Atle Ivar Olsen

Development of Production Technology of Juvenile *Artemia* Optimal for Feeding and Production of Atlantic Halibut Fry

NTNU Trondheim
Norges teknisk-naturvitenskapelige universitet

Doktor ingeniøravhandling 1999: 135
Institutt for bioteknologi

ACKNOWLEDGEMENTS

The work presented in this thesis was performed as a part of the project "Helårlig storskalaproduksjon av kveiteyngel" supported by the Norwegian Research Council and Stolt Sea Farm A.S. The experimental work with halibut larvae took place at SSF Øye in Kvinesdal during some spring months in 1995 and 1996, and the rest of the work was performed at SINTEF Fisheries and Aquaculture in Trondheim.

I have many persons to thank for help and advice during my work with this thesis.

First, I thank my supervisors: Professor Arne Jensen who first leaded me into the field of aquaculture in 1993, and Professor Yngvar Olsen. They are both to be thanked for their help for me to accomplish my work. Without them, there would be no thesis.

I am greatly thankful to all the employees and students I have had the pleasure to share my time with here at Brattøra. Thanks especially to Jan Ove Evjemo, who first introduced me to the world of *Artemia*, and to Kjell Inge Reitan and Ingrid Overrein.

I am also grateful to the people and staff at Stolt Sea Farm Øye, in Kvinesdal, especially Yngve Attramadal, and to all co-workers on the halibut project. Also, thanks to all my co-authors.

Thanks to the Norwegian Research Council for financial support for my work and to the Department of Biotechnology at NTNU for financial support during the last two months. Also, thanks to Jorunn Skjermo and José Rainuzzo for their support during the last year, and to Ingrid Salvesen for our "competition" to finish first – you won!

Finally, I thank my family, and especially my wife Cecilia, who has been my greatest inspiration and support during the completion of my work.

Atle Lars Olsen
Trondheim 20.12.99

CONTENTS

SUMMARY

The goal of the studies that are reported in this thesis was to develop adequate methods for the production of good quality fry of Atlantic halibut (*Hippoglossus hippoglossus* L.). In this connection, prey preferences of the Atlantic halibut larvae is important. When given the choice between rotifers and short term (ST) enriched nauplii, the Atlantic halibut larvae showed no preferences for any of these the first two days of the live feed period. From day 3 and onwards the larvae preferred ST nauplii. When the larvae had the opportunity to select between different size classes of *A. franciscana*, the preferred size was the newly hatched nauplii during the first 3 weeks. Around day 20 at 3-4 mg dry weight (DW), the larvae showed a pronounced shift in their preferred size of *A. franciscana*, and they started to select for individuals of 1.2-1.4 mm length or longer. This size corresponds to *A. franciscana* cultivated for 3 days.

The larvae apparently digested the newly hatched nauplii better than the ST nauplii during the first weeks, this presumably because of the high lipid content of the latter. Also, the bigger size classes of *A. franciscana* seemed to be more efficiently digested than the ST nauplii by larvae of less than 2.4 mg DW. Bigger larvae showed almost equal and very efficient digestion of all size classes of *A. franciscana*. The larvae never selected positively for ST nauplii, and use of newly hatched nauplii for some time may be beneficial based on the selection and digestion patterns of the larvae. However, the newly hatched nauplii do not contain DHA (docosapentaenoic acid) and are therefore nutritionally inadequate for the larvae. This size class of *Artemia* may be beneficially used for a few days after onset of exogenous feeding provided that the nutritional status of the larvae at the onset of exogenous feeding is adequate and that the nutritional quality of the feed used later is satisfactory.

The larvae selected positively for larger *A. franciscana* in a period before they reached the size when they normally start to accept formulated food. Prey of the size 1.2-1.4 mm (i.e. 3-day-old *A. franciscana*) seemed to be the optimal size for the larvae after about day 20 in the live feed period. It was possible to produce 3-day-old *A. franciscana* with 18 mg DHA g^{-1} DW and a DHA/EPA ratio > 1.0 by using a diet containing more than 39% lipids and 10% DHA, respectively. A high DHA/EPA ratio (> 1.0) is needed after the production because *A. franciscana* appears to transform DHA to EPA (eicosapentaenoic acid) relatively efficiently.

Attempts were made to stabilise the content of DHA in *A. franciscana* in the larval tanks by incubating them with the DHA rich microalgae *I. galbana*. However, 6 mg C l^{-1} or higher levels of this alga was negative for the DHA content of ST *A. franciscana* which then decreased more rapidly during the first 24 h than at lower algal concentrations. This was most likely due to a replacement of lipids by algal cells in the gut of the nauplii. In the case of 3- and 4-day-old *A. franciscana*, the effect of addition of *I. galbana* on the DHA level in the animals was small for the algal levels normally used in the larval tanks (1-2 mg C l^{-1}).

The microalgae *Tetraselmis* sp. was used to reduce bacterial level and change bacterial flora associated with the live feed. During a 4 h incubation of 2-day-old *A. franciscana* with this alga, the numbers of associated bacteria and *Vibrio* spp. were reduced significantly. This effect was even more pronounced after transfer to first feeding conditions. The composition of the microbial flora associated with the animals became more diverse, and less dominated by *Vibrio alginolyticus*, which was the dominating species associated with 2-day-old *A. franciscana*. The results also showed that the algae indirectly affected the larvae through changing the associated bacterial flora of the live feed. Larvae which received algal-treated 2-day-old *A. franciscana* had significantly lower numbers of Vibrios and bacteria with haemolytic activity compared to larvae fed non-treated feed. In another first feeding experiment, 4-day-old *A. franciscana* had high numbers of associated bacteria, and especially of bacteria exhibiting haemolytic activity, even if the procedure for algal incubation was used. Differences in bacterial flora associated with the live feed may lead to differences in growth of larvae.

Atlantic halibut juveniles with better quality was obtained with 20% perfect larvae when juvenile *A. franciscana* was used as feed compared to about 4% with the use of ST nauplii. The 3-day-old *A. franciscana* seems to be the optimal size for the larvae from about day 20 after first feeding because this size has many useful properties. It is the most preferred size by the larvae from this time in the live feed period, it is efficiently digested by the larvae and it can be produced with a relatively high DHA content (> 18 mg g^{-1} DW). The main challenge is to improve the microbial quality of this feed.

LIST OF PAPERS

Paper 1: Olsen, A. I., Attramadal, Y., Reitan, K. I. and Olsen, Y. 1999. Food selection and digestion characteristics of Atlantic halibut (*Hippoglossus hippoglossus*) larvae fed cultivated prey organisms. *Aquaculture* 181: 293-310.

Paper 2: Olsen, A. I., Evjemo, J. O. and Reitan, K. I. 1999. Production of juvenile *Artemia franciscana* with high DHA content. Submitted *Aquaculture International.*

Paper 3: Olsen, A. I., Jensen, A., Evjemo, J. O. and Olsen, Y. 1997. Effect of algal addition on stability of fatty acids in enriched *Artemia franciscana*. *Hydrobiologia* 358: 205-210.

Paper 4: Olsen, A. I., Mæland, A., Waagbø, R. and Olsen, Y. 1999. Effect of algal addition on stability of fatty acids and some water-soluble vitamins in juvenile *Artemia franciscana*. Submitted *Aquaculture Nutrition.*

Paper 5: Olsen, A. I., Olsen, Y., Attramadal, Y., Birkbeck, T. H., Skjermo, J. and Vadstein, O. 1999. Effect of short term feeding on microalgae on bacterial level and composition associated with juvenile *Artemia franciscana*. Submitted *Aquaculture.*

Paper 6: Olsen, A. I., Attramadal, Y., Jensen, A. and 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.

1. INTRODUCTION

The Atlantic halibut (*Hippoglossus hippoglossus* L.) is believed to be the next important species in Norwegian fish farming, and major research efforts to develop Atlantic halibut for this purpose were initiated more than a decade ago (Olsen *et al.*, 1999). Like many other fish species the Atlantic halibut needs live feed to initiate or to facilitate exogenous feeding, and regular production of adequate live feed represents one of the major obstacles in the commercial farming of this species. The studies reported in the present thesis represent efforts to develop adequate production technology that will secure live feed of high quality for the growth and survival of halibut larvae in commercial fish farming.

The Atlantic halibut larvae have an extended yolk sack stage (45 days at 6°C) and are relatively big at the onset of exogenous feeding with about 0.9 mg dry weight (DW) and a standard length of about 11 mm. The larvae increase their weight exponentially after a few days of feeding, and a specific growth rate of 6-12% daily weight increase (DWI) is normal (Olsen *et al.*, 1999). To maintain the optimal growth rate, the larvae must be fed adequate feed. Some important factors in this context are believed to be the prey size and microbiological as well as nutritional quality.

Much work has focused on the content of lipids of the live feed, especially that of essential fatty acids (EFA) like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Watanabe, 1982; Rainuzzo, 1993; Sargent *et al.*, 1999). The DHA/EPA ratio has been shown to be of importance for pigmentation in flatfish larvae such as turbot (*Scophthalmus maximus* L., Rainuzzo, 1993; Reitan *et al.*, 1994), and it has been suggested that excess EPA give larval mortality, as shown for yellowtail (*Seriola quinqueradiata*, Watanabe, 1993). Feeding has in many cases been initiated with natural zooplankton, often harvested from fertilised ponds (Holmefjord *et al.*, 1989). The early stages of zooplankton species used as feed for Atlantic halibut larvae typically have a low lipid content (about 10% of dry weight), a high DHA content (30-40% of total fatty acids) and a DHA/EPA ratio of 1.5-2 (Evjemo and Olsen, 1997; Olsen *et al.*, 1999). Since zooplankton is generally low in total lipids and carbohydrates, they have a high protein content. Zooplankton in shallow waters at higher latitudes may have a protein/lipid ratio of about 5-6 (Båmstedt, 1986), which may be very important for the growing larvae (Øie *et al.*, 1997).

The sizes of zooplankton found in nature show great variation (Hunter, 1981) and the fish larvae therefore have a broad spectrum of prey sizes to select among as they grow. They can thereby optimise their feed uptake. The size distribution of prey organisms has been shown to be more important than the total number of preys available, both for growth and for survival of some fish larvae (Frank and Leggett, 1986; Bremigan and Stein, 1994). As the mouth width of the larvae increases as the larvae grow, their ability to ingest bigger prey sizes also increases and the size range of possible prey increases. The largest prey a larva can ingest is limited by the mouth width and a steady increase in preferred prey sizes for the growing larvae has been reported for several species (Detwyler and Houde, 1970; Hunter, 1981; Dabrowski and Bardega, 1984; Fernandez-Diaz *et al.*, 1994). The smallest preys a larvae will ingest may be set by metabolic relations such as a minimum caloric value of each prey item needed for the larvae to have a net gain by ingesting it (Hunter, 1981).

