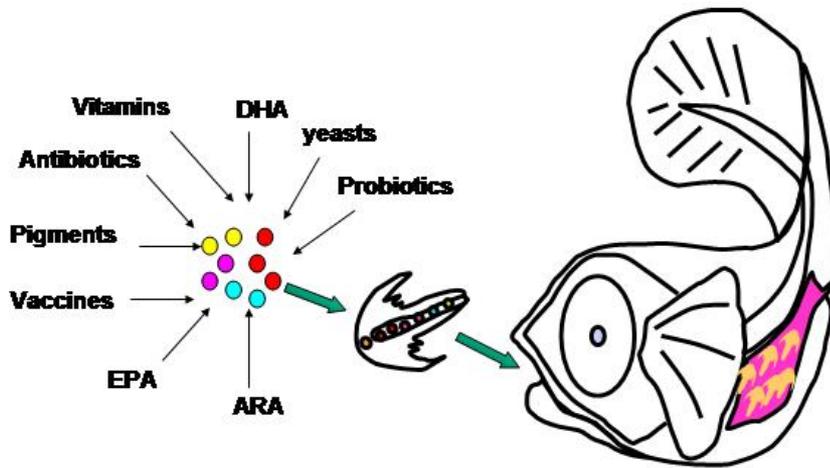


Handbook of Protocols and Guidelines for Culture and Enrichment of Live Food for Use in Larviculture



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Introduction

Larviculture, more particularly the start feeding of early larval stages, appears to be the major bottleneck for the industrial upscaling of the culture of fish and shellfish. Evolutionary, larvae of most fish and crustaceans are fixed on the scheme of motile prey organisms and encounter problems to accept inert/dry diets. Even if they accept the diets, their poor enzymatic activity and not functional stomach will not allow them to digest the existing formulated diets (Pedersen et al., 1987, Pedersen and Hjelmeland, 1988). Improving the acceptance of dry diets for fish larvae and formulate more digestible and less polluting diets remains thus still a central task for aquaculturists. Before this is achieved, live food (phyto- and zooplankton) will remain an important food source for the start feeding of early larval stages.

Among the important starter feeds used in larviculture are newly hatched nauplii of *Artemia* and marine rotifer *Brachionus plicatilis*. The successful development of commercial farms in the Mediterranean area has been made possible by several improvements in the production techniques of this live food (Candreva et al., 1996; Dehasque et al., 1998).

The nutritional aspects of *Artemia* and rotifers have received major attention in larviculture and several commercial products have been launched to increase the lipid and vitamin content in nauplii and rotifers (Coutteau and Sorgeloos, 1997).

Hatching protocol of Artemia cyst and Purification of newly hatched nauplii

Disinfection of Artemia cysts with liquid bleach (Van Stappen 1996)

- Prepare 200 ppm hypochlorite solution: ± 20 ml liquid bleach (NaOCl) 10 l^{-1}
- Soak cysts for 30 min. at a density of ± 50 g cysts. l^{-1} ;
- Wash cysts thoroughly with tap water on a 125 μm screen;
- Cysts are ready for hatching incubation.

Procedures for the decapsulation of Artemia cysts (Van Stappen 1996)

HYDRATION STEP

- Hydrate cysts by placing them for 1 h in water (≤ 100 g. l^{-1}), with aeration, at 25°C.

DECAPSULATION STEP

- Collect cysts on a 125 μm mesh sieve, rinse, and transfer to the hypochlorite solution.
- The hypochlorite solution can be made up (in advance) of either liquid bleach NaOCl (fresh product; activity normally =11-13% w/w) or bleaching powder $\text{Ca}(\text{OCl})_2$ (activity normally $\pm 70\%$) in the following proportions:
 - * 0.5 g active hypochlorite product (activity normally labeled on the package, otherwise to be determined by titration) per g of cysts; for procedure see further;
 - * an alkaline product to keep the $\text{pH} > 10$; per g of cysts use:
 - 0.15 g technical grade NaOH when using liquid bleach;
 - either 0.67 NaCO_3 or 0.4 g CaO for bleaching powder; dissolve the bleaching powder before adding the alkaline product; use only the

supernatants of this solution;

· seawater to make up the final solution to 14 ml per g of cysts.

· Cool the solution to 15-20°C (i.e. by placing the decapsulation container in a bath filled with ice water). Add the hydrated cysts and keep them in suspension (i.e. with an aeration tube) for 5-15 min. Check the temperature regularly, since the reaction is exothermic; never exceed 40°C (if needed add ice to decapsulation solution). Check evolution of decapsulation process regularly under binocular.

WASHING STEP

· When cysts turn grey (with powder bleach) or orange (with liquid bleach), or when microscopic examination shows almost complete dissolution of the cyst shell (= after 3-15 min.), cysts should be removed from the decapsulation suspension and rinsed with water on a 125 µm screen until no chlorine smell is detected anymore. It is crucial not to leave the embryos in the decapsulation solution longer than strictly necessary, since this will affect their viability.

DEACTIVATION STEP

· Deactivate all traces of hypochlorite by dipping the cysts (< 1 min.) either in 0.1 N HCl or in 0.1% Na₂S₂O₃ solution, then rinse again with water. Hypochlorite residues can be detected by putting some decapsulated cysts in a small amount of starch-iodine indicator (= starch, KI, H₂SO₄ and water). When the reagent turns blue, washing and deactivation has to be continued.

USE

· Incubate the cysts for hatching, or store in the refrigerator (0-4°C) for a few days before hatching incubation. For long term storage cysts need to be dehydrated in saturated brine solution (1 g of dry cysts per 10 ml of brine of 300 g NaCl.l⁻¹). The brine has to be renewed after 24h.

Artemia hatching

- Use transparent or translucent cylindro-conical containers
- Supply air through open aeration line down to the tip of the conical part of the tank; oxygen level should be preferably maintained about 4 g.l^{-1} , apply strong aeration
- A valve at the tip of the tank will facilitate harvesting, in small containers hatched larvae may be siphoned by a clean pipet connected to a transparent rubber tube
- Use preheated, filtered (*e.g.* with a filter bag) natural seawater ($\pm 33 \text{ g.l}^{-1}$) as hatching medium
- Hatching temperature varies strain to strain, optimum is about 28°C for most strains
- pH should be 8-8.5; if necessary add dissolved sodium bicarbonate (up to 2 g.l^{-1} technical grade NaHCO_3) or sodium carbonate solution drop by drop
- Apply minimum illumination of 2000 lux at the water surface, (*i.e.* by means of fluorescent light tubes close to water surface)
- Disinfect cysts prior to hatching incubation
- Incubate cysts at density of 2 g.l^{-1} ; for smaller volumes ($<20\text{l}$) a maximal cyst density of 5 g.l^{-1} can be applied. Required amount of cysts depends on hatching efficiency of cyst batch (number of nauplii per gram, see further) and required amount of nauplii
- Incubate for 24 hr (the incubation period may be shorter (18-20 hr) for fast hatching cysts)
- Stop aeration and harvest the newly hatched nauplii sunked to the bottom of the containers by opening the valve or siphoning by a pipet or clean transparent rubber tube.

