged to Phenon 1, sharing the *V. alginolyticus*-like properties of *A* isolates, but the *TA* group had a higher diversity ($J' = 0.40$, Table 3) than *A* with representatives in Phena 2, 3 and 5 (Fig. 1).

Isolates in Phenon 2 were more diverse than in Phenon 1, being non-pigmented (18/19), motile, oxidase positive (18/19), swarming (15/19), Hugh and Leifson fermentative (18/19) and sucrose utilising on TCBS agar (13/16). These organisms were also closely related to *V. alginolyticus* but it was noticeable that none of the *A* isolates were members of this phenon. Phenon 3 contained 14 isolates with more varied properties than other groups. They were Gram-negative, non-pigmented, motile and non-swarming; in the Hugh and Leifson O/F test 7/14 failed to grow and the remainder were fermentative. They did not grow on TCBS agar, and were negative in the Voges-Proskauer and indole tests. Such properties are characteristic of the *Aeromonas* and *Pseudomonas* genera. Isolates grouped in Phena 4 and 5 belonged to the *Pseudomonas* or *Alcaligenes* genera, being motile, non-pigmented (except for 4/20 of Phenon 4), unable to grow in Hugh and Leifson medium, and unreactive in indole and ONPG tests. The phena differed mainly in motility (14/20 and 12/14 motile in Phena 4

Table 3
Bacteria isolated from 2-day-old *A. franciscana* and in *Tetraselmis* sp. culture. The relative diversities ($J'$, ranging between 0 and 1, i.e. low and high diversity) calculated using Shannon's index are also given

| Strains | *A* | *TA* | *A24* | *TA24* | *Tsp* |
|---|---|---|---|---|---|
| *V. alginolyticus* | 32 | 24 | 10 | 5 | |
| Other *Vibrio* sp. | | 2 | 1 | 1 | |
| *Aeromonas* | 1 | 3 | | 2 | 1 |
| Enterobacteriaceae | 1 | | | 1 | 1 |
| *Pseudomonas/ Alcaligenes* | 3 | 1 | 9 | 3 | 26 |
| *Flavobacterium/ Cytophaga* | | | 13 | 1 | 2 |
| *Flavobacterium/ Xanthomonas* | | 1 | | | 8 |
| *Moraxella* | | | 1 | | |
| *Acinetobacter* | 2 | | | | |
| Gram + ve coccus | 1 | | | | |
| Isolates not tested further | 0 | 10 | 5 | 3 | 9 |
| Relative diversity ($J'$) | 0.17 | 0.40 | 0.62 | 0.83 | 0.51 |

and 5, respectively) and in oxidase tests (14/20 and 1/14 motile in Phena 4 and 5, respectively). Phenon 6 was comprised of isolates from *A24* and *Tetraselmis*, and these bacteria were pigmented, non-motile, swarming (10/14) and oxidase positive (11/14). These were considered members of the *Cytophaga* or *Flavobacterium* genera. Isolates from Phenon 7 were pigmented, oxidase negative, unable to grow on Hugh and Leifson medium, and were assigned to the *Flavobacterium/Xanthomonas* genera.

After the 24-h simulation experiment, the bacterial concentration in the *TA24* sample was reduced considerably and the population was the most diverse ($J' = 0.83$, Table 3), representing a combination of the *TA* and *Tsp* populations. As with the *TA24* microbial flora, the composition of the *A24* group changed markedly from the initial *A* flora to become more diverse ($J' = 0.62$), with only 3% (1/36) of isolates showing identity with Phenon 1 and containing organisms from six different phena as well as 5 of the 15 isolates not assigned to the seven phena. The population was distinctly different from that of *TA24* with members of two phena not present in *Tsp*.

The identification results are summarised in Table 3. With the exception of the *V. alginolyticus* isolates, which dominated both the *A* and *TA* populations, the identity of most other organisms was uncertain beyond genus level. For *A* and *TA*, those organisms which were Voges-Proskauer-negative are nevertheless closely related to the Voges-Proskauer-positive *V. alginolyticus* strains. The *Tsp* isolates were diverse and appeared to be representatives of the genera *Pseudomonas*, *Flavobacterium*, *Moraxella*, *Acinetobacter* and *Aeromonas*.