Collected natural zooplankton which is suitable as live feed, is available only during a part of the year, and we have little control with nutritional quality, size distribution, and the associated microflora. To have a steady predictable production of Atlantic halibut fry, preferentially all round the year, we have to cultivate the live feed. Feeding has been initiated with enriched rotifers (*Brachionus plicatilis*) and short term (ST) enriched *Artemia* nauplii (Holmefjord *et al.*, 1989). Later in the live feed period, which may last for 60 days, Atlantic halibut larvae have also been fed larger stages such as 2-day-old or even bigger *Artemia* (Reitan *et al.*, 1993a; Stoss, J., Stolt Sea Farm Øye, personal communication). The cultivated live feed may be used either as the sole live feed or as a supplement in combination with natural zooplankton when available. The nutritional composition of the cultivated live feed can easily be manipulated as shown for both rotifers and *Artemia* (Léger *et al.*, 1986; Lavens and Sorgeloos, 1991; Olsen *et al.*, 1993a; Dhert *et al*, 1993; Dhont and Lavens, 1996; Coutteau and Sorgeloos, 1997). A relevant model for nutritional composition of the cultivated live feed is the natural prey for Atlantic halibut, which we may assume is the naupliar stages of marine copepods.

The crustacean *Artemia* (Class: *Crustacea*; Subclass: *Branchiopoda*; Order: *Anostraca*; Family: *Artemiidae* and Genus: *Artemia*), named brine shrimp, has become very important in aquaculture world-wide. Different species of *Artemia* (e.g. *franciscana*, *sinica*, and *parthenogenetica*) have been evaluated for use as feed at different developmental stages or sizes: decapsulated cysts, newly hatched nauplii, lipid enriched nauplii, juveniles, adults, as well as dried and freeze dried material (Lèger *et al.*, 1986). *Artemia* has a low content of EFA compared to marine copepods. To meet the EFA demands of the Atlantic halibut larvae the nauplii have to be enriched with emulsified lipids with a high content of EFA prior to the use as live feed. The short term enrichment (12-24 h) of *Artemia* nauplii leads to lipid levels of 25-30% of dry weight (DW) in the live feed, which results in a concomitant low protein to lipid ratio. The juvenile *Artemia* have some advantages: they are bigger and would give more energy per prey consumed and thereby less energy expenditure for the larvae to catch enough feed, and the size produced can be adjusted according to the size preferences of the Atlantic halibut larvae. The nutritional composition of larger *Artemia* appears in many ways more similar to that of natural zooplankton compared to ST nauplii, and would thereby presumably be better for the Atlantic halibut larvae. The juvenile *Artemia* contains less lipids (<18% of DW) and thereby more protein than the ST nauplii (Léger *et al.*, 1986; Lavens and Sorgeloos, 1991). The digestibility of the live feed is also of great importance, and it has been suggested that bigger stages of *Artemia* are better digested than the nauplii because of the thin exoskeleton of the former (Léger *et al.*, 1986; Dhert *et al.*, 1992). In addition, the high lipid content of the ST *Artemia* is believed to be unfavourable for the larval digestion system (Conceicão, 1997). Gara *et al.* (1998) reported an inverse correlation between lipid content the liver of Atlantic halibut larvae and pigmentation success.

The price for *Artemia* cysts has increased considerably during the last years because of higher demands and variations in availability. Because of this, it is economical to produce and feed the larvae with juvenile *Artemia*. Fewer individuals would be required and the number of cysts needed is therefore reduced. A reduction in cyst costs of 60% has been reported with the use of ongrown *Artemia* as larval feed for two different species (Lavens and Sorgeloos, 1991). The survival of the *Artemia* has to be acceptable during production, and the animals have to be produced with high growth rate to obtain the desired sizes in short time with minimum work. To obtain a satisfactory nutritional value of *Artemia*, i.e. a satisfactory content of DHA, it is necessary to know what levels

of lipids and DHA are needed in the food for *Artemia* to obtain the desired DHA content in the animals. General culture conditions as feeding techniques and regimes, washing or water exchange have to be optimised in order to improve the growth rate, survival, and microbial and nutritional quality. The content of DHA is unstable in *Artemia* (Danielsen *et al.*, 1995; Evjemo *et al.*, 1999), and it has been suggested that the animals use DHA for energy production (Dhert *et al.*, 1993). It is important that the nutritional value of the live feed is satisfactory when the larvae ingest it. A part of the live feed is normally stored some hours before it is given to the larvae and the feed may remain in the larval tanks a period before being eaten by the larvae. Knowledge of the kinetics of fatty acid metabolism during and after the production, and which factors influence it, is necessary to be able to secure a satisfactory nutritional quality when the larvae eat the feed.

The associated microflora of the live feed is transferred to the larvae during cultivation of marine fish larvae (Muroga *et al.*, 1987; Perez Benavente and Gatesoupe, 1988; Nicolas *et al.*, 1989). A flora dominated by fast growing opportunistic bacteria may cause problems (Skjermo and Vadstein, 1999), and high numbers of bacteria have caused mass mortality during first feeding of marine fish larvae (Perez Benavente and Gatesoupe, 1988). High numbers of haemolytic bacteria associated with the live feed may also cause larval mortality as these bacteria are believed to be detrimental to the fish larvae (Nicolas *et al.*, 1989). The production of live feed in dense cultures, at high food densities and at elevated temperatures with heavy aeration, create an environment which promotes growth of fast growing opportunistic bacteria. The bacterial load may be reduced by addition of antibiotics, by disinfection, or by washing with fresh water (Rodríguez *et al.*, 1991). However, reducing the load non-selectively could lead to an unstable situation promoting the growth of opportunistic bacteria at a later stage. It is therefore desirable to develop techniques and procedures that could reduce the number of bacteria associated with the live feed and at the same time give the feed a stable flora (Vadstein *et al.*, 1993; Skjermo and Vadstein, 1999). Probiotic bacteria have been tested for used for early stages of fish larvae (Skjermo and Vadstein, 1999), and has been used during the production of juvenile *Artemia* with promising results (Verschuere, 1999).

The title of this thesis is "Development of production technology of juvenile *Artemia* optimal for feeding and production of Atlantic halibut fry". The goal of the work was to develop adequate methods and procedures for production of *Artemia* to secure satisfactory survival, high growth rate and good quality of the Atlantic halibut fry. The work was started by investigated the prey preferences of the Atlantic halibut larvae for two species of cultivated live feed, *A. franciscana* and *B. plicatilis*, and thereafter the preferences of the larvae for different size classes of *A. franciscana* (Paper 1). When the preferred size classes had been determined, the next step was to find methods to produce the preferred sizes of *A. franciscana* with a satisfactory nutritional value (a high content of DHA and a high protein/lipid ratio) for Atlantic halibut larvae (Paper 2). The nutritional value of the cultivated live feed is not constant after production and the effect on *A. franciscana* of adding a DHA rich microalgae to the fish tanks on the nutritional quality for the actual size classes of *A. franciscana* during first feeding was examined (Papers 3 and 4). Problems encountered in the production of marine larvae, like high mortalities and low growth rates of the larvae, are often ascribed to bacteria or other microorganisms. The use of microalgae to change and improve the bacterial flora associated with the juvenile *A. franciscana* was examined. Preliminary experiments were performed with fish larvae

to investigate the effect of this (Paper 5). A first feeding experiment was carried out to evaluate some of the effects on Atlantic halibut larvae of using juvenile *A. franciscana* compared to the commonly used short term enriched nauplii (Paper 6).

2. OPTIMAL PREY SIZES FOR ATLANTIC HALIBUT LARVAE

It is of great importance to know the size preferences of the larvae during the live feed period in order to optimise the feeding regime for Atlantic halibut larvae when they are fed the cultivated live feed organisms *Artemia* and rotifers. Feeding was initiated at 285°d (day degrees) in the present work (Paper 1). It was found that the larvae had a slight preference (not significant) for rotifers the first day (Fig 2.1). From day 3 onward the larvae selected strongly for ST nauplii. Guibrandsen (1993) performed experiments at a larval age of 260°d and he found that the larvae had no preferences for rotifers or *Artemia* nauplii the first days and concluded that the larvae were non-selective the first 2 days when offered *Artemia* nauplii and rotifers. He also stated that the ingestion of larger prey increased slightly after 6 days.

When offered different size classes of *A. franciscana*, the larvae selected for the smallest preys for a long time as is evident from Fig. 2.2. Higher contours (high values for α-indices) indicate stronger preference, and two main ridges are evident in the plot. From the start of feeding at an individual larval dry weight (DW) of about 0.9 mg and to about 3.5 mg DW, there is a ridge in the plot suggesting that the larvae preferred the newly hatched nauplii of about 0.5 mm length. The selection for bigger *A. franciscana* became apparent at around day 20 after first feeding (larval weight about 3.5 mg DW), as indicated by a new ridge for the α-index for *A. franciscana* of the size class 1.2-1.4 mm length. Outside of the two ridges, there were only small areas with α-indices over 0.2 for some size classes of *A. franciscana* longer than 1.8 mm.

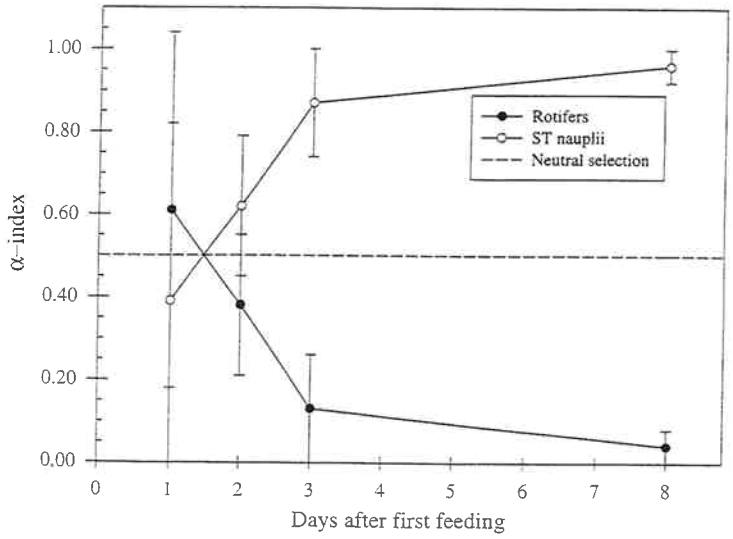

Figure 2.1 Selection between rotifers and ST enriched nauplii. Preference is given as α-indices (Chesson, 1983; Paper 1), ranging between 0 and 1. Neutral selection corresponds to an α-index of 0.5. Higher values indicate positive selection.