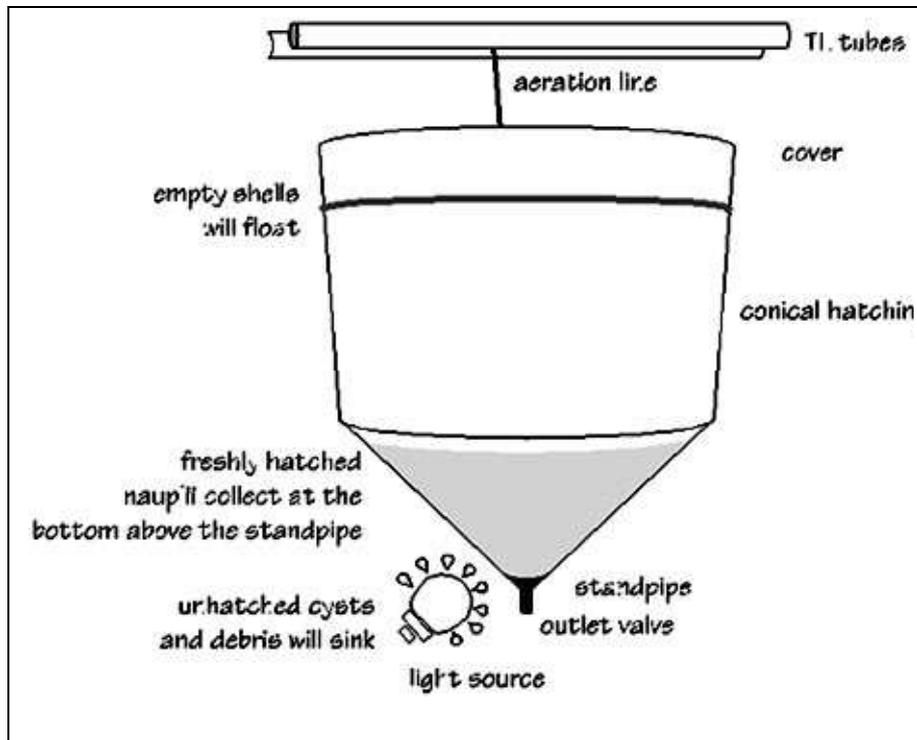
Determination of hatching percentage, hatching efficiency and hatching rate (Van Stappen 1996)

- Incubate exactly 2 g of cysts in exactly 1000 ml 33 g.l^{-1} seawater under continuous illumination (2000 lux) at 28°C in a cilindroconical tube (preferentially) or in a graduated cylinder; provide aeration from bottom as to keep all cysts in suspension (aeration not too strong as to prevent foaming); run test in triplicate.

- After 24 h incubation take 6 subsamples of 250 µl out of each cone.
- Pipet each subsample into a small vial and fixate nauplii by adding a few drops of lugol solution.
- Per cone ($i = 6$ subsamples), count nauplii (n_i) under a dissection microscope and calculate the mean value (N), count umbrellas (u_i) and calculate mean value (U).
- Decapsulate unhatched cysts and dissolve empty cyst shells by adding one drop of NaOH solution (40g.100 ml⁻¹ distilled water) and 5 drops of domestic bleach solution (5.25% NaOCl) to each vial.
- Per cone ($i = 6$), count unhatched (orange colored) embryos (e_i) and calculate mean value (E).
- Hatching percentage $H\% = (N \times 100):(N + U + E)^{-1}$
- Calculate $H\%$ value per cone and calculate mean value and standard deviation of 3 cones = final value
- Hatching efficiency $HE = (N \times 4 \times 1000):(2)^{-1}$
- calculate HE value per cone and calculate mean value and standard deviation of 3 cones = final value
- Eventually leave hatching tubes for another 24 h, take subsamples again and calculate $H\%$ and HE for 48 h incubation.
- Hatching rate (HR): start taking sub-samples and calculating $H\%$ & HE from 12 h incubation in seawater onwards (follow procedure above). Continue sampling/counting procedures until mean value for $H\%$ & HE remains constant for 3 consecutive hours. The mean values per hour are then expressed as percentage of this maximal $H\%$ & HE . A hatching curve can be plotted and T_{10} , T_{90} etc. Extrapolated from the graph. A simplified procedure consists in sample taking e.g. every 3 or more hours.

Harvesting and distribution

After hatching and prior to feeding the nauplii to fish/crustacean larvae, they should be separated from the hatching wastes (empty cyst shells, unhatched cysts, debris, microorganisms and hatching metabolites). Five to ten minutes after switching off the aeration, cyst shells will float and can be removed from the surface, while nauplii and unhatched cysts will concentrate at the bottom (Fig. 1).



[Figure 1: Hatching container at harvest](#) (After Van Stappen, 1996)

Since nauplii are positively phototactic, their concentration can be improved by shading the upper part of the hatching tank (use of cover) and focusing a light source on the transparent conical part of the bottom. Nauplii should not be allowed to settle for too long (*i.e.*, maximum 5 to 10 min.) in the point of the conical container, to prevent dying off due to oxygen depletion. Firstly, unhatched cysts and other debris that have accumulated underneath the nauplii are siphoned or drained off when necessary (*i.e.* when using cysts of a lower hatching quality). Then the nauplii are collected on a filter using a fine mesh screen ($< 150 \mu\text{m}$), which should be submerged all the time so as to prevent physical damage of the nauplii. They are then rinsed thoroughly with water in order to remove possible contaminants and hatching metabolites like glycerol (Van Stappen, 1996).

Instar I nauplii completely thrive on their energy reserves; therefore should be harvested and fed to the fish or crustacean larvae in their most energetic form, (*i.e.* as soon as possible after hatching). For a long time farmers have overlooked the fact that an *Artemia* nauplius in its first stage of development can not take up food and thus consumes its own energy reserves. At the high temperatures applied for cyst

incubation, the freshly-hatched *Artemia* nauplii develop into the second larval stage within a matter of hours. It is important to feed first-instar nauplii to the predator rather than starved second-instar meta-nauplii which have already consumed 25 to 30% of their energy reserves within 24 h after hatching. Moreover, instar II *Artemia* are less visible as they are transparent, are larger and swim faster than first instar larvae, and as a result consequently are less accessible as a prey. Furthermore they contain lower amounts of free amino acids, and their lower individual organic dry weight and energy content will reduce the energy uptake by the predator per hunting effort. All this may be reflected in a reduced growth of the larvae, and an increased *Artemia* cyst bill as about 20 to 30% more cysts will be needed to be hatched to feed the same weight of starved meta-nauplii to the predator (Van Stappen, 1996).