The percentage of *Vibrio* spp. obtained from the identification agreed well with the percentage obtained on the TCBS agar (*A*: 85% vs. 69%, *TA*: 54% vs. 47%, *A24*: 2% vs. 6% and *Tsp*: 0% vs. 0.002%). The only exception was *TA24* (12.5% vs. 43%), but here the number of colonies on the plates was low (only 16 isolates, Table 1).

### 3.3. First feeding experiment with Atlantic halibut

The data from the first feeding experiment (Table 4) showed similar results for 2-day-old *A. franciscana* to those reported for the other experiments. The numbers of CFU on TCBS and haemolytic counts were similar to the counts reported in Table 2, with a significant reduction in CFU on TCBS during the 4-h incubation, but not for haemolytic counts. Samples taken from the fish tanks after 1 day showed a similar reduction to that found in the 24-h simulation experiment, with significant reductions in numbers, and *2A* sampled from the tanks were similar to *T2A* after the 4-h incubation. The ST nauplii also showed great reductions after the transfer to the fish tanks with about 95% reduction of CFU on TCBS and haemolytic counts.

The development of counts in tank water and on larvae was similar over the first 8 days, with very low counts on day 0 (before addition of live feed). The feed seemed to have a major impact on microbial conditions in the water and the larvae. On day 8 and 16 the TCBS and haemolytic counts were higher in both water and on larvae in the tanks receiving standard 2-day-old *A. franciscana* (*2A*). The differences between the two treatments were statistically significant for all water and larvae samples, except for haemolytic counts in water on day 8. For larvae, the numbers increased at all sampling points, whereas for water the numbers decreased again from day 8 to 16 for CFU on

Table 4
Bacteriological data from first feeding experiment for short-term enriched *A. franciscana* (*STA*), 2-day-old *A. franciscana* incubated with algae (*T2A*) or not (*2A*) (CFU individual$^{-1}$), Atlantic halibut larvae (CFU larvae$^{-1}$) given the different feeds (*STA-L*, *T2A-L* and *2A-L*), and water in tanks (CFU ml$^{-1}$), from blood agar and TCBS agar. $n = 2$–6, ±S.D

| Sample | CFU on TCBS (individual$^{-1}$ or ml$^{-1}$) | Haemolytic (CFU individual$^{-1}$ or ml$^{-1}$) |
|---|---|---|
| *STA*, before feeding day 0 | $414 \pm 89^{a}$ | $1163 \pm 588^{a}$ |
| *STA*, in fish tank day 2 | $22 \pm 9^{b}$ | $63 \pm 28^{b}$ |
| *STA*, in fish tank day 6 | $22 \pm 12^{b}$ | $63 \pm 38^{b}$ |
| *T2A*, before feeding day 7 | $1138 \pm 188^{a}$ | $763 \pm 313$ |
| *2A*, before feeding day 7 | $3750 \pm 375^{c}$ | $1013 \pm 63^{b}$ |
| *T2A*, in tank day 8 | $266 \pm 62^{b}$ | $477 \pm 68^{a}$ |
| *2A*, in tank day 8 | $728 \pm 381^{a}$ | $550 \pm 125^{a,b,c}$ |
| *T2A*, in tank day 16 | $204 \pm 49^{b}$ | $163 \pm 13^{c}$ |
| *2A*, in tank day 16 | $685 \pm 20^{a}$ | $1125 \pm 225^{a,b}$ |
| Water day 0 | $275 \pm 85^{a}$ | $50 \pm 20^{a}$ |
| Water day 2 | $1982 \pm 349^{b}$ | $32{,}450 \pm 13{,}650^{b}$ |
| Water (*T2A*) day 8 | $4070 \pm 653^{c}$ | $2800 \pm 600^{c}$ |
| Water (*2A*) day 8 | $8700 \pm 200^{d}$ | $4350 \pm 750^{c}$ |
| Water (*T2A*) day 16 | $650 \pm 95^{a}$ | $750 \pm 150^{d}$ |
| Water (*2A*) day 16 | $2515 \pm 95^{e}$ | $1650 \pm 50^{e}$ |
| *ST-L* day 0 | $3 \pm 3^{a}$ | $35 \pm 15^{a}$ |
| *ST-L* day 2 | $775 \pm 301^{b}$ | $1475 \pm 994^{b}$ |
| *T2A-L* day 8 | $3600 \pm 100^{c}$ | $11{,}500 \pm 1500^{c}$ |
| *2A-L* day 8 | $14{,}375 \pm 5475^{d}$ | $20{,}750 \pm 1750^{d}$ |
| *T2A-L* day 16 | $13{,}000 \pm 800^{d}$ | $25{,}000 \pm 2000^{e}$ |
| *2A-L* day 16 | $125{,}000 \pm 7000^{e}$ | $80{,}000 \pm 7000^{f}$ |