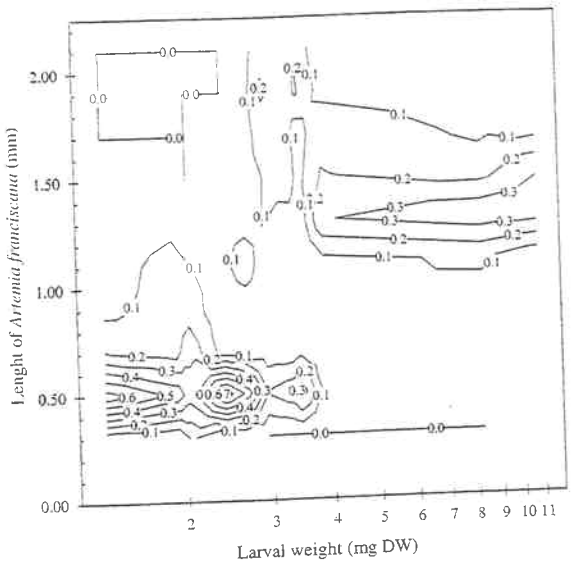

Figure 2.2 Selection of different size classes of *A. franciscana* during the live feed period of Atlantic halibut larvae. Preference is given as α-indices (Chesson, 1983; Paper 1). Neutral selection is 0.100-0.143.

Most likely, the larvae ingested rotifers the first days because these are easy to catch. As the larvae gained some more experience, they switched to the bigger nauplii. The results suggested that the 1-day-old nauplii (ST nauplii, 0.84 mm length; Paper 1) were slightly bigger than the most preferred size at onset of feeding, and the larvae in fact never selected positively for the ST nauplii. Figures 2.1 and 2.2 suggest that the larvae may prefer a prey organism of a size between the size of rotifers and ST nauplii at the onset of feeding (285°d), and newly hatched nauplii (< 0.6 mm) may be an optimal size. The larvae ingested this smallest size class of *A. franciscana* throughout the whole live feed period. The abrupt change at around day 20 after first feeding is different from the more common pattern of gradually increase in preferred size reported for growing fish larvae, as fish larvae often increases their preferred sizes according to their gradual increase in mouth width (Hunter, 1981). The reason for the selection pattern found here for Atlantic halibut larvae, may be due to differences between the developmental stages of *A. franciscana* used. The juvenile stages of *A. franciscana* have higher swimming speed and the larvae may need to reach a certain size to be able to catch these stages. The colour of the feed has been reported to affect feed selection (Arthur, 1976; Dendrinos *et al.*, 1984), as well as taste and odour of the feed (Hunter, 1981; Dabrowski, 1984; Nicolas *et al.*, 1989), and this may differ between the different stages of *A. franciscana*.

3. PRODUCTION AND NUTRITIONAL VALUE OF *A. FRANCISCANA*

Artemia species are non-selective filter feeders, and the main food sources in nature are algae, detritus and bacteria (Sorgeloos *et al.*, 1986; Gorospe *et al.*, 1996). Growth has been reported on the bacteria *Flexibacter* Inp3 alone, although at a low growth rate (Intriago and Jones, 1993). The size range of food ingested by *Artemia* is between a few microns and 25-30 µm in nauplii, and up to 50 µm for adults (Dobbeleir *et al.*, 1980). As long as the food is within these sizes, several different foods can support growth. Many of the reported production trials of juvenile or adult stages of *Artemia* sp. have been performed with cheap agricultural by-products, for example rice bran (Dobbeleir *et al.*, 1980; Dhert *et al.*, 1992; Gorospe *et al.*, 1996). Good growth has been achieved with microalgae, like *Isochrysis galbana,* by Evjemo and Olsen (1999) and with several different microalgae used by other authors (Rosowski, 1989; Abreu-Grobois *et al.*, 1991; Correa-Sanoval *et al.*, 1994; Arriaga Haro and Re Araujo, 1997; Fábregas *et al.*, 1998).

The nutritional value of juvenile and adult *Artemia* given agricultural by-products is considered good with respect to overall protein and total lipid content (Dobbeleir *et al.*, 1980; Léger *et al.*, 1986; Lavens and Sorgeloos, 1991; Dhert *et al.*, 1992). However, when intended as feed for marine cold water species such as Atlantic halibut, the nutritional value is not considered adequate because of the low content of n-3 HUFA and the frequent absence of DHA (Léger *et al.*, 1986). The growth rate of *Artemia* is often low when algae are not included, and the low culture densities that have been used (normally < 10 individuals ml^{-1}) result in a very space demanding, extensive production.

Given the high costs of producing *Artemia* on algal diets, our work was concentrated on a commercial fish meal (SSF microfeed). This fish meal contains 75% protein, has particles in the size range of 5-50 µm, and has high contents of the fatty acids DHA and EPA (22 and 9 mg g^{-1} DW, respectively). The resulting n-3 HUFA level in the juvenile *A. franciscana* fed only fish meal was still not satisfactory, and further addition of lipids to the diet was necessary, especially to increase the content of DHA in the animals. Dhont and Lavens (1996) reported better results for long term enrichment of *Artemia* juveniles than for a short term enrichment on day 7. In preliminary experiments for short term enrichment of 5-day-old *A. franciscana*, satisfying levels of DHA were not obtained in the animals (Unpublished results, Kjell Inge Reitan). Therefore, a procedure for long term enrichment with DHA was developed. The work was concentrated on deciding the levels of lipid that were needed in the diet to give satisfactory contents in the animals (Paper 2). Production strategies were established (Paper 2) and the stability of the DHA content after ST enrichment or production (long term enrichment) was examined (Paper 3 and 4).

3.1 Production of juvenile *A. franciscana* with a satisfactory nutritional value

As mentioned in the introduction, the content of DHA and the protein to lipid ratio of naupliar stages of marine copepods differ distinctly from those of *Artemia*. Reitan and Olsen (1999) have reported increased growth and survival rate of Atlantic halibut larvae when the DHA level of the ST nauplii used as feed was increased from 5.5 to 15.5 mg g^{-1} DW. Based on knowledge of the content of DHA in zooplankton, it is likely that a further increase in the DHA content would be beneficial. However, to retain a high protein to lipid ratio, the lipid level must be kept low and preferentially

below 20% lipid of the DW. The ratio between DHA and EPA in the tissue of 12-day-old larvae has been shown to be of importance for pigmentation of turbot larvae (Rainuzzo, 1993; Reitan *et al.*, 1994). Recent findings suggest that a similar relationship exists also for Atlantic halibut larvae (personal communication Rainuzzo, J.R., SINTEF Fisheries and Aquaculture). This, however, deserves further investigation.

To obtain 16 mg DHA g^{-1} DW in 3-day-old *A. franciscana*, it was necessary to add at least 39% lipids and 10% DHA to the fish meal (Paper 2). The following procedure for production of 3-day-old *A. franciscana* was suggested (Paper 2):

- Food level: 0.09 g l^{-1} fish meal and 0.054 g l^{-1} fish oil high in DHA (e.g. Pronova TG 1040), giving 47% lipid in the diet.
- Feeding rate: 3 times a day (2 on day 0) to maintain the food level.
- *Artemia* density: Maximum 20 ind. ml^{-1} at start of production.
- Washing: No washing during the cultivation period.
- Temperature: 26-28°C.
- Oxygen concentration: > 2.5 mg O_2 l^{-1}.

The composition of juvenile *A. franciscana* produced according to this and a slightly modified procedure used in Paper 4 is given in Table 3.1.

Table 3.1. Composition of juvenile *A. franciscana* fed a diet of fish meal and added lipids according to the suggested procedure. Data from 3 separate experiments.

Size class (days)	Lipid (%)	DHA (mg g^{-1} DW)	DHA/EPA	Length (mm)	DW (µg ind^{-1})
3[a]	18.4	20.7	1.03	1.32±0.12	8.24±0.60
3[b]	18.8±0.1	18.5	1.33	1.24±0.06	4.94±0.52
3[c]	18.6±0.8	17.5	1.08	1.30±0.11	5.01±0.41
4[b]	17.7±0.7	12.9	0.70	1.29±0.08	6.57±0.49
4[c]	10.8±0.9	2.6	0.31	1.42±0.16	6.68±0.44
6[a]	12.9	3.7	0.25	2.22±0.25	24.18±0.76

[a]Paper 2, [b]Paper 4, [c]Paper 6 (reduced lipid level in diet for this 4-day-old *A. franciscana*).

The survival of *A. franciscana* reported in Paper 2 was low. However, when we used this procedure later (Paper 6), a survival of 85±5% was obtained (N=4 production cycles, unpublished results), but the animals obtained were smaller (Table 3.1). A slightly modified feeding strategy was also tested for the production of 3- and 4-day-old *A. franciscana* (Paper 4). The food level and lipid addition on day 0 and 1 were reduced to 0.06 g l^{-1} and 0.03 g l^{-1}, respectively. This resulted in a survival of 93% until day 3 (unpublished results). The biochemical composition and size of the animals were similar to those reported in Paper 6, and this suggested that it is possible to obtain the same nutritional composition of the 3-day-old *A. franciscana* as shown in Paper 2, with higher survival rates.

A. franciscana cultivated for 4 days or more had DHA/EPA ratios below 1 (Table 3.1), and the highest DHA levels obtained in 6-day-old *A. franciscana* was 3.7 mg g^{-1} DW (Paper 2, Table 3.1). *Artemia* juveniles and adults contain small amounts of DHA and EPA irrespectively of their diet (Léger *et al.*, 1986). The DHA and EPA contents reported in Table 3.1 for 3- and 4-day-old *A. franciscana* produced with the recommended procedure, are higher than most of those reported by other authors for 7-day-old or older *Artemia* (Table 3.2).

Table 3.2. Content of DHA and EPA in ongrown *Artemia* cultivated on different food as reported by other authors.

Authors	Food	Size (days)	DHA (mg g^{-1} DW)	EPA (mg g^{-1} DW)
Intriago and Jones (1993)	Combinations of bacteria and microalgae	8	0.1-0.8	1.1-4.0
Lavens and Sorgeloos (1991)	Mixture of maize bran and soybean-pellets	Pre-adults	4.6	2.8
Dobbeleir *et al.* (1980)	Rice bran and soybean meal	Adults	-	Trace
Dhont and Lavens (1996)	YM20 for 7 days, then 4h with Dry Selco	7	4.4	5.8
Dhont and Lavens (1996)	Added Dry Selco continuously	7	16.5	44.2

The only authors that found high amounts of DHA were Dhont and Lavens (1996). They reported a DHA content for 7-day-old *Artemia* similar to that given in Table 3.1 for 3-day-old *A. franciscana*. However, the EPA content of the former was much higher giving a low DHA/EPA ratio of 0.37.