Cold storage of newly hatched nauplii

Molting of the *Artemia* nauplii to the second instar stage may be avoided and their energy metabolism greatly reduced by storage of the freshly-hatched nauplii at a temperature below 10°C in densities of up to 8 million per liter. Only a slight aeration is needed in order to prevent the nauplii from accumulating at the bottom of the tank where they would suffocate. In this way nauplii can be stored for periods up to more than 24 h without significant mortalities and a reduction of energy of less than 5%. Applying 24-h cold storage using styrofoam insulated tanks and blue ice packs or ice packed in closed plastic bags for cooling, commercial hatcheries are able to economize their *Artemia* cyst hatching efforts (i.e., reduction of the number of hatchings and harvests daily, fewer tanks, bigger volumes). Furthermore, cold storage allows the farmer to consider more frequent and even automated food distributions of an optimal live food. This appeared to be beneficial for fish and shrimp larvae as food retention times in the larviculture tanks can be reduced and hence growth of the *Artemia* in the culture tank can be minimized. For example, applying one or maximum two feedings per day, shrimp farmers often experienced juvenile *Artemia* in their larviculture tanks competing with the shrimp postlarvae for the algae. With poor hunters such as the larvae of turbot *Scophthalmus maximus* and tiger shrimp *Penaeus monodon*, feeding cold-stored, less active *Artemia* furthermore results in much more efficient food uptake Léger *et al.* (1986).

Importance of naupliar size

The nutritional effectiveness of a food organism is in the first place determined by its ingestibility and, as a consequence by its size and form. Naupliar size, varying greatly from one geographical source of *Artemia* to another, is often not critical for crustacean larvae, which can capture and tear apart food particles with their feeding appendages. For marine fish larvae that have a very small mouth and swallow their prey in one bite the size of the nauplii is particularly critical. For example, fish larvae that are offered oversized *Artemia* nauplii may starve because they cannot ingest the prey. Fish that produce small eggs, such as gilthead seabream, turbot and grouper must be fed rotifers as a first food because the nauplii from any *Artemia* strain are too large. In these cases, the size of nauplii (of a selected strain) will determine when the fish can be switched from a rotifer to an *Artemia* diet. As long as prey size does not interfere with the ingestion mechanism of the predator, the use of larger nauplii (with a higher individual energy content) will be beneficial since the predator will spend less energy in taking up a smaller number of larger nauplii to fulfill its energetic requirements. Data on biometrics of nauplii of enriched and non-enriched *Artemia urmiana* are presented in Table 1.

Table 1: Biometry of non-enriched, enriched with fatty acid emulsions, and fed with *Dunaliella tertiolecta* 0-72 hours after enrichment at cold storage (size in mm)

hour after enrichment	Non-enriched nauplii	Enriched with FA emulsion	Enriched with <i>D. tertiolecta</i>
0	0.517	0.715	0.827
12	0.534	0.743	0.848
24	0.539	0.745	0.840
36	0.530	0.773	0.843
48	0.527	0.763	0.834
60	0.522	0.762	0.817
72	0.521	0.728	0.813

Nutritional quality

Another important dietary characteristic of *Artemia* nauplii was identified in the late 1970s and early 1980s, when many fish and shrimp hatcheries scaled up their production and reported unexpected problems when switching from one source of *Artemia* to another. Japanese, American and European researchers studied these problems

and soon confirmed variations in nutritional value when using different geographical sources of *Artemia* for fish and shrimp species. The situation became more critical when very significant differences in production yields were obtained with distinct batches of the same geographical origin of *Artemia* (Van Stappen, 1996)

Studies in Japan and the multidisciplinary International Study on *Artemia* revealed that the concentration of the essential fatty acid (EFA) 20:5n-3 eicosapentaenoic acid (EPA) in *Artemia* nauplii was determining its nutritional value for larvae of various marine fishes and crustaceans (Léger *et al.*, 1986). Various results were obtained when different batches of the same geographical *Artemia* source, containing different amounts of EPA were used to feed shrimp larvae. Levels of this EFA vary tremendously from strain to strain and even from batch to batch (Table 2, Figure 2), the causative factor being the fluctuations in biochemical composition of the primary producers available to the adult population. Following these observations, appropriate techniques have been developed for improving the lipid profile of deficient *Artemia* strains. Commercial provisions of *Artemia* cysts containing high EPA levels are limited and consequently, these cysts are very expensive. Therefore, the use of the high-EPA cysts should be restricted to the feeding period when feeding of freshly-hatched nauplii of a small size is required (Van Stappen, 1996).

Table 2. Intra-strain variability of 20:5n-3 (EPA) content in *Artemia*. Values represent the range (area percent) and coefficient of variation of data as compiled by Léger *et al.* (1986).

Cyst source	20:5n-3 range (area %)	Coefficient of variation (%)
San Francisco Bay, CA-USA	0.3-13.3	78.6
Great Salt Lake (South arm), UT-USA	2.7-3.6	11.8
Great Salt Lake (North arm), UT-USA	0.3-0.4	21.2
Chaplin Lake, Canada	5.2-9.5	18.3
Macau, Brazil	3.5-10.6	43.2
Bohai Bay, PR China	1.3-15.4	50.5
Urmia Lake, Iran	1.2-15.1	50.2

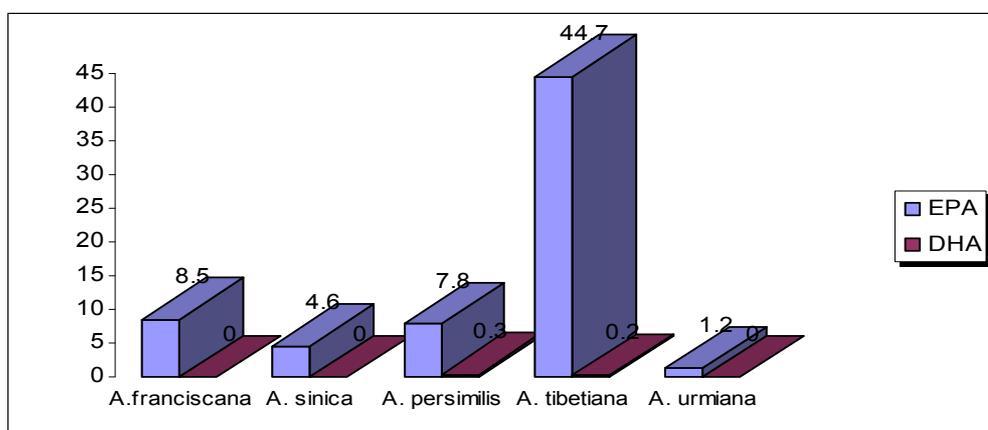


Figure 2: EPA & DHA levels in various strains of *Artemia* nauplii

In an experiment performed on enrichment of *Artemia urmiana* with fatty acid emulsions and unicellular algae *D. tertiolecta* between and preserved for 72 h. after enrichment indicates that EPA dose not change considerably when the enriched *Artemia* are preserved in cold incubator at 4°C (tables 2 & 3) (Manaffar, 2002).

Table 2: Change in EPA level in enriched and non-enriched nauplii preserved in cold, 0-72 hours after enrichment

hour after enrichment	Non-enriched nauplii	Enriched with FA emulsion	Enriched with <i>D. tertiolecta</i>
0	1.47	14	3.3
12	1.35	10.2	2.9
24	1.38	13.1	3.2
36	1.4	12.4	4.1
48	1.2	14	3.5
60	1.1	12.3	3.6
72	1.15	11.3	3.4

Table 3: Change in DHA level in enriched and non-enriched nauplii preserved in cold, 0-72 hours after enrichment

hour after enrichment	Non-enriched nauplii	Enriched with FA emulsion	Enriched with <i>D. tertiolecta</i>
-----------------------	----------------------	---------------------------	-------------------------------------

0	0	17.5	0
12	0	14.4	0
24	0	12.1	0
36	0	10.1	0
48	0	9.3	0
60	0	3.11	0
72	0	0.9	0

In contrast to fatty acids, the amino acid composition of *Artemia* nauplii seems to be remarkably similar from strain to strain, suggesting that it is not environmentally determined in the manner that the fatty acids are.