Different superscript letters in columns indicate significant different numbers ($P < 0.05$, tested in-between *STA* (three samples), between all 2-day-old *A. franciscana*, between all water samples and all larvae samples, respectively).

TCBS and after day 2 (very high counts) for haemolytic counts. This was most likely due to the increased water exchange (Olsen et al., 2000).

## 4. Discussion

The procedure for incubation of *A. franciscana* with *Tetraselmis* sp. for 4 h changed the live feed both with respect to total numbers and to composition of associated bacteria flora, particularly for vibrios. When the animals were first incubated for 4 h, the changes during the next 24 h under simulated first feeding conditions were more pronounced with significant reductions for all three categories of bacteria. In the animals not incubated for 4 h, no changes in haemolytic counts were observed during the 24-h incubation, whereas total numbers and TCBS counts were reduced significantly. Thus, the incubation with algae not only reduced the numbers of associated bacteria, but also made the animals more susceptible to further reductions in numbers under first-feeding

conditions. This was confirmed by the more pronounced changes observed in bacterial composition during the 24 h incubation, especially for the previously algal-treated animals (Fig. 1 and Table 3). This suggests that a lower initial level of bacteria associated with the *A. franciscana* enhanced the algal effect. The microbial flora of *Tetraselmis* was quite different from that of 2-day-old *A. franciscana* (*A*) and was more diverse (relative diversity: $J' = 0.51$). The value reported here for the relative diversity of the bacterial flora in *Tetraselmis* sp. culture is similar to the value of 0.50 found in a culture grown at $0.6\mu_{max}$, but lower than found for other algal species (Salvesen et al., 2000). The relative diversity of 2-day-old *A. franciscana* was initially low, but increased both through the 4-h treatment, and even more during the subsequent 24 h reaching a high value (0.83) in *TA24*. This indicates a major shift in the microbial population structure in *A. franciscana* during the 24-h incubation with *Tetraselmis*, and the shift was greatest for the animals given the 4-h algal treatment.

In a study of the microbiology of *Artemia,* Austin and Allen (1982) showed that although the bacterial concentration in newly hatched *Artemia* was relatively low, the concentration in culture water increased to $> 10^7$ ml$^{-1}$ within 24 h. Less than 25% of the microbial flora was characterised as Gram-negative rods with Gram-positive rods and cocci constituting the majority of the flora, which is in contrast to experience in the aquaculture industry, and the results presented here. This probably occurred because the *Artemia* cysts used by Austin and Allen (1982) were not decapsulated using the normal alkaline or oxidising agents, which are strongly bactericidal. A study by Straub and Dixon (1993) of adult *Artemia* from hypersaline ponds in California showed the presence of *V. alginolyticus*, *V. fluvialis*, *V. metschnikovii*, *V. mimicus* and others at high salinity levels. At lower salinities other enteric bacteria dominated and, in contrast to earlier studies (Austin and Allen, 1982; Gilmour et al., 1975), the flora was comprised mainly of Gram-negative rods. The associated bacteria flora of juvenile *A. franciscana* reported in this paper is in broad agreement with those reported by Gorospe and Nakamura (1996) and Muroga et al. (1987).