In experiment 3 (Paper 2), a linear relation between increase in length and time was found, and for the increase in dry weight an exponential relation with time was found. Because of differences in animal densities, temperature, food and feeding regimes, it is not easy to compare these results with growth data obtained by other authors. However, Evjemo and Olsen (1999) obtained about 3.6 µg DW $ind.^{-1}$ in 3-day-old *A. franciscana* using *I. galbana* at maximum growth rate at otherwise similar conditions. This is lower than the dry weights reported in Table 3.1 for 3-day-old *A. franciscana*, showing that the diet used in the present experiments gave good growth also compared to the use of an algal diet.

To summarise, the presented procedure for production of 3-day-old *A. franciscana* gave a satisfactory lipid level and fatty acid composition (19% lipid, about 19 mg DHA g^{-1} DW), and a protein to lipid ratio of 3.4 (Paper 6). Even if the survival was low in one experiment, later experiments suggested that it can be kept high (> 85%) during the 3 day production cycle, especially if the food rations (including lipid addition) are reduced by 50% on day 0 and 1 compared to the procedure suggested in Paper 2. The length of the 3-day-old *A. franciscana* produced is the preferred size for the larvae from day 20 and onwards, and the biomass per prey item is more than 2 times higher than that of ST nauplii (Paper 6). Finally, it was apparently difficult to produce 4-day-old, and older, juveniles with a high DHA content (Papers 2, 4 and 6).

3.2 Stability of fatty acids in *A. franciscana*

The production of live feed normally takes place in 24 h cycles, i.e. the feed is freshly produced and ready for use once a day. Some of the feed will then have to be stored before it can be given to the fish larvae. In addition, the retention time in the fish

tanks before ingestion by the fish larvae may be several hours. The nutritional value of the feed may be reduced during starvation, e.g. the content of essential fatty acids may decrease rapidly (Danielsen *et al.*, 1995) and the protein content can be reduced. as demonstrated for rotifers (Makridis and Olsen, 1999).

Various techniques for preservation of the nutritional value have been tested. A linear relationship between loss rates and temperature has been reported for ST enriched nauplii for lipids and fatty acids (Evjemo *et al.*, 1999), and a reduction of the storage temperature after production seems to be an efficient way to reduce the losses of DHA after enrichment. Evjemo *et al.* (1999) reported a loss of DHA of 99% during 24 h post enrichment at 30°C, while at 12 degrees the loss was reduced to 67% for the same period. A close connection between the loss rate of lipids and temperature has also been reported for rotifers (Olsen *et al.*, 1993b). Even at a temperature of 12°C in the fish tanks (for Atlantic halibut larvae), high losses in the live feed will persist. For rotifers, however, adequate stabilisation of the DHA content may be achieved by addition of microalgae rich in DHA (e.g. *I. galbana*; 1-2 mg C l^{-1}) to the fish tanks (Reitan *et al.*, 1993b). This treatment will also stabilise the protein content of the rotifers (Makridis and Olsen, 1999).

An attempt to stabilise the lipid and fatty acid content in ST nauplii by addition of *I. galbana* gave the opposite effect. Higher losses were found during the first 24 h (calculated as loss rates, L (d^{-1}) in Paper 3) at high algal concentrations (from 6 mg C l^{-1}), most likely due to a rapid expelling of lipids from the gut by the ingested algal cells. However, between 48 and 72 h of the incubation period, a certain stabilisation of the content of DHA as well as other lipids, was observed. This indicated that microalga can be used to stabilise the DHA content, and other fatty acids and lipids, of juvenile *A. franciscana*. The experiment with 3- and 4-day-old *A. franciscana* reported in Paper 4, indicated that some stabilisation was obtained, especially for 4-day-old *A. franciscana*. The loss rates of DHA as reported in these two experiments, are given in Figure 3.1 (coefficients of the equations are given in Paper 3 and 4). A positive correlation between the loss rate (L) and algal concentration indicated a stabilising effect of the algae on the DHA content in the animals. For the ST enriched *A. franciscana* this correlation was accordingly negative between 0 and 24 h after enrichment but positive for the same nauplii after 48 to 72 h. The same stabilising effect was observed for juvenile *A. franciscana*, although it was not statistically significant for 3-day-old *A. franciscana* ($p = 0.26$). It was necessary to use a much higher concentration of *I. galbana* for *A. franciscana* than for rotifers, for this method to have any effect. This can be ascribed to the difference in incipient limiting concentration, the algal concentration needed by the animal to maintain maximum feeding rate, between the two organisms. For *A. franciscana* this concentration is reported to be 7-10 mg C l^{-1} (Evjemo and Olsen, 1999) while for rotifers a value of 1-2 mg C l^{-1} has been reported (Korstad *et al.*, 1989).

It is interesting to note that the net loss of DHA between day 3 and 4 of cultivation was 30% (Paper 4). This indicated that even if the animals were given a diet high in DHA, they could not accumulate more DHA after day 3, and they thereafter had a net loss. When 3-day-old *A. franciscana* were transferred to 12°C under starving conditions, they still contained 13.1 mg DHA g^{-1} DW after 24 h, the same content of DHA as the animals (4-day-old *A. franciscana*) cultivated for additional 24 h at 26°C.

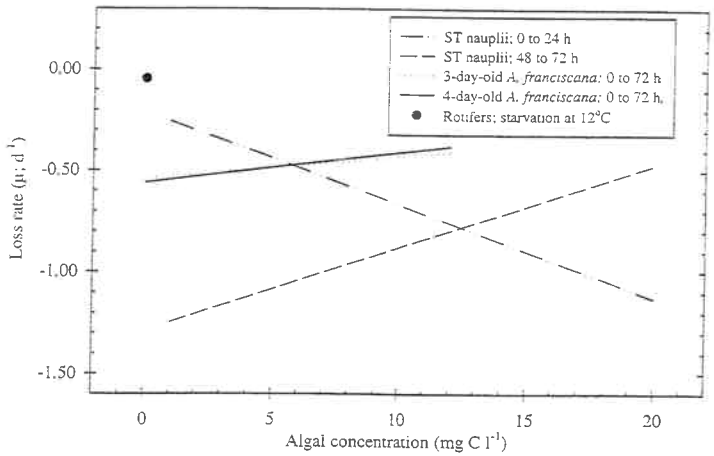

Figure 3.1 Loss rates of DHA (d^{-1}) in *A. franciscana* as a function of algal concentration. Regression coefficients (±STD) for the equations: $L_Q = aC + b$ (d^{-1}) are taken from Papers 3 and 4. The loss rate value for rotifers at 12°C is taken from Olsen *et al.* (1993b). ($P < 0.05$ except for 3-day-old *A. franciscana* where $P = 0.26$).

The absolute increase in the content of EPA in the 3-day-old animals after transfer to starving conditions, as well as in the presence of algae (Paper 4), indicated that DHA was converted to EPA as a part of the metabolism. This has formerly been suggested to take place in *Artemia* nauplii by Watanabe (1993) and Evjemo *et al.* (1997), and has been demonstrated for *Artemia* nauplii by Navarro *et al.* (1999).

In conclusion, for the ST nauplii the addition of the alga *I. galbana* at concentrations as high as 6 mg C l^{-1} to the tanks had an adverse effect on the stability of the DHA content. However, such high algal concentration is not used in larval tanks. For the juvenile *A. franciscana*, an addition of less than 6 mg C l^{-1} of *I. galbana* gave small effects on the stability of the DHA content. It had, however, another effect on juvenile *A. franciscana* because the content of the water soluble vitamin ascorbic acid (AA) showed an increase already at 3 mg C l^{-1} of *I. galbana*, and even more at higher algal concentrations (Paper 4). This is, however, a higher algal concentration than the normally used addition of 1-2 mg C l^{-1}.

Important factors that may contribute to ensure a high DHA content of *A. franciscana* for Atlantic halibut larvae are:

- Secure high nutritional value at the end of the production. This will allow a slight reduction in the content of especially DHA, and the feed still will have an adequate nutritional value when ingested by the larvae.
- Reduce the storage temperature immediately after production. A storage temperature of 8-12°C has been suggested for ST nauplii (Danielsen *et al.*, 1995).
- Develop procedures for production and feeding that reduce storage time after production and the retention time of *A. franciscana* in the fish tanks. All "old"

feed should be eaten or removed through water exchange before the larvae are fed the next batch.

4. MANIPULATION OF THE MICROBIAL FLORA OF *A. FRANCISCANA*

High mortalities during first feeding of marine fish larvae have in many cases been ascribed to microbial activity (Perez Benavente and Gatesoupe, 1988). The problems often seem to be proliferation of opportunistic pathogenic bacteria rather than specific obligate pathogenic bacteria (Vadstein *et al.*, 1993). In most cases, the live feed is believed to be the main vector for transfer of bacteria to the larvae (Muroga *et al.*, 1987; Perez Benavente and Gatesoupe, 1988; Nicolas *et al.*, 1989). Various means have been found to reduce the number of bacteria associated with the live feed (Rodríguez *et al.*, 1991; GomezGil-RS *et al.*, 1994). However, non-selective reduction of the bacterial load normally obtained by these methods, tend to create an unstable environment that is ideal for new growth of opportunistic bacteria.

It is assumed that it is advantageous for fish larvae to have an associated bacteria flora dominated by non-opportunistic slow growing bacteria (Salvesen *et al.*, 1999). Two strategies that have been suggested to improve the associated microflora of early larval stages are microbial maturation of the water, a slow re-colonisation of water after antibacterial treatment, or the use of probiotic bacteria (Skjermo and Vadstein, 1999). Selected bacterial strains have also been shown to be beneficial for juvenile *Artemia* during cultivation (Verschuere, 1999). When the juvenile *Artemia* were cultivated in the presence of one of the beneficial strains (LVS9) or a beneficial mixture of 9 strains, improved appetite of postlarvae of the shrimp *Litopenaeus vannamei* was found with no adverse effects observed (Verschuere, 1999).

Microalgae are normally added to the larval tanks, and this algal addition has been found to affect the microflora of larvae (Skjermo and Vadstein, 1993; Bergh *et al.*, 1994). Rotifers grown in the presence of algae have been reported to contain reduced bacterial numbers (Rodríguez *et al.*, 1991; Øie *et al.*, 1994). The microalga *Tetraselmis* sp. is commonly used in larval tanks of Atlantic halibut, and this algal species has been shown to exhibit bactericidal effects against some bacteria (Duff and Bruce, 1966; Kellam and Walker, 1989; Austin and Day, 1990; Austin *et al.*, 1992).