The presence of several proteolytic enzymes in developing *Artemia* embryos and *Artemia* nauplii has led to the speculation that these exogenous enzymes play a significant role in the breakdown of the *Artemia* nauplii in the digestive tract of the predator larvae. This has become an important question in view of the relatively low levels of digestive enzymes in many first-feeding larvae and the inferiority of prepared feeds *versus* live prey (Van Stappen, 1996).

A stable form of vitamin C (ascorbic acid 2-sulphate) is present in *Artemia* cysts. This derivative is hydrolysed to free ascorbic acid during hatching, the -ascorbic acid levels in *Artemia* nauplii varying from 300 to 550 $\mu\text{g g}^{-1}$ DW. The published data would appear to indicate that the levels of vitamins in *Artemia* are sufficient to fulfill the dietary requirements recommended for growing fish. However, vitamin requirements during larviculture, are still largely unknown, and might be higher due to the higher growth and metabolic rate of fish and crustacean larvae (Van Stappen, 1996).

Enrichment with nutrients

As mentioned previously, an important factor affecting the nutritional value of *Artemia* as a food source for marine larval organisms is the content of essential fatty acids, eicosapentaenoic acid (EPA: 20:5n-3) and even more importantly docosahexaenoic acid (DHA: 22:6n-3). In contrast to freshwater species, most marine organisms do not have the capacity to biosynthesize these EFA from lower chain unsaturated fatty acids, such as linolenic acid (18:3n-3). In view of the fatty acid deficiency of *Artemia*, research has been conducted to improve its lipid composition by prefeeding with (n-3) highly unsaturated fatty

acid (HUFA)-rich diets. It is fortunate in this respect that *Artemia*, because of its primitive feeding characteristics, allows a very convenient way to manipulate its biochemical composition. Thus, since *Artemia* on molting to the second larval stage (*i.e.* about 8 h following hatching), is non-selective in taking up particulate matter, simple methods have been developed to incorporate lipid products into the brine shrimp nauplii prior to offering them as a prey to the predator larvae. This method of bioencapsulation, also called *Artemia* enrichment or boosting (Fig. 3), is widely applied at marine fish and crustacean hatcheries all over the world for enhancing the nutritional value of *Artemia* with essential fatty acids (Van Stappen 1996).

Figure 3. Schematic diagram of the use of *Artemia* as vector for transfer of specific components into the cultured larvae.

British, Japanese, French and Belgian researchers have also developed other enrichment products, including unicellular algae, w-yeast and/or emulsified preparations, compound diets, micro-particulate diets or self-emulsifying concentrates. Apart from the enrichment diet used, the different techniques vary with respect to hatching conditions, pre-enrichment time (time between hatching and addition of enrichment diet), enrichment period, and temperature. Highest enrichment levels are obtained when using emulsified concentrates (Table 4) (Van Stappen 1996).

Table 4. Enrichment levels (mg.g⁻¹ DW) in *Artemia* nauplii boosted with various products

Commercial HUFA emulsions	DHA	EPA	(n-3) HUFA
Super Selco (INVE Aquaculture NV)	14.0	28.6	50.3
DHA Selco (INVE Aquaculture NV)	17.7	10.8	32.7

Superartemia (Catvis)	9.7	13.2	26.3
SuperHUFA (Salt Creek)	16.4	21.0	41.1

The Selco diet is a self-dispersing complex of selected marine oil sources, vitamins and carotenoids. Upon dilution in seawater, finely dispersed stable microglobules are formed which are readily ingested by *Artemia* and which bring about EFA-enrichment levels which largely surpass the values reported in the literature (Léger *et al.*, 1986). For enrichment the freshly-hatched nauplii are transferred to an enrichment tank at a density of 100 (for enrichment periods that may exceed 24 h) to 300 nauplii.ml⁻¹ (maximum 24-h enrichment period); the enrichment medium consisting of disinfected seawater maintained at 25°C. The enrichment emulsion is usually added in consecutive doses of 300 mg.l⁻¹ every 12 h with a strong aeration (using airstones) being required so as to maintain dissolved oxygen levels above 4 mg.l⁻¹ (the latter being necessary to avoid mortalities). The enriched nauplii are harvested after 24 h (sometimes even after 48 h), thoroughly rinsed and then fed directly or stored at below 10°C so as to minimize the metabolism of HUFA prior to administration, *i.e.* HUFA levels being reduced by 0-30% after 24 h at 10°C, Fig. 4.3.9. By using these enrichment techniques very high incorporation levels of EFA can be attained that are well above the maximal concentrations found in natural strains. These very high enrichment levels are the result not only of an optimal product composition and presentation, but also of proper enrichment procedures: *i.e.* the nauplii being transferred or exposed to the enrichment medium just before first feeding, and opening of the alimentary tract (instar II stage). Furthermore, size increase during enrichment will be minimal: *Artemia* enriched according to other procedures reaching > 900 µm, whereas here, high enrichment levels are acquired in nauplii measuring 660 µm (after 12-h enrichment) to 790 µm (after 48-h enrichment, Fig. 4.3.10.). Several European marine fish hatcheries apply, therefore, the following feeding regime, switching from one *Artemia* diet to the next as the fish larvae are able to accept a larger prey: only at the start of *Artemia* feeding is a selected strain yielding small freshly-hatched nauplii with a high content of EPA (10 mg g⁻¹ DW) used, followed by 12-h and eventually 24-h (n-3) HUFA enriched *Artemia* meta-nauplii. Average (n-3) HUFA levels in enriched *Artemia* varies from 15 to 28% or 22 to 68 mg.g⁻¹ DW and 16 to 30% or 32 to 64 mg.g⁻¹ DW, respectively (Van Stappen, 1996).

In view of the importance of DHA in marine fish species a great deal of effort has been made to incorporate high DHA/EPA ratios in live

food. To date, the best results have been obtained with enrichment emulsions fortified with DHA (containing a DHA/EPA ratio up to 7), yielding *Artemia* meta-nauplii that contain 33 mg DHA.g⁻¹ DW. Compared to enrichment with traditional products, a maximum DHA/EPA ratio of 2 instead of 0.75 can be reached using standard enrichment practices (Van Stappen, 1996).

The reason for not attaining the same ratio is the inherent catabolism of DHA upon enrichment within the most commonly used *Artemia* species (i.e. *A. franciscana*). The capability of some Chinese *Artemia* strains to reach high DHA levels during enrichment and to maintain their levels during subsequent starvation might open new perspectives to provide higher dietary DHA levels and DHA/EPA ratios to fish and crustacean larvae (Van Stappen, 1996).