The numbers of bacteria associated with the 2-day-old *A. franciscana* (*A*) were high, but were consistent with numbers in *Artemia* grown for 48 h with the diet Protein Selco (Rodríguez et al., 1991). The numbers and semi-qualitative composition after 24 h in algae (*TA24*), were similar to that found in another experiment with 2-day-old *A. franciscana* treated in the same way and sampled from larval tanks with Atlantic halibut larvae (Olsen et al., 1999). In that experiment, counts of 385, 173 and 165 CFU *A. franciscana*$^{-1}$ were found, compared to 660, 170 and 240 counts for M65, TCBS and haemolytic counts, respectively, in the present study. Also, in the first feeding experiment reported here, the numbers of bacteria associated with *A. franciscana* were low in the tanks, and significantly lower when the feed was incubated with algae before addition to the tanks. It can therefore be concluded that the simulation experiments gave a realistic picture of the changes that take place in the live feed after transfer to larval tanks. For *Artemia* cultivated with other food, varying numbers of associated bacteria have been reported, varying from $7 \times 10^3$ to $3.7 \times 10^6$ CFU *Artemia*$^{-1}$ (Gorospe and Nakamura, 1996; Muroga et al., 1987; Verschuere et al., 1997).

The variability reported for numbers and composition of the bacterial flora of *Artemia* reflects the different growth conditions and foods. The results reported here and

by Olsen et al. (2000) for *A. franciscana* grown under similar conditions and with the same food indicate that the flora is a product of the food for *A. franciscana*. The immediate effect of the algal incubation is probably to change the gut content by expelling the faeces and remains of fishmeal and oil. By removing this very rich growth substrate of disintegrated particulate matter, including some of the bacterial content of the gut, the bacterial load of the animals was significantly reduced. Similar results have been obtained using *Isochrysis galbana* for rinsing of 2-day-old *A. franciscana* (Atle I. Olsen, unpublished data). With *I. galbana* a 70% reduction of total numbers from $3.6 \times 10^4$ CFU *A. franciscana*$^{-1}$, 79% reduction in CFU on TCBS from $1.8 \times 10^4$ CFU *A. franciscana*$^{-1}$, and 90% reduction in haemolytic counts from 500 CFU *A. franciscana*$^{-1}$ was obtained. This suggests that the ingestion of food particles like algae does not encourage growth of saprophytic bacteria to the same degree as fish meal, oil and lipid products and yeast. Øie et al. (1994) reported lower bacterial counts for rotifers cultivated with algae than with other foods such as yeast. Other rinsing procedures can be used to reduce the numbers of bacteria associated with the live feed, but if this only reduces the numbers, and does not diversify the flora as well as removing bacterial growth substrates, this may lead to an unstable situation. Other effects of the algae such as the reported production of anti-microbial components from *Tetraselmis* sp. (Duff and Bruce, 1966; Kellam and Walker, 1989; Austin and Day, 1990; Austin et al., 1992) could also contribute to the changes in the associated flora to *A. franciscana*, but this requires further examination.

Microalgae have been suggested to affect the behaviour of larvae (Naas et al., 1992), and to affect the larval microbiology (Skjermo and Vadstein, 1993b; Bergh et al., 1994). The further advantageous effect of algal addition shown here in reducing the content of bacteria that are unfavourable to fish larvae could prove extremely beneficial in larval rearing.

## 5. Conclusions

The results presented here showed that the numbers of associated bacteria could be reduced by, on average, 75% during 4-h incubation, and the diversity of the associated bacteria flora increased. The explanation of this effect was probably the expulsion and replacement of the gut contents by algae, thus removing the substrate for growth of opportunistic bacteria. Further incubation under first feeding conditions gave further decreases in numbers, especially in the 2-day-old *A. franciscana* first incubated for 4 h, and the diversity of the bacteria flora increased even more. The data from the first feeding experiment showed that the bacterial flora of the live feed was directly transferred to the larvae. We believe that it would be advantageous to change and diversify the associated flora of the live feed before feeding to the larvae, especially to reduce the number of haemolytic bacteria associated with the *A. franciscana*.