The aim of the experiments described in Paper 5 was to investigate if algae could be used to improve and change the flora associated with juvenile *A. franciscana*. The gut of *A. franciscana* contains food remains, faeces and bacteria after cultivation. By replacing this rich substrate with integrated food particles such as algal cells, reduced bacterial growth was expected. Another possible effect of the algae could be to change the flora associated with *A. franciscana*. The algal culture contains other bacteria and a more diverse flora that might colonise and change the gut flora of *A. franciscana*. It was expected that by reducing the number of associated bacteria and maybe changing and diversifying the flora associated with the juvenile *A. franciscana*, the quality of the live feed would be improved.

4.1 Microbial flora associated with *A. franciscana* – effect of microalgae

Preliminary experiments have shown small effects of 2 h incubation of 2-day-old *A. franciscana* with *Tetraselmis* sp., and varying number of associated bacteria after a period of 24 h, at algal concentrations of 5-20 mg C l^{-1}. Promising reductions in bacterial numbers were found after 4 and 8 h of incubation with *Tetraselmis* sp. at 5-

10 mg C l^{-1}, and the following procedure was developed for 2-day-old *A. franciscana* (Paper 5):

- Algal species: *Tetraselmis* sp.
- Algal concentration at start: 7 mg C l^{-1}
- Temperature: 20±1°C
- Duration: 4 h
- Density of *A. franciscana*: 100±10 ind. ml^{-1}

The animals were washed thoroughly after incubation and stored at 14-15°C until used as feed for the Atlantic halibut larvae. Because the microalgae may influence the associated bacterial flora of the live feed also in the larval tanks, both algal treated and untreated 2-day-old *A. franciscana* were incubated at simulated first feeding conditions (algal concentration 1-2 mg C l^{-1} of *Tetraselmis* sp.), to examine possible effects. The results are summarised in Fig 4.1.

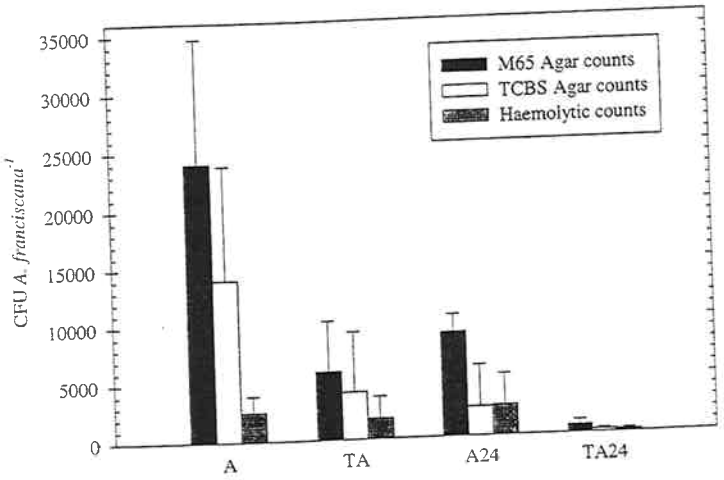

Figure 4.1. Semi-qualitative and quantitative composition of bacterial flora associated with 2-day-old *A. franciscana* (Bars are STD, n = 4 separate experiments). *A*: 2-day-old *A. franciscana*, *TA*: after 4 h incubation with *Tetraselmis* sp., and *A24* and *TA24* represent *A* and *TA* after 24 h, respectively, at simulated first feeding conditions (data from Paper 5).

The algal batches used varied in the number of associated bacteria between $1 \cdot 10^5$ and $13 \cdot 10^5$ colony forming units (CFU) ml^{-1}. Vibrios (estimated on TCBS agar) and bacteria with haemolytic activity remained, however, always below 1% of the total counts.

The initial bacterial load associated with the 2-day-old *A. franciscana* was high and variable; e.g., total numbers were in the range of 11,000-37,000 CFU $ind.^{-1}$. During

the 4 h incubation, the total number became reduced on average by 75%, and also the number of Vibrios was significantly reduced. Bacteria with haemolytic activity showed less reduction, and after the 24 h incubation under first feeding conditions the numbers in fact increased in the *A* group (Fig. 4.1). The most pronounced changes were observed for the *TA* group, which contained less than 3% of the total numbers and less than 1% of Vibrios and haemolytic bacteria of total CFU in group *A*.

4.2 Composition of the bacterial flora associated with 2-day-old *A. franciscana*

A number of 156 isolates from the four *A. franciscana* samples presented in Fig. 4.1 and from *Tetraselmis* sp. (*Tsp*) were examined by cluster analyses based on 33 morphological, biochemical and antibiotic sensitivity tests (Paper 5). The dendrogram could be divided into two roughly equal groups, the first containing most of the *A* and *TA* isolates, and the second all of the *Tsp* isolates and the majority of the *A24* isolates. The isolates from *TA24* were more evenly distributed. At a level of 85% similarity based on the 33 criteria, 10 groups of 4 or more isolates could be recognised. Some of the isolates were further identified to genus and a few to the species level (Paper 5).

The flora associated with the 2-day-old *A. franciscana* (*A*) showed very little diversity, and 34 out of 40 isolates (85%) were *Vibrios* with *Vibrio alginolyticus* as the dominating species (14 isolates). This species dominated the *TA* sample as well with 19 isolates, and in this sample, *Vibrios* constituted 22 out of 41 isolates. No *Vibrios* were isolated from the *Tetraselmis* sp. culture. The bacterial flora of this culture was diverse and appeared to have representatives from the genera *Pseudomonas*, *Flavobacterium*, *Moraxella*, *Acinetobacter* and *Aeromonas*. *Vibrio alginolyticus* was also found in *A24* (2 isolates) and *TA24* (1 isolate). These animals had, however, a much more diverse flora resembling that of the algal culture, although *A24* had some groups of isolates distinctly different from those found in the algal culture (Paper 5). The diversity indices, calculated as relative diversity J' (Zar, 1996) ranging between 0 (low) and 1 (highest), revealed that the initial flora associated with *A. franciscana* had very low diversity with a value of 0.17 (Paper 5). The value for the algal culture was 0.51, and after the 4 h incubation, the value for *A. franciscana* had increased to 0.40. The values increased further during the incubation at first feeding conditions and was as high as 0.83 in *TA24*.

It can be concluded that incubation with *Tetraselmis* sp. had great impact on the flora associated with the 2-day-old *A. franciscana*. Reductions in total CFU and counts of Vibrios could be observed already after the first 4 h incubation. To obtain reduction in the amount of bacteria with haemolytic activity, *A. franciscana* had to be maintained for 24 h more at first feeding conditions (12°C, algal concentration 1-2 mg C l^{-1}).

4.3 Impact of algae on the bacterial flora associated with *A. franciscana*

When the juvenile *A. franciscana* was sampled about 24 h after transfer to larval tanks, a reduction in number of associated bacteria was always observed (Paper 5 and 6; Table 4.1). The bacterial counts were quite similar to those obtained in the simulation experiment reported in Chapter 4.1. The same effect was observed for ST nauplii 24 h after transfer to larval tanks, as shown in Table 4.1. The numbers found for ST nauplii varied considerably, but Vibrios and bacteria with haemolytic activity always constituted around 100% of total CFU before transfer to the larval tanks.

The amount of bacteria and composition of the flora associated with the live feed seemed to have great impact on both the number and the composition of the bacterial flora associated with Atlantic halibut larvae, as shown in both Papers 5 and 6. Larvae fed different size classes of *A. franciscana* showed 2.5 times higher total CFU on day 45 and about 2 times more Vibrios and bacteria with haemolytic activity compared to larvae fed ST nauplii (Paper 6). In another experiment, larvae fed 2-day-old *A. franciscana* from day 7 to 16 showed significantly lower numbers of associated Vibrios and haemolytic bacteria when the feed was treated with algae before fed to the larvae (Paper 5).

Table 4.1 Quantitative and semi-qualitative composition of bacterial flora associated with *A. franciscana* used as feed for Atlantic halibut larvae. All juvenile *A. franciscana* were sampled from the larval tanks. Data were compiled from Paper 5 and 6, and data for ST nauplii from two more experiments (n=3, except for 2-day-old not incubated, and 4-day-old where n=2 and n=1, respectively; mean ± STD). Vibrios are estimated on TCBS agar.

A. franciscana	Total	Vibrio	Haemolytic
ST nauplii[a] (after enrichment)	781±552	666±98	786±197
ST nauplii[a] (in tank)	246±80	29±15	78±43
2-day-old (not incubated[c])	(9000±1550)[b]	707±95	838±129
2-day-old (incubated[c])	385±0	214±30	268±23
4-day-old (incubated[c])	1440±191	808±243	1144±561

[a]Enriched with DHA Selco as described in Paper 6. [b]Data from Fig 4.1. [c]Incubated refer to the 4 h incubation with *Tetraselmis* sp.

Larvae fed the algal treated 2-day-old *A. franciscana* contained 13,000 CFU larvae^{-1} of Vibrios compared to 125,000 for the other treatment. For bacteria with haemolytic activity, the numbers were 25,000 and 80,000 CFU larvae^{-1}, respectively (Paper 5). In both experiments, the amount of bacteria and the composition of the associated larval flora reflected the numbers associated with the live feed. Both reduced bacterial numbers and a more diverse flora associated with the live feed may be important for larval performance. An observation during first feeding under similar conditions revealed that the larvae apparently preferred 2-day-old *A. franciscana* previously incubated with algae. Larvae previously fed algal treated 2-day-old *A. franciscana* showed reduced appetite and stopped ingesting live feed when offered 2-day-old *A. franciscana* which had not been treated with algae. This is in agreement with Nicolas *et al.* (1989) who reported that turbot larvae did not ingest rotifers with high numbers of bacteria, possibly due to odours from the live feed. The great changes in the associated bacteria flora of *A. franciscana* after the transfer to first feeding conditions (Chapter 4.1 and 4.2) indicated that the algae can have indirect effects on the fish larvae by changing the associated microflora of the live feed.

The procedure reported in Paper 5 for 4 h incubation of 2-day-old *A. franciscana* with algae was used for both 3- and 4-day-old *A. franciscana* in the experiment reported in Paper 6. Bacterial numbers from 4-day-old *A. franciscana* sampled from larval tanks in that experiment are given in Table 4.1. The data suggested that the procedure was not that effective at least not for 4-day-old *A. franciscana*. These size classes have a higher ingestion rate of algae (Evjemo and Olsen, 1999), and this may have influenced the results. This needs, however, further examination.

5. *A. FRANCISCANA* AS FEED FOR LARVAE OF ATLANTIC HALIBUT

To produce adequate feed for Atlantic halibut larvae, factors such as nutritional value, prey size, digestibility and microbial quality have to be considered. It is also important to account for the fact that Atlantic halibut larvae have a daily weight increase of 6-12% (Olsen *et al.*, 1999) and that they undergo great changes during the first weeks of exogenous feeding until they have passed metamorphosis (Govoni *et al.*, 1986). It is likely that the optimal food will change during these first weeks, and the most critical factors therefore have to be examined during the whole live feed period.