Apart from EFA, other nutrients such as vitamins and pigments can be incorporated in *Artemia*. Fat soluble vitamins (especially vitamin A and vitamin E) were reported to accumulate in *Artemia* over a short-term (9 h) enrichment period with vitamin A levels increasing from below 1 IU.g⁻¹ (WW basis) to over 16 IU.g⁻¹ and vitamin E levels increasing from below 20 µg.g⁻¹ to about 250 µg.g⁻¹. Recently tests have also been conducted to incorporate ascorbic acid into live food. Using the standard enrichment procedure and experimental self-emulsifying concentrates containing 10, 20 and 30% (on a DW basis) of ascorbyl palmitate (AP) in addition to the triglycerides, high levels of free ascorbic acid (AA) can be incorporated into brine shrimp nauplii. For example, a 10%-AP inclusion in the emulsion enhances AA levels within freshly-hatched nauplii by 50% from natural levels (500 µg g⁻¹ DW). By contrast, however, a 20 or 30% addition increases AA levels in *Artemia* 3-fold and 6-fold respectively after 24 h enrichment at 27°C; with (n-3) HUFA levels remaining equal compared to normal enrichment procedures. Moreover, these AA concentrations do not decrease when the enriched nauplii are stored for 24 h in seawater (Van Stappen, 1996).

Standard Method for enrichment of *Artemia* nauplii with HUFA emulsions (triplicate enrichment on 1 l scale):

· Seawater disinfection

- add 1 mg. l⁻¹ NaOCl (100 µl bleach solution 10 l⁻¹ of 0.45 µm filtered seawater)
- incubate 1 h
- aerate strongly overnight
- add 0.5 g.l⁻¹ NaHCO₃ (dissolved in deionized water and GF filtered)

· Cyst disinfection

- use cylindroconical container
- 4 g cysts. l⁻¹ tapwater
- 20 min at 200 mg.l⁻¹ NaOCl (±2.0 ml bleach solution.l⁻¹)
- harvest and rinse well, weigh out 2 × 50%

· Hatching

- 2 cylindroconical containers
- add 1/2 of the cysts per litre disinfected natural/artificial seawater
- incubate for 24 h, at 28°C, 2000 lux light, strong aeration
- separate nauplii from debris if needed in an aquarium in seawater
- make nauplii suspension of about 300 N/ml, count accurately (3 × 250 µl samples).

· Enrichment (triplicate)

- transfer volume containing 200,000 nauplii to a sieve
- rinse them well with filtered seawater
- stock in 1 l cone with point aeration at 200 nauplii. ml⁻¹
- count initial density (3 × 250 N.ml⁻¹) add 2 × 0.2 g of emulsion (2 × 2 ml of 5 g. 50 ml⁻¹ diluted emulsion) over 24 h (t = 0 h and t = 10-12 h)
- incubate for 24 h at 28°C, strong aeration, monitor O₂ and pH regularly!

· Harvesting

- count survival, *i.e.* count dead nauplii (no lugol) and total nauplii (+lugol) from 3 × 250 µl sample per cone
- remove all aeration
- concentrate nauplii using light
- siphon nauplii on sieve

- rinse well with tapwater
- dry sieve on paper towel
- transfer nauplii into vial and freeze at -30°C

· **Results**

- survival percentage during enrichment
- fatty acid composition of enriched *Artemia*

Standard Method for Enrichment of *Artemia* nauplii with Vitamin C:

Enrichment procedure:

Enrichment with vitamin C is a secondary step to enrichment with HUFAs. Therefore in order to enrich the live food with vitamins, fatty acid emulsion is used as carrier of this component.

- Prepare fatty acid emulsion as explained earlier
- add 10% to 20% Ascorbyl palmitate w/w to the fatty acid emulsion
- Mix properly using an electric mixer until the vitamin C is dissolved (dilute with DDFW if necessary).
- Preserve the emulsion containing vitamin C in refrigerator before use.
- Nauplii are enriched with 2 doses of above mixture at 0.0 h and at 10-12 h during the process of enrichment
- Enrichment process is same as explained for HUFA.
- Stop aeration after 24 h and siphon the nauplii into a clean beaker containing filtered sea water.
- Preserve the enriched *Artemia* in a cold incubator with gentle aeration

Sample preparation:

-Samples should be stored at **-80°C** for **Vit C** analysis before you begin the procedure or should be processed immediately after enrichment.

-Bring your live sample (e.g. enriched *Artemia*) over a sieve.

-Rinse the sample very well with tap water.

-Dry the bottom side of the sieve using paper.

- Cut sample into small pieces

- Transfer 0.5-1 g of the sample into a plastic test tube

- Add 100 µl of internal standard (Iso-Ascorbic Acid) into the test tube

- Add 2 ml of standard solution to the sample [1 mM EDTA + 2 mM Hemocystein in 500 ml double distilled filtered water (DDFW) for fish sample **OR** 1 g MPA + 1 ml Acetic Acid + 0.3774 g EDTA + 500 ml DDFW for *Artemia* sample]

- Homogenize the sample for 1-2 minutes at 4°C

- Transfer the supernatant to a clean test tube

- Add again 2 ml of above solution to the sample and repeat earlier steps

- Once again add 1 ml of standard solution and repeat the earlier steps in order to have about 5 ml supernatant

- Centrifuge the homogenized sample for 5-10 minutes at 10000 rpm at 4°C
- Filter the sample through cartridge powder already conditioned with methanol, DDFW and standard solution
- Sample is ready for injection
- Preferentially 3 samples should be made available for each analysis

Standard Method for Enrichment of *Artemia* nauplii with Antibiotics:

Antibiotics are also incorporated into HUFA emulsion for enrichment of nauplii.

Procedure:

- Prepare fatty acid emulsion according to standard procedure as explained earlier
- Calculate the amount of antibiotic required for enrichment of the fish/shrimp larvae (it may differ according to the weight, size or species)
- Add the calculated amount of antibiotic (e.g 10% w/w) to the fatty acid emulsion
- Mix properly using an electric mixer until the antibiotic is dissolved in the fatty acid emulsion
- Preserve the emulsion containing antibiotic in refrigerator before use.
- Nauplii are enriched with 2 doses of this mixture at 0.0 h and at 10-12 h during the process of enrichment
- Enrichment process is same as explained for HUFA.
- Stop aeration after 24 h and siphon the nauplii into a clean beaker containing filtered sea water.
- Preserve the enriched *Artemia* in a cold incubator with gentle aeration

Standard Method for Enrichment of *Artemia* nauplii with Probiotics and Prebiotics:

1. procedure for enrichment with probiotics

- Introduce newly hatched nauplii in 500 ml bottles
- Prepare a solution with 150 mg l⁻¹ DHA and 50 mg l⁻¹ of Probiotic preparation (commercial preparation) or bacterial suspension at a concentration of 10⁷ – 10⁸

CFU l⁻¹ (if the probiotic have been isolated by your own)

- Add this solution to the bottle containing nauplii
- Incubate for 14 to 20 hours (1st step enrichment)
- Count bacteria associated to 1dph *Artemia* on selected media
- If larvae have to be fed with *Artemia*, a second step enrichment is needed. To this end 1dph *Artemia* are further enriched with DHA (50 mg l⁻¹) and probiotic preparation (50 mg l⁻¹).
- Distribute enriched *Artemia* with a peristaltic pump

2. Procedure for enrichment with prebiotic

- Introduce newly hatched nauplii in 500 ml bottles
- Prepare a solution with 150 mg l⁻¹ DHA and 10, 30 or 60 mg l⁻¹ of prebiotic powder
- Add this solution to the bottle containing nauplii
- Incubate for 14 to 20 hours (1st step enrichment)
- Count bacteria associated to 1dph *Artemia* on selected media
- If larvae have to be fed with *Artemia*, a second step enrichment is needed. To this end 1dph *Artemia* are further enriched with DHA (50 mg l⁻¹) and prebiotic powder (50 mg l⁻¹).
- Distribute enriched *Artemia* with a peristaltic pump

Kjeldahl Method for determination of Crude protein

Protein content (%of dry matter) was determined from duplicated samples by the Kjeldahl method, with a semi-automated distillation unit and digester.