## Acknowledgements

We thank Marthe Schei and Kristin Bonaunet for technical assistance. This study was supported by the Research Council of Norway and by Stolt Sea Farm.

## References


Austin, B., Allen, D.A., 1982. Microbiology of laboratory-hatched brine shrimp (*Artemia*). Aquaculture 26, 369–383.

Austin, B., Day, J.G., 1990. Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture 90, 389–392.

Austin, B., Baudet, E., Stobie, M., 1992. Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. J. Fish Dis. 15, 55–61.

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

Cowan, S.T., 1974. Cowan and Steel's Manual for the Identification of Medical Bacteria. 2nd edn. Cambridge Univ. Press, Cambridge.

Danielsen, T.L., Evjemo, J.O., Olsen, Y., 1995. Stability of short-time enriched n-3 fatty acids in *Artemia* during starvation at different temperatures. In: Lavens, P., Jaspers, E., Roelants, I. (Eds.), LARVI '95. Fish and Shellfish Larviculture Symposium. European Aquaculture Society, Special Publication 24pp. 128–131.

Duff, D.C.B., Bruce, D.L., 1966. The antibacterial activity of marine planktonic algae. Can. J. Microbiol. 12, 877–884.

Furniss, A.L., Lee, J.V., Donovan, T.J., 1979. The vibrios. Public Health Service Monograph Series No. 11 HMSO, London, 58 pp.

Gilmour, A., McCallum, M.F., Allan, M.C., 1975. Antibiotic sensitivity of bacteria isolated from the canned eggs of the CAn brine shrimp (*Artemia salina*). Aquaculture 6, 221–231.

GomezGil-RS, B., Abreu-Grobois, F.A., Romero-Jarero, J., De Los Herrera-Vega, M., 1994. Chemical disinfection of *Artemia* nauplii. J. World Aquacult. Soc. 25, 579–583.

Gorospe, J., Nakamura, K., 1996. Associated bacterial microflora in *Artemia*-rice bran culture. Isr. J. Aquacult. 48, 99–107.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms: I. *Cyclotella nana* Husted, and *Deutonula confervacea* (Cleve) Gran. Can. J. Microbiol. 8, 229–239.

Kellam, S.J., Walker, J.M., 1989. Antibacterial activity from marine microalgae in laboratory culture. Br. Phycol. J. 24, 191–194.

Keskin, M., Keskin, M., Rosenthal, H., 1994. Pathways of bacterial contamination during egg incubation and larval rearing of turbot, *Scophthalmus maximus*. J. Appl. Ichthyol. 10, 1–9.

Makridis, P., Fjellheim, A.J., Skjermo, J., Vadstein, O., 2000. Control of the bacterial flora of the live feed organisms *Brachionus plicatilis* and *Artemia franciscana*. Aquaculture 185, 207–218.

Munro, P.D., Henderson, P.J., Barbour, A., Birkbeck, T.H., 1999. Partial decontamination of rotifers with ultraviolet radiation: the effect of change in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot. Aquaculture 170, 229–244.

Muroga, K., Higashi, M., Keitoku, H., 1987. The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegi*) at larval and juvenile stages. Aquaculture 65, 79–88.

Naas, K.E., Næss, T., Harboe, T., 1992. Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus* L) in green water. Aquaculture 105, 143–156.

Nicolas, J.L., Robic, E., Ansquer, D., 1989. Bacterial flora associated with a trophic chain consisting of microalgae, rotifers and turbot larvae: influence of bacteria on larval survival. Aquaculture 83, 237–248.

Nimura, Y., 1989. Shortest gut passage time and gut content volume of *A. franciscana*. Nippon Suisan Gakkaishi 55, 2209.

Øie, G., Reitan, K.I., Olsen, Y., 1994. Comparison of rotifer culture quality with yeast plus oil and algal-based cultivation diets. Aquacult. Int. 2, 225–238.

Oliver, J.D., 1982. Taxonomic scheme for the identification of marine bacteria. Deep-Sea Res. 29, 795–798.