5.1 Digestion of different size classes of *A. franciscana*

The relative digestion efficiency of the most actual sizes of *A. franciscana* as feed for Atlantic halibut larvae, based on the selection results presented in Chapter 2, are given in Fig. 5.1 (details in Paper 1). Data for ST enriched nauplii are also included because this is the most commonly used live feed for Atlantic halibut larvae (Olsen *et al.*, 1999).

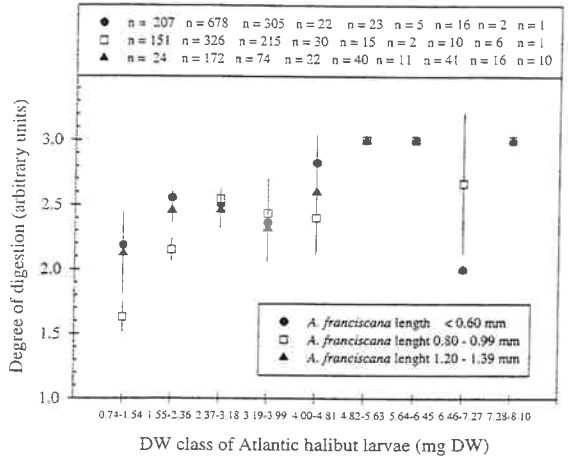

Figure 5.1 Relative digestion efficiencies of size classes of *A. franciscana* actual for use as live feed for Atlantic halibut larvae as a function of larval size. Degree of digestion is in the range 1 (low) to 3 (high). The size classes correspond to newly hatched nauplii (< 0.60 mm), Short Term (ST) enriched nauplii (0.80-0.99 mm) and 3-day-old *A. franciscana* (1.20-1.39 mm). Data from Paper 1.

Most strikingly, the ST nauplii were least digested by the smallest larvae (< 2.37 mg DW larva^{-1}). This was also generally the least digested size class in Atlantic halibut larvae (Paper 1). The newly hatched nauplii and juveniles of 1.20-1.39 mm length were better digested than ST nauplii by the smaller larval size classes, which, as may be expected, showed poorer digestion efficiency than the bigger larval stages. Larvae of bigger size classes (> 2.37 mg DW) showed equal and more complete digestion of the different size classes of *A. franciscana*, and larvae of the size 4.82 mg DW and bigger obtained digestion values close to 3 (Paper 1). The smaller larvae have a

poorly developed digestion system and may have been dependent on autolysis of the live feed for digestion as has been suggested for marine fish larvae (Dabrowski, 1984). Shields *et al.* (1999) observed that enriched *Artemia* nauplii apparently passed through the gut of Atlantic halibut larvae largely undigested, whereas copepods (*Eurytemora velox*) were more completely assimilated, early in the live feed period.

5.2 Quality of Atlantic halibut juveniles

The main challenge so far has been to produce enough Atlantic halibut larvae for weaning. As the production increases and the numbers satisfies the demand of the marked, the quality will be more important (Næss and Lie, 1998; Olsen *et al.*, 1999). Common quality defects are incomplete pigmentation or that the larvae are pigmented on both sides, incomplete eye migration, or various other deformities (Næss *et al.*, 1995; Paper 6).

Good quality larvae should consequently have a completely pigmented upper (ocular) side, no pigmentation on the lower (blind) side, complete eye migration and no visible deformities (Paper 6). Larvae fed ST *A. franciscana* as the only feed do frequently not satisfy these requirements, but the quality can be improved by feeding them natural zooplankton (Næss *et al.*, 1995; Næss and Lie, 1998; Shields *et al.*, 1999). In the present study (Paper 6), more than 20% of the juvenile Atlantic halibut satisfied the quality requirements when they were fed juvenile *A. franciscana* from day 14 and all through the weaning period. Feeding ST nauplii the whole live-feed period resulted in 4% of perfect larvae (Table 5.1). Similar results were obtained by Shields *et al.* (1999) when they fed the larvae either enriched *Artemia* nauplii or natural zooplankton (*Eurytemora velox*). The group fed enriched nauplii had 4% perfect larvae on day 71 compared to 40% perfect larvae in the zooplankton group.

Table 5.1. Quality evaluation of the largest larvae on day 71 and the remaining smaller larvae on day 89 (Paper 6). Different subscript letters within a column indicate significant differences ($P<0.05$).

Larvae	Number evaluated	Perfect (%)	Complete pigmentation (%)	Complete pigmentation upper side (%)	Complete/ correct metamorphosis (%)
Small fed juvenile	100	17^a	39^a	71^a	33^a
Small fed ST	100	4.0^b	6.0^b	23^b	23^a
Large fed Juvenile	64	28^a	33^a	42^c	75^b
Large fed ST	59	3.4^b	8.5^b	15^b	47^c

The larvae fed increasing size groups of *A. franciscana* in the live feed period showed significantly higher proportions of perfect larvae, and the pigmentation was also more complete. The differences were less pronounced for metamorphosis. However, the largest larvae that were fed juvenile *A. franciscana* also performed best during metamorphosis. Some of the differences in pigmentation success can possibly be ascribed to the high lipid content of ST nauplii compared to that of juvenile *A. franciscana*, as suggested in Paper 6 and by Gara *et al.* (1998).

5.3 Feeding regimes with *Artemia*

The use of larger size classes of *A. franciscana* as feed can be advantageous for nutritional as well as for energetic reasons. Faster growth and developmental rates may be achieved by larvae which ingest a low number of large prey items per unit of time than for larvae which consume a high number of mall prey (Hunter, 1981; Lavens and Sorgeloos, 1991). Feeding regimes using larger size classes of *Artemia* have frequently been applied in the cultivation of marine larvae (for a review see Léger *et al.*, 1986). The Atlantic halibut larvae increase their ingestion rate of ST *Artemia* nauplii in an exponential way and van der Meeren (1995) has reported a predicted ingestion rate of more than 2500 nauplii per day by larvae of 21.8 mm standard length. This high number suggests that the ST nauplii probably are too small to sustain optimal growth of the larvae. The 3-day-old juveniles have more than twice the dry weight of the ST nauplii (Paper 6), and consequently contain more energy and protein per feed item. Juvenile *A. franciscana* had a higher percentage of protein with 64-69% of DW, compared to 56% in the ST nauplii (Paper 6). It is therefore reasonable to expect that the use of juvenile *A. franciscana* would result in faster growth than with use of ST nauplii. Improved growth rate of Atlantic halibut larvae offered larger prey was not obtained in the experiment reported in Paper 6. Other experiments, however, have demonstrated this potential of growth improvement also for Atlantic halibut. A group of Atlantic halibut larvae fed 4-day-old *Artemia* from day 20 instead of ST nauplii, reached the double dry weight on day 70 compared to the larval group that continued with ST nauplii as feed for the whole period (personal communication, Ingrid Lein, AKVAFORSK).

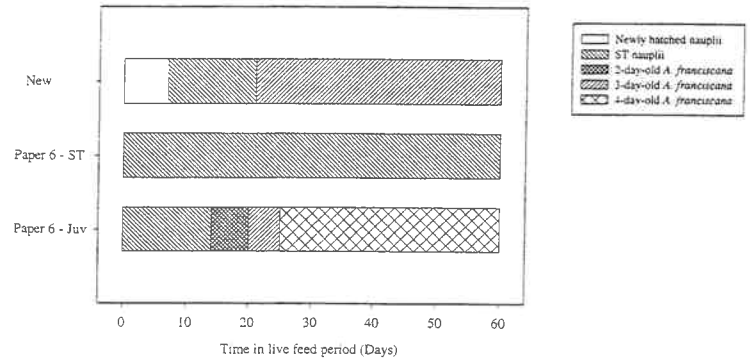

Figure 5.2. Feeding regimes used for Atlantic halibut larvae in Paper 6 and a suggested regime based on use of newly hatched nauplii for some of the first days of the live feed period. Algae were added during the first 4-5 weeks (1-2 mg C l^{-1}).

Some feeding regimes for Atlantic halibut larvae based on cultivated live feed are shown in Fig. 5.2. ST nauplii are commonly used for the whole live feed period in Norway. Besides this, natural zooplankton and combinations of zooplankton and ST nauplii added as supplement when needed are also common (Olsen *et al.*, 1999). In addition, the use of only cultivated live feed all through the larval period, except for a short time believed to be critical for pigmentation, has been suggested (Ness *et al.*, 1995; Næss and Lie, 1998).

High survival and complete pigmentation were obtained when newly hatched nauplii were used until day 19 followed by natural zooplankton (Næss *et al.*, 1995). A feeding regime used in UK for Atlantic halibut larvae involve use of newly hatched nauplii until day 9 followed by enriched nauplii, and this procedure has given quite good survival, growth and pigmentation (Gara *et al.*, 1998).

Newly hatched nauplii are preferred before ST nauplii by the larvae and are more efficiently digested compared to ST nauplii by Atlantic halibut larvae (Paper 1). This suggests that newly hatched nauplii may advantageously be used for some time after initiating exogenous feeding. The dry weight of the newly hatched nauplii is the same as that of the ST enriched nauplii (ca 2.3-2.5 µg ind.$^{-1}$), but the newly hatched nauplii will provide more dietary protein the first period. The larvae would probably gain from a higher protein content and better digestibility these first days. Gara *et al.* (1998) reported an inverse relation between lipid levels of the liver of the larval groups and percentage perfect metamorphosis, suggesting that overall lipid intake affect the metamorphosis success, as also was suggested in Paper 6. The success of feeding the larvae newly hatched nauplii would be dependent on the nutritional status of the larvae when they start to ingest feed and the nutritional value of the feed used after the period with newly hatched nauplii.

The data presented in Chapter 2.1 show that the prey size offered to the larvae should be increased from about day 20 (at about 3.5 mg DW), from small nauplii to *A. franciscana* > 1.2 mm length. Larger feed sizes later in the live feed period, like 4-day-old and older *A. franciscana* may give even better growth, provided that the microbial quality is satisfactory and that the DHA level is not to low. The larvae have no problems to ingest larger feed as they ingested a wide range of sizes when given the opportunity (Paper 1). However, 3-day-old *A. franciscana* may be the optimal size to produce, regarding nutritional value, satisfactory survival rate through the production period and probably microbial quality. Weaning of Atlantic halibut is normally initiated around day 50, but an earlier provision of formulated food is desired because of nutritional reasons and the costs of producing live feed (Olsen *et al.*, 1999). The larvae exhibited an abrupt shift in preference and digesting ability at around day 20. At this stage of development, the larvae change their behaviour and undergo great physiological changes. It may therefore also be a good opportunity for an early introduction of formulated food. This deserves further investigation.