- **Digestion phase:**
 - Transfer about 1.0 g of sample to the digestion tube
 - Add 20 ml of concentrated sulfuric acid
 - Add 2 catalyst agent tablet (selenium mixture)
 - Set the heating apparatus at a temperature of 420°C for a period of 30-40 minutes.
 - At the end of digestion phase usually you will have a clear and transparent solution. In this phase N₂ in the sample is converted to (NH₄)₂SO₄. Remove the tubes from heating device, cool them with water.

- **Distillation phase:**
 - Transfer the tube containing digested material to the distillation unit
 - Place an erlenmeyer containing 25 ml Boric Acid at the end point of the distillation unit
 - The nitrogen converted to ammonium sulfate is then distilled in the presence of 25 ml of 40% sodium hydroxide for a period of 4 minutes
 - resulting in the liberation of ammonia, which was absorbed in a solution of boric acid

- **Titration phase:**
 - Titrate the solution with a standard solution of 0.1 M hydrochloric acid until the color of solution is turned to blugreen color.

Calculating of protein content:

$$\% \text{Protein} = 6.25 * \frac{\text{Wolume of Hcl(ml) * normality of HCl * 1400}}{\text{Dry weight of sample(mg)}}$$

Determination of Crude fat

Two methods are used for extraction of total fats:

1. Simple method by means of diethyle ether at 40°C
 - Transfer two replicates of 1.00 g from each sample to 15 ml screw capped glass tubes
 - Add 10 ml diethyl ether on each sample
 - Close the cap air tight and keep the tubes in an incubator at 40°C for 12 hrs.
 - Transfer the tubes to room temperature until they are acclimatized
 - Open the cap slowly and carefully, siphone the supernatant from each tube by pasture pipet to pre-weighted 50 ml pear-shaped flasks
 - Add another 10 ml diethyl ether on each sample and repeat the above steps
 - Siphone the supernatant for the second time into pear-shaped flasks containing the supernatant of earlier phase
 - Evaporate the solvents on a rotavapor at 35 °C, flush the remaining solvents with nitrogen gas, and weigh the pearshaped flasks again.
 - Weight the flask again, the difference of weight indicats the total lipid of our sample
 - Calculate the percentage and average of the two replicates in order to find out the final percentage of crude fat in the sample.

2. Soxhlet method:

- Weight duplicate samples of about 2.5 grams of each sample
- Wrap the samples in filter paper and place them in the extraction chamber of Soxhlet apparatus
- Add approximately 175 ml of anhydrous ether to a pre-weighed Soxhlet flask
- On the heater and continue the fat extraction for about 6h
- Evaporate the solvents by rotavapor at 35 °C
- Flush the remaining solvents with nitrogen
- Weight the completely dried Soxhlet flask containing the fat
- was oven dried overnight at 60°C and weighted, the difference of weight indicates the total lipid of our sample
- Calculate the percentage and average of the two replicates in order to find out the final percentage of crude fat in the sample

Calculation of fat content:

$$\% \text{Fat} = 100 * \frac{\text{Weight of fat}}{\text{Dry weight of sample(mg)}}$$

Determination of Fatty acid profile:

- **Method 1: Esterification of extracted lipids using iso-octane or heptane and methanolic KOH (Lieboritz, et al. 1987) and preparing the sample for injection to Gas Chromatography**

Procedure:

- Transfer 0.1 g of extracted lipid to a 5 ml glass tube
- Add 1 ml iso-octane and 0.05 ml of 2 M methanolic KOH on lipid
- Shake the tube very strongly for 15 minutes
- Keep the tube undisturbed for few minutes until released glycerol sedimented
- siphon the supernatant containing fatty acid methyl esters to a clean 3 ml tube
- inject 0.4 μ l to GC

- **Method 2: FAME-Preparation by Direct Esterification**

Amount of product used for FAME-analysis between 50-100 mg (dry) and 200-500 mg (wet) sample.

Amount of product used for dry weight measurement: 3 x 50 mg (dry), 3 x 100 mg (wet).

Sample	Amount of product	IS
Enr. or Non-Enr. Artemia	0.20 g – 0.25 g	100 μ l 20:2n6 - 5 mg/ml
Algae	0.10 g - 0.15 g	100 μ l 22:2n6 – 15 mg/ml
Rotifers	0.35 g – 0.40 g	100 μ l 20:2n6 - 5 mg/ml
Emulsion	\pm 0.050 g	100 μ l 22:2n6 – 15 mg/ml
Oil	\pm 0.010 g	100 μ l 22:2n6 – 15 mg/ml
Fish meal	0.05 g – 0.07 g	100 μ l 20:2n6 - 5 mg/ml
Macrobrachium Larvae	0.15 g – 0.20 g	100 μ l 20:2n6 - 5 mg/ml
Macrobrachium Eggs	0.04 g- 0.05 g	100 μ l 20:2n6 - 5 mg/ml

Procedure

- Put the sample in a 35 ml glass tube with a teflon lined screw cap
- Add 5 ml of methanol/toluene mixture (3:2 v/v) Add exactly 0.100 ml of the internal standard solution (containing 4.78255 mg/ml 20:2(n-6) or 4.79995 mg 22:2(n-6) fatty acid dissolved in iso-octane). Add 5 ml of freshly prepared acetylchloride/methanol mixture (1:20 v/v) as the esterification reagent.
- Flush the tube with nitrogen gas and close tightly.
- Shake tube carefully, make sure that the product doesn't stick too high up the wall of the glass tube (to avoid incomplete reaction).
- Put the glass tube in a boiling water bath (100°C) for one hour, shaking the tubes regularly (every 10 min) – but carefully.
- After one hour, cool down tubes, add 5 ml of distilled water and 5 ml hexane.
- Centrifuge the tube for five minutes, and transfer the upper (hexane) layer into a teflon tube. Repeat the hexane extraction two more times with 3 ml of hexane.
- Dry the combined hexane phases by filtering in a pearshaped flask of a known weight over an anhydrous sodiumsulphate filter. Evaporate the solvents on a rotavapor at 35 °C, flush the remaining solvents with nitrogen gas, and weigh the pearshaped flask again.
- The FAME's are finally dissolved in 0.5 ml iso-octane and transferred in a 2 ml glass vial with teflon lined screw cap. The vial is flushed with nitrogen and the sample is stored in a freezer at -30 °C until injection.
- For the actual GC analysis, inject 0.25-0.4 µl of a dilution in iso-octane, containing ± 2 mg FAME's/ml. The dilution can be calculated from the difference between the two weighings of the pear-shaped flask; The individual FAME-amounts are calculated using the (known) amount of the internal standard as a reference.