Olsen, A.I., Attramadal, Y., Jensen, A., Olsen, Y., 1999. Influence of size and nutritional value of *Artemia franciscana* on growth and quality of halibut larvae (*Hippoglossus hippoglossus*) during the live feed period. Aquaculture 179, 475–487.

Olsen, A.I., Attramadal, Y., Reitan, K.I., Olsen, Y., 2000. Food selection and digestion characteristics of Atlantic halibut (*Hippoglossus hippoglossus*) larvae fed cultivated prey organisms. Aquaculture 181, 293–310.

Perez Benavente, G., Gatesoupe, F.J., 1988. Bacteria associated with cultured rotifers and *Artemia* are detrimental to larval turbot, *Scophtalmus maximus* L. Aquacult. Eng. 7, 289–293.

Reitan, K.I., Rainuzzo, J.R., Øie, G., Olsen, Y., 1993. Nutritional effects of algal addition in first feeding of turbot (*Scophtalmus maximus* L.) larvae. Aquaculture 118, 257–275.

Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. J. Phycol. 30, 972–979.

Rodríguez, J.L., Planas, M., Otero, J.J., 1991. Microflora and antibacterial treatments of rotifers and *Artemia*. In: Lavens, P., Sorgeloos, P., Jaspers, E., Ollevier, F. (Eds.), LARVI '91. Fish and Crustacean Larviculture Symposium. European Aquaculture Society, Special Publication 15 pp. 403–405.

Salvesen, I., Reitan, K.I., Skjermo, J., Øie, G., 2000. Microbial environments in marine larviculture: impacts of algal physiological conditions on the bacterial load in 6 microalgae. Aquacult. Int., in press.

Skjermo, J., Vadstein, O., 1993a. Characterization of the bacterial flora of mass cultivated *Brachionus plicatilis*. Hydrobiologia 255/256, 185–191.

Skjermo, J., Vadstein, O., 1993b. The effect of microalgae on skin and gut bacterial flora of halibut larvae. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinnereim, K. (Eds.), Proceedings of the First International Conference on Fish Farming Technology. A.A. Balkema, Rotterdam, pp. 61–67.

Skjermo, J., Vadstein, O., 1999. Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177, 333–343.

Slaatebræk, G., 1975. Grunnleggende metoder ved undersøkelser av den mikrobielle flora i et forurenset marint miljø. Cand. Scient. Thesis (in Norwegian), University of Bergen, Norway.

Sneath, P.H.A., Sokal, R.R., 1973. Numerical Taxonomy. Freeman, San Francisco.

Sokal, R.R., Michener, C.D., 1958. A statistical method for evaluating systematic relationships. Univ. Kans. Sci. Bull. 38, 1409–1438.

Sorgeloos, P., Lavens, P., Léger, P., Tackaert, W., Versichele, D., 1986. Manual for the culture and use of the brine shrimp *Artemia* in aquaculture, *Artemia* Reference Center. Faculty of Agriculture, State University of Ghent, Belgium, 319 pp.

Straub, D.V., Dixon, B.A., 1993. Bacteriological flora of the brine shrimp (*Artemia franciscana*) from a hypersaline pond in San Francisco Bay, CA. Aquaculture 118, 309–313.

Støttrup, J.G., Gravningen, K., Norsker, N.H., 1995. The role of different algae in the growth and survival of turbot larvae (*Scophtalmus maximus* L.) in intensive rearing systems. ICES Mar. Sci. Symp. 201, 173–186.

Verschuere, L., Dhont, J., Sorgeloos, P., Verstraete, W., 1997. Monitoring Biolog patterns and r/K-strategists in the intensive culture of *Artemia* juveniles. J. Appl. Microbiol. 83, 603–612.

Walne, P.R., 1966. Experiments in the large-scale culture of the larvae of *Ostrea edulis* L. Fish. Invest. London, Ser. 2 25, 1–55.

Zar, J.H., 1996. Biostatistical analysis. 3rd edn. Prentice-Hall, Englewood Cliffs, NJ, p. 662.