Rotifers have been used as a first feed for Atlantic halibut larvae and they are readily ingested although not selected for positively by the larvae (Paper 1). It is nevertheless possible that the larvae could benefit from ingesting rotifers during the first days of exogenous feeding, and the rotifers may be used along with *Artemia* nauplii. The rotifers are probably more easy to catch than nauplii and thereby give the larvae experience in capturing prey, and they seemed to be more easily digested than ST nauplii (Paper 1). Also, by ingesting the rotifers the digestion efficiency of the larvae for nauplii later may be improved. The digestion process of herring larvae has been shown to be triggered by the ingestion of small inert particles (Hjelmeland *et al.*, 1988), and for early larval stages of anchovy, both increased enzyme production and retention of the digesting enzymes after defaecation after ingestion of meals were observed (Pedersen, B.H., 1989).

6. CONCLUSIONS

When the Atlantic halibut larvae were given the choice between rotifers and ST nauplii, the larvae showed no preferences for any of these the first two days (Paper 1). From day 3 and onwards the larvae preferred ST nauplii. If the larvae had the opportunity to select between different size classes of *A. franciscana*, the preferred size was the newly hatched nauplii the first 3 weeks. This is therefore most probably the preferred feed size of *Artemia* (400-500 µm) in this period. Around day 20 (3-4 mg DW), the larvae showed a pronounced shift in their preferred size of *A. franciscana*, and they started to select for individuals of 1.2-1.4 mm length or longer. This size normally corresponds to *A. franciscana* cultivated for 3 days.

The digestion of rotifers seemed to be very efficient, and the larvae apparently digested the newly hatched nauplii better than the ST nauplii during the first weeks, presumably because of the high lipid content of the latter (25-28% total lipid of DW is normal). Also, the bigger size classes of *A. franciscana* seemed to be more efficiently digested than the ST nauplii by larvae weighing less than 2.4 mg DW. Bigger larvae showed almost equal and very efficient digestion of all size classes of *A. franciscana* (Paper 1).

The larvae selected positively for the larger *A. franciscana* in a period before they reached the size when they normally accepted formulated food and prey of the size 1.2-1.4 mm (i.e. 3-day-old *A. franciscana*) seemed to be the optimal size for the larvae. This size class can be produced with 18 mg DHA g^{-1} DW and a DHA/EPA ratio > 1.0 by using the culture conditions recommended and summarised in Chapter 3.3. The experiments showed that in 2-day-old, or older, *A. franciscana*, the lipid and DHA levels seldom reached 200 and 20 mg g^{-1} DW, respectively.

A. franciscana appears to transform DHA to EPA relatively efficiently and a high initial DHA/EPA ratio (> 1.0) is therefore needed. Because of the instability of DHA and other lipids in *A. franciscana* after enrichment (Paper 3), and in juveniles after the production (Paper 4), it is important that *A. franciscana* is nutritionally adequate after production, as well as when the larvae ingest the feed. The DHA content of ST *A. franciscana* decreased more rapidly during the first 24 h in animals kept at concentrations of 6 mg C l^{-1} or higher of *I. galbana*, than in animals kept at lower algal concentrations (Paper 3, Fig. 3.1). This was most likely due to a replacement of lipids by algal cells in the gut of the nauplii. In the case of 3- and 4-day-old *A. franciscana*, the effect of addition of *I. galbana* on the DHA content in the animals was small for the algal levels normally used in the larval tanks (1-2 mg C l^{-1}). The best way to preserve the nutritional value post enrichment is to store the animals at lower temperatures (e.g. 8-12°C).

The experiments reported in Paper 5 showed that microalgae can be used to manipulate bacterial level and the composition associated with the live feed when juvenile stages of *Artemia* are raised on fish meal and fish oil. During a 4 h incubation of 2-day-old *A. franciscana* with the alga *Tetraselmis* sp., the numbers of associated bacteria and *Vibrio* spp. were significantly reduced. This effect was even more pronounced after a subsequent transfer to first feeding conditions. The composition of the microbial flora associated with the animals became more diverse, and less dominated by *Vibrio alginolyticus*, which was the dominating species associated with 2-day-old *A. franciscana* (Paper 5). The results also showed that the algae indirectly affected the

larvae through changing the associated bacterial flora of the live feed. Larvae which received algal-treated 2-day-old *A. franciscana* had significantly lower numbers of Vibrios and bacteria with haemolytic activity (Paper 5) compared to larvae fed non-treated feed. In the experiment with feeding of bigger *A. franciscana* juveniles to the larvae (Paper 6), 4-day-old *A. franciscana* had high numbers of associated bacteria, and especially of bacteria with haemolytic activity, even if the procedure for algal incubation was used. Differences in bacterial flora associated with the live feed may lead to differences in growth of larvae. Improvement of the microbial quality of juvenile *A. franciscana* is one of the main obstacles for the use of juvenile *A. franciscana* as feed for the larvae, and both a further development of the algal incubation method or the use of probiotic bacteria during cultivation seem to be promising.

Another challenge in live feed formulation is to determine exactly the DHA demands of the Atlantic halibut larvae throughout the live feed period. ST nauplii are the most commonly used cultivated live feed for Atlantic halibut larvae. The fact that the larvae never selected positively for ST nauplii and that this also was the least digested size class in case of the earliest larval stages of Atlantic halibut, suggests that it may be beneficial to use newly hatched nauplii for some days after first feeding. However, the newly hatched nauplii do not contain DHA and are therefore nutritionally inadequate for the larvae and research is needed to determine if the total effect of using this size class for some days in the beginning of the live feed period would be beneficial. Also the nutritional status of the larvae at the onset of exogenous feeding and the nutritional quality of the feed used later will be of importance for the success of using newly hatched nauplii for the larvae for some days as suggested in Paper 6.

Furthermore, the optimal lipid level of the feed and thereby the protein/lipid ratio, is an important topic for future research, as a higher protein level of the live feed is believed to improve the quality of the fry. Exact demands, however, are mostly unknown. The 3-day-old *A. franciscana* has many useful properties and better quality of the Atlantic halibut juveniles was obtainable with the use of 3- and 4-day-old *A. franciscana* as feed, compared to ST nauplii (Paper 6). If the microbial quality can be improved, better growth of the Atlantic halibut larvae should be obtainable with this feed. Faster growth rate means a shorter live feed period, which is important for the economy of the production. Furthermore, with variation in the availability of cysts and increasing prises, increasing the biomass produced per cyst may reduce the total live feed costs. The use of juvenile *A. franciscana* as feed will presumably be advantageous as long as weaning is performed around day 50-60.

7. REFERENCES

Abreu-Grobois, F.A., Briseno-Duenas, R., Herrara, M.A. and Malagon, M.L. 1991. A model for growth of *Artemia franciscana* based on food ratio-dependent gross growth efficiencies. *Hydrobiologia* 212: 27-37.

Arriaga Haro, V.M. and Re Araujo, A.D. 1997. Uso de microalgas vivas e inertes como alimento para *Artemia franciscana* (Anostraca: Artemiidae). *Rev. Biol. Trop.* 45 (2): 803-811.

Arthur, D. K., 1976. Food and feeding of larvae of three fishes occurring in the California Current, *Sardinops sagax*, *Engraulis mordax* and *Trachurus symmetricus*. *U.S. Fish. Bull.* 74: 517-530.

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

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

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

Bremigan, M. T. and Stein, R. A. 1994. Gape-dependent larval foraging and zooplankton size: Implications for fish recruitment across systems. *Can. J. Fish Aquat. Sci.* 51: 913-922.

Båmstedt, U. 1986. Chemical composition and energy content. In: E.D.S. Coroner and S.C.M. O'Hara (Eds.) The biological chemistry of marine copepods. Clarendon press, Oxford. pp. 1-58.

Chesson, J. 1983. The estimation and analysis of preferences and its relationships to foraging models. *Ecology* 64: 1297-1304.

Conceicão, L. E. C. 1997. Growth in early life stages of fishes. An explanatory model. PhD. Thesis. Wageningen Agricultural University, The Netherlands.

Correa-Sadoval, F., Voltolina-Lobina, D., Bückle-Ramírez, L.F. and Cordero-Esquiel, B. 1994. The growth rates of four populations of *Artemia franciscana* (Anostraca: Artemiidae). *Rev. Biol. Trop.* 42 (3): 605-609.

Coutteau, P. and Sorgeloos, P. 1997. Manipulations of dietary lipids, fatty acids and vitamins in zooplankton cultures. *Freshwater biology* 38: 501-512.

Dabrowski, K. 1984. The feeding of larvae: present "state of the art" and perspectives. *Repr. Nutr. Develop.* 24 (6): 807-833.

Dabrowski, K. and Bardega, R. 1984. Mouth size and predicted food size preference of larvae of three cyprinid fish species. *Aquaculture* 40: 41-46.

Danielsen, T. L., Evjemo, J. O. and Olsen, Y. 1995. Stability of short-time enriched n-3 fatty acids in *Artemia* during starvation at different temperatures. In: LARVI '95. Fish and Shellfish Symposium. Lavens, P., E. Jaspers & I. Roelants, (Eds.). European Aquaculture Society. Special Publication NO. 24: 128-131.

Dendrinos, P. Dewan, S. and Thorpe, J.P. 1984. Improvements in the feeding efficiency of larval, post larval and juvenile Dover sole (*Solea Solea* L.) by the use of staining to improve the visibility of *Artemia* used as food. *Aquaculture* 38: 137-144.

Detwyler, R. and Houde, E. D. 1970. Food selection by laboratory-reared larvae of the scaled sardine *Harengula pensacolae* (Pisces, Clupeidae) and the bay anchovy *Anchoa mitchilli* (Pisces, Engraulidae). *Mar. Biol.* 7: 214-222.

Dhert, P., Bombeo, R. B., Lavens, P. and Sorgeloos, P. 1992. A simple semi flow-through culture technique for the controlled super-intensive production of *Artemia* juveniles and adults. *Aquacult. Eng.* 11: 107-119.

Dhert, P., Sorgeloos, P. and Devresse, B. 1993. Contributions toward a specific DHA enrichment in the live food *Brachionus plicatilis* and *Artemia* sp. In: Proceedings of the First International Conference on Fish Farming Technology (Reinertsen, H., Dahle, L.A, Jørgensen, L. & Tvinnereim, K. eds.), Trondheim, Norway, 9-12 August 1993, pp. 109-115. A.A. Balkema, Rotterdam, Netherlands.