Gas Chromatography conditions for FAME analysis:

Quantitative determination is done by a gas chromatograph equipped with an autosampler and a TPOCI (temperature programmable on-column injector). Injections (0.4 μ l) are performed on-column into a polar 30-50 m capillary column, with a diameter of 0.32 mm and a layer thickness of 0.25 μ m which may be connected to a pre-column. The carrier gas used could be H₂ or N₂, at a pressure of 100 kPa and the detection mode FID. The oven programming may vary for different samples with different origins. A sample programme which has been used for analysis of fatty acids in *Artemia* nauplii by many laboratories is as follows: rise from the initial temperature of 85°C to 150°C at a rate of 30°C/min, from 150°C to 152°C at 0.1°C/min, from 152°C to 172°C at 0.65°C/min, from 172°C to 187°C at 25°C/min and to stay at 187°C for 7 min. The injector was heated from 85°C to 190°C at 5°C/sec and stayed at 190°C for 30 min. Identification was based on standard reference mixtures (Nu-Chek-Prep, Inc., U.S.A.). Integration and calculations were done on computer with a software program.

Protocol for hatching Rotifer cyst and its culture

Procedure for hatching:

1. Transfer very low amount of rotifer cysts (100 μg) to a falcon tube of 50 ml
2. Add about 30 ml of 25 ppt autoclaved water of 25 °C
4. Expose the falcon to light (1000 lux)
5. Collect the hatched rotifers after 24 hrs and following procedure for culture

Rotifer culture up to 15 L bottles

The stock culture for rotifers is kept in a thermo-climatised room (25 \pm 1°C). The vials (50 ml conical centrifuge tubes) are previously autoclaved and disposed on a rotator (4 rpm) which, at each rotation the water mixed with the enclosed air, supplying oxygen to the rotifers. The vials on the rotator are exposed to the light of two fluorescent tubes at a distance of 20 cm (light intensity of 3000 lux on the tubes). The culture water (seawater mixed with tap water to a salinity of 25 ppt) should be prefiltered on a 1 μm capsule membrane filter and treated overnight with 5 mg l^{-1} NaOCl. The next day the excess of NaOCl was neutralised with sodium thiosulphate and the water is filtered over a 0.45 μm filter. Inoculation of the tubes is performed at a density of 2 rotifers ml^{-1} . The food consisted of marine *Chlorella* centrifuged and concentrated to $1\text{-}2 \times 10^8$ cells ml^{-1} before feeding to the rotifers. The algal concentrate is stored at 4 °C in a refrigerator for a maximum period of 7 days. The algal concentrate should be homogenised by shaking and 200 μl is given to each of the tubes. The rotifer density increased from 2 to 200 individuals ml^{-1} after one week. The rotifers are then rinsed, a small part is used for maintenance of the stock, and the remaining rotifers were used for the starter culture. Starter cultures consists of a static system with erlenmeyers of 500 ml, placed at 2 cm from the fluorescent light tubes (5000 lux). In the erlenmeyers the temperature is maintained more or less constant at (28 °C). The rotifers are stocked at a density of 50 individuals ml^{-1} and fed with freshly harvested algae (*Chlorella* 1.6×10^6 ml^{-1}). Approximately 50 ml of algal suspension is added every day to supply enough food. The rotifer concentration increased to 200 individuals ml^{-1} within 3 days. During this short rearing period no aeration is applied. Once the rotifers reached a density of 200-300 individuals ml^{-1} they are rinsed on a submerged filter consisting of 2 filter screens. The upper mesh size (200 μm) retained large waste particles, while the lower sieve (50 μm) collected the rotifers. The

concentrated rotifers are then distributed in 15 l bottles filled with 2 l at a density of 50 individuals ml⁻¹. A mild aeration is provided. Every other day the cultures were cleaned (double-screen filtration) and restocked at densities of 200 rotifers ml⁻¹. Fresh algae (*Chlorella* 1.6×10⁶ ml⁻¹) are supplied daily. After adding algae for approximately one week the 15 l bottles are used for the inoculation of rotifers in small and large scale experiments.

Mass culture of rotifers

Culture conditions

Temperature

Temperature is one of the most important environmental variables for all aquatic organisms (Alzieu, 1990). It influences the oxygen content of the water, the primary product which is the source of food in the open sea and the reproduction and growth of all species. The tolerance limits of every organism to temperature are different for every species, and depend on the physiology of the animal.

The optimal culture temperature for rearing rotifers is strain dependent. Each species or rotifer strain has a different range of temperature requirement. However, the type of physiological changes that occur at high temperatures are likely to be similar amongst strains (Nogrady *et al.*, 1993). Increasing the temperature, until a certain limit, generally results in an increased reproduction activity. Rearing rotifers below their optimal temperature slows down the population growth considerably. Hirayama and Rumengan (1993) reported that *B. rotundiformis* grow best at higher temperature (>25°C) while *B. plicatilis* shows a greater tolerance to below 20°C. Optimal temperature for *B. plicatilis* is 25°C (Lubzens *et al.*, 1985), and for *B. rotundiformis* reproduction stops under 15°C, whereas *B. plicatilis* is still reproducing at this temperature. Hagiwara and Lee (1991) stated that at culture temperatures ranging from 23.1-30.6°C, the L-type rotifers produced more resting eggs at the lowest temperature (23.1°C) and the S-type produced more at a higher temperature (28.2 and 30.6°C).

Salinity

In general, salinity has an effect on reproduction, nutrition and growth of aquatic organisms. Growth may be optimal at a restricted salinity range depending on the species. The rotifer *B. plicatilis* is able to tolerate a wide range of salinities (euryhaline organism) from 1 up to 97 ppt (Walker, 1981). Optimal reproduction, however, can only

take place at salinities below 35 ppt (Lubzens, 1987). At high salinity (20 - 30 ppt) filtration rate as well as food assimilation (Lebedeva and Orlenko, 1995) is reduced.

Although *B. plicatilis* has a very wide salinity range tolerance, transferring of the rotifers directly from low to high salinity may cause stress and immobilization of the rotifers (Øie and Olsen, 1993), and can even result in a high mortality rate. This should be taken into consideration when rotifers have to be fed to predators which are being reared at different salinity (± 5 ppt higher). Therefore, it is safe to acclimatize them by gradually increasing the salinity level (Nogrady *et al.*, 1993; Sorgeloos and Lavens, 1996).

Dissolved Oxygen

Of all the dissolved gases, oxygen plays the most important role in determining the potential biological quality of the water used in rearing operations. It is essential for respiration, helps the breakdown of organic detritus, and enables the completion of biochemical pathways. The oxygen sources in the water are diffusion from the atmosphere into the water and the photosynthetic activity of phytoplankton and other plants.