Dhont, J. and Lavens, P. 1996. Tank production and use of ongrown *Artemia*. In: P. Lavens and P. Sorgeloos (Eds.) Manual on the production and use of live food for aquaculture. FAO Fisheries technical paper 361, Rome.

Dobbeleir, J., Adam, N., Bossuyt, E., Bruggeman, E. and Sorgeloos, P. 1980. New aspects of the use of inert diets for high density culturing of brine shrimp. In: The brine shrimp *Artemia* - Ecology, Culturing and use in Aquaculture, Vol. 3. Persoone, G., Sorgeloos, P., Roels, O. and Jaspers, E. Wetteren, (Eds.) Belgium: Universa press. pp. 165-174.

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

Evjemo, J. O. and Olsen, Y. 1997. Lipid and fatty acid content in cultivated live feed organisms compared to marine copepods. In: Hagiwara, A., Snell, T. W., Lubzens, E., Tamaru, S. (Editors), Live Food in Aquaculture. *Hydrobiologia* 358: 159-162.

Evjemo, J. O., Cotteau, P., Olsen, Y. and Sorgeloos, P. 1997. The stability of docosahexaenoic acid (DHA) in two *Artemia* species following enrichment and subsequent starvation. *Aquaculture* 155: 135-148.

Evjemo, J.O. and Olsen, Y. 1999. Effect of food concentration on the growth and production rate of *Artemia franciscana* feeding on algae (T. *iso*). *J. Exp. Mar. Biol. Ecol.* 242: 273-296.

Evjemo, J.O., Danielsen, T.L. and Olsen, Y. 1999. Stability of lipid, protein and n-3 fatty acids in enriched *Artemia franciscana* starved at different temperatures. Submitted *Aquaculture*.

Fábregas, J., Otero, A., Morales, E.D., Arredondo-Vega, B.O. and Patiño, M. 1998. Modification of the nutritive value of Phaeodactylum tricornutum for *Artemia* sp. in semicontinuous cultures. *Aquaculture* 169: 167-176.

Fernández-Diaz, C., Pascual, E. and Yúfera, M. 1994. Feeding behaviour and prey size selection of gilthead seabream, *Sparus aurata*, larvae fed on inert and live food. *Mar. Biol.* 118: 323-328.

Frank, K. T. and Leggett, W. C. 1986. Effect of prey abundance and size on the growth and survival of larval fish: an experimental study employing large volume enclosures. *Mar. Ecol. Prog. Ser.* 34: 11-22.

Gara, B., Shields, R.J. and McEvoy, L. 1998. Feeding strategies to achieve correct metamorphosis of Atlantic halibut, *Hippoglossus hippoglossus* L, using enriched *Artemia*. *Aquacult. Res.* 29: 935-948.

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

Gorospe, J.N., Nakamura, K., Abe, M and Higashi, S. 1996. Nutritional contribution of *Pseudomonas* sp. in *Artemia* culture. *Fish. Sci.* 62 (6): 914-918.

Govoni, J.J, Boehlert, G.W, and Watanabe, Y. 1986. The physiology of digestion in fish larvae. *Environ. Biol. Fishes* 16 (1-3): 59-77.

Gulbrandsen, J. 1993. Prey consumption of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae: selectivity or availability? *Aquaculture* 116: 159-170.

Hjelmeland, K., Pedersen, B.H and Nilsen, E.N. 1988. Trypsin content in intestines of herring larvae, *Clupea harengus*, ingesting inert polystyrene spheres or live crustacea prey. Mar. Biol. 98: 331-335.

Holmefjord, I., Bolla, S. and Reitan, K. I. 1989. Start feeding of Atlantic halibut (*Hippoglossus hippoglossus* L.) on enriched rotifers and *Artemia* compared with collected plankton. *Rapp. P-v. Réun. Cons. int. Explor. Mer.* 191: 474-483.

Hunter, J. R. 1981. Feeding ecology and predation of marine fish larvae. In: Lasker E. (Ed.), Marine fish Larvae. Morphology, Ecology and Relation to Fisheries. Washington Sea Grant Program, Seattle, WA. pp. 34-77.

Intriago, P. and Jones, D.A. 1993. Bacteria as food for *Artemia*. Aquaculture 113: 115-127.

Kellam, S.J. and Walker, J.M. 1989. Antibacterial activity from Marine Microalgae in Laboratory culture. *Br. Phycol. J.* 24: 191-194.

Korstad, J., Vadstein, O. and Olsen, Y. 1989. Feeding kinetics of *Brachionus plicatilis* fed *Isochrysis galbana*. Hydrobiologia 186/187: 51-57.

Lavens, P. and Sorgeloos, P. 1991. Production of *Artemia* in culture tanks. In: R.A. Browne, P. Sorgeloos, C.N.A. Trotman (eds.), *Artemia* Biology. CRC Press, pp. 317-350.

Léger, P., Bengtson, D. A., Simpson, K. L. and Sorgeloos, P. 1986. The use and nutritional value of *Artemia* as a food source. *Oceanogr. Mar. Biol. Ann. Rev.* 24: 521-623.

Makridis, P. and Olsen, Y. 1999. Protein depletion of the rotifer *Brachionus plicatilis* during starvation. *Aquaculture* 174: 343-353.

Muroga, K., Higashi, M. and 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.

Navarro, J.C, Henderson, R.J, McEvoy, L.A., Bell, M.B. and Amat, F. 1999. Lipid conversion during enrichment of *Artemia*. *Aquaculture* 174: 155-166.

Nicolas, J.L., Robic, E. and 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.

Næss, T., Germain-Henry, M. and Naas, K.E. 1995. First feeding of Atlantic halibut (*Hippoglossus hippoglossus*) using different combinations of *Artemia* and wild zooplankton. *Aquaculture* 130: 235-250.

Næss, T. and Lie, Ø. 1998. A sensitive period during first feeding for the determination of pigmentation pattern in Atlantic halibut, *Hippoglossus hippoglossus* L., juveniles: the role of diet. *Aquacult. Res.* 29: 925-934.

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

Olsen, Y., Rainuzzo, J.R., Reitan, K.I. and Vadstein, O. 1993a. Manipulations of lipids and ω3 fatty acids in *Brachionus plicatilis*. In: Proceedings of the First International Conference on Fish Farming Technology (Reinertsen, H., Dahle, L.A, Jørgensen, L. & Tvinnereim, K. eds.), Trondheim, Norway, 9-12 August 1993, pp. 101-108. A.A. Balkema, Rotterdam, Netherlands.

Olsen, Y., Reitan, K.I. and Vadstein, O. 1993b. Dependence of temperature on loss rates of rotifers, lipids and ω3 fatty acids in starved *Brachionus plicatilis* cultures. *Hydrobiologia* 255/256: 13-20.

Pedersen, B.H. 1989. Secretion of protease into the gut of herring larvae digesting copepods (abstract). *Rapp. P-v. Réun. Cons. int. Explor. Mer.* 191: 474-483.

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

Rainuzzo, J.R. 1993. Lipids in early stages of marine fish. PhD. Thesis. University of Trondheim. Norway.

Reitan, K.I., Evjemo, J.O, Olsen, Y. and Øie, G. 1993a. Biomass growth in Atlantic halibut (*Hippoglossus hippoglossus* L.) from eggs to metamorphosis. 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, Netherlands, pp. 417-420.

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

Reitan, K.I., Rainuzzo, J.R and Olsen, Y. 1994. Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. *Aquacult. Int.* 2: 33-48.

Reitan, K.I. and Olsen, A.I. 1999. Importance of dietary polyunsaturated fatty acids in first feeding of halibut larvae (*Hippoglossus hippoglossus* L.). World Aquaculture '99, The annual International Conference and exhibition of the world Aquaculture society, 26 April – 2 May 1999. Sydney, Australia.

Rodríguez, J.L., Planas, M. and Otero, J.J. 1991. Microflora and antibacterial treatments of rotifers and *Artemia*. In: LARVI '91. Fish and Shellfish Symposium. (Eds. P. Lavens, E. Jaspers, I. Roelants) European Aquaculture Society, Special Publication 24, pp. 403-405.

Rosowski, J.R. 1989. Rapid growth of the Brine shrimp, *Artemia franciscana* Kellogg, in xenic cultures of *Chlorella* sp. (*Chlorophyceae*). Aquaculture 81: 185-203.

Salvesen, I., Skjermo, J. and Vadstein, O. 1999. Growth of turbot (*Scophthalmus maximus* L.) during first feeding in relation to the proportion of *r/K*-strategists in the bacterial community of the rearing water. *Aquaculture* 175: 337-350.

Sargent, J., Bell, G., McEvoy, L., Tocher, D. and Estevez, A. 1999. Recent developments in the essential fatty acid nutrition of fish. *Aquaculture* 177: 191-199.

Shields, R.J., Bell, J.G., Luizi, F.S., Gara, B., Bromage, N.R. and Sargent, J.R. 1999. Natural copepods are superior to enriched *Artemia* nauplii as feed for halibut larvae. *J. Nutr.* 129: 1186-1194.

Skjermo, J. and Vadstein, O. 1993. 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. and Vadstein, O. 1999. Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* 177: 333-343.

Sorgeloos, P., Lavens, P., Léger, P., Tackaert, W. and 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.

Vadstein, O., Øie, G., Olsen, Y., Salvesen, I., Skjermo, J. and Skjåk-Bræk, G. 1993. A strategy to obtain microbial control during larval development of marine fish. In: Reinertsen, H., L. A., Dahle, Jørgensen, L., Tvinnereim, K. (Eds.), Proceedings of the First International Conference on Fish Farming Technology. A. A. Balkema, Rotterdam, pp. 69-75.

van der Meeren, T. 1995. Feed consumption and gut evacuation in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. In: LARVI '95. Fish and Shellfish Symposium. Lavens, P., E. Jaspers & I. Roelants, (Eds.). European Aquaculture Society, Special Publication NO. 24: 381-384.

Verschuere, L. 1999. Microbial community analysis and control in the culture of *Artemia* juveniles for aquaculture and pet shop industry. Ph.D. Thesis. University of Gent, Belgium.

Watanabe, T. 1982. Lipid nutrition in fish. *Comp. Biochem. Physiol.* 73B: 3-15.

Watanabe, T. 1993. Importance of Docosahexaenoic Acid in Marine Larval Fish. *J. World Aquacult. Soc.* Vol. 24 (2): 152-161.

Zar, J.H. 1996. Biostatical analysis. 3rd ed. Prentice-Hall, Englewood Cliffs, New Jersey, pp. 662.

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

Øie, G., Makridis, P., Reitan, K.I. and Olsen, Y. 1997. Protein and carbon utilisation of rotifers (*Brachionus plicatilis*) in first feeding of turbot larvae (*Scophthalmus maximus* L.). *Aquaculture* 153: 103-122.