In rotifer cultures, dissolved oxygen is also one of the most important chemical characteristics. Most rotifers can survive in water containing as low as 2 mg.l^{-1} of dissolved oxygen (Sorgeloos and Lavens, 1996). Some rotifers, however, can tolerate anaerobic or nearly anaerobic conditions for short periods.

The oxygen solubility in culture water depend on the temperature, the salinity, the rotifer density and the type of food. Oxygen solubility correlates inversely with temperature and salinity. Increasing temperature results in decreasing dissolved oxygen concentration in culture water, whereas at high temperature the demand for dissolved oxygen increases due to the increased rotifers' metabolic rate. In a high density culture of rotifers ($>10^3$ individuals. ml^{-1}) the supply of oxygen is crucial and it is difficult to maintain an optimum dissolved oxygen level (Yoshimura *et al.*, 1996a).

Ammonia

All aquatic organisms, particularly fauna, provide a source of organic nitrogen through their excretory products, the by-product of metabolism, and the breakdown of dead cells and tissues. Under the action of proteolytic bacteria, organic nitrogen is transformed to NH_4^+ . The concentration of unionized ammonia (NH_3) is largely a function of

NH_4^+ , temperature and pH. The toxicity of ammonia for rotifers is not very clear. Although a high ammonia concentration is generally found in rotifer rearing tanks, rotifers seem to be resistant to it (Coves *et al.*, 1990), and no correlation has yet been found between high levels of total ammonia and abnormal behavior of rotifers. However, the excretion of ammonia becomes a significant problem once density reaches an order of 10^3 - 10^4 rotifers. ml^{-1} (Yoshimura *et al.*, 1994). Therefore, it is essential that cultures are rinsed before they are distributed as food for larvae which are themselves sensitive to levels of around 1 ppm total ammonia in the water (Coves *et al.*, 1990). The setting up of a nitrifying microflora in tanks also leads to the formation of nitrites and nitrates which may also be toxic to rotifers.

pH

Most aquatic organisms can tolerate a pH range of 6-9 which is a far wider range than that encountered in their normal natural environment (Coves *et al.*, 1990). Fukusho (1989) stated that rotifers can survive in an environment having a pH range from 5 to 9. In their natural environment rotifers live at pH levels above 6.6, and in culture conditions the best results are obtained at a pH above 7.5 (Sorgeloos and Lavens, 1996). In a high density culture of rotifers a pH 7.0 is optimal for rotifer population growth (Yoshimura *et al.*, 1995). The pH level is related to the toxicity of excretion products i.e. NH_3 .

Microbial aspects

In high density rotifer cultures a high concentration of organic matter is measured. These high concentrations of organic matter favour the development of large numbers of bacteria. Coves *et al.* (1990) measured the number of bacteria of around 10^7 bacteria. ml^{-1} and 10^3 - 10^4 bacteria in the digestive tract of each *Brachionus*. By means of scanning electron microscopy (SEM), Munro *et al.* (1993) stated that the majority of the bacterial strains associated with rotifers were located on the external surface.

The number of bacteria in the gut of rotifers is related to the bacterial population in the environment through the grazing process. Nicolas *et al.* (1989) reported that accumulation of bacteria in the gut of rotifers resulted from grazing rather than from internal multiplication. The composition of the bacterial stock is also affected by the diets given to the rotifers. As mentioned by Øie *et al.* (1994) the use of yeast enriched with capelin oil resulted in a considerably higher number of both suspended and rotifer-associated bacteria than algal diets.

Bacterial microflora is therefore an important element in the successful culture of rotifers. They are responsible for the levels of ammonia, for recycling part of the organic matter, and for making up deficiencies in the food supply, probably causing diseases (Coves *et al.*, 1990). Contamination of bacterial flora and protozoan in rotifer culture have resulted in the sudden collapse of rotifer cultures (Hagiwara *et al.*, 1995b; Hino, 1993; Maeda and Hino, 1991). Balompapung (1994) found that bacterial strains such as *Plavobacterium*, *Aeromonas* and *Vibrio* sp. isolated from the unstable or collapsing rotifer cultures showed toxicity for the rotifer population. Shiri-Harzevilli *et al.* (1997) stated that a *Vibrio anguillarum* strain TR27 caused a negative growth rate in sub-optimal rotifer cultures. Culture collapses can not be avoided unless bacterial environments in the rotifer cultures are properly managed, which is not an easy task.

Although most bacteria are not pathogenic for rotifers their proliferation should be avoided since a real risk of accumulation and transfer via the food chain can cause detrimental effects on the predator (Sorgeloos and Lavens, 1996). Besides the risk for contamination, the bacteria are able to recycle the organic matter by multiplying or through producing dissolved compounds. The bacteria can thus supply substances which are deficient in certain diets, especially simple ones (yeast) (Coves *et al.*, 1990). They are known to synthesize B groups vitamins, particularly B₁₂ which are necessary for *Brachionus* to reproduce. A recent study by Lee *et al.* (1997) revealed that a certain bacteria strain can be used as food for *B. plicatilis* to enhance the growth rate. They compared four kinds of rotifer feed that is PSB (Purple Nonsulfur Bacteria), *Chlorella* sp., baker's yeast, and an aerobic photosynthetic bacterium *Erythrobacter* sp. S-pi-I. The rotifers fed on this bacteria showed better growth rates than those fed on other feed.

Diets used in rotifer cultures

Algae

In their natural environment rotifers live on micro-algae, bacteria, yeast and protozoa (Fukusho, 1989). Micro-algae are used to produce mass quantities of zooplankton (rotifers, copepods and brine shrimps) which serve in turn as food for larval and early-juvenile stages of crustacean and fish (Sorgeloos and Lavens, 1996). For the cultivation of rotifers, food that can be produced in a large amount under artificial cultivation conditions and can be effectively utilized by rotifers is most desirable, since rotifers have very fast filtration capacity. Undoubtedly, marine micro-algae are the best diet for rotifers

and very high yields can be obtained if sufficient algae are available and an appropriate management is followed.

The most common algae used in rotifer cultures is *Nannochloropsis oculata* (Lubzens, 1987; Hirayama *et al.*, 1989; Fukusho, 1989) with a size of 2-3 μm in diameter and a relatively high content in 20:5n-3 fatty acid (EPA), *Tetraselmis tetrahele* or *T. suicica* which have a cell diameter of 20-30 μm and high EPA content, *Isochrysis galbana* containing high level of 22:6n-3 fatty acid (DHA). Some other micro-algae including *Dunaliella tertiolecta*, *Pavlova lutheri*, *Chlorella* sp. and *Stichococcus* sp. have also been used as food for rotifer cultures.

Micro-algae are believed to play a role in stabilizing the water quality, nutrition of the larvae, and microbial control. Caric *et al.* (1996) reported that at the exponential phase of growth, the highest lipid content was found in rotifers fed on *Dunaliella tertiolecta*, *Paedactylum tricornutum*, *Nannochloropsis* sp., and nannoplankton. At the stationary phase of growth the *Nannochloropsis*-fed rotifers had a significantly higher lipid content. It is well known that lipids are important elements of cell structure and a major energy source in most zooplankton organisms and marine fish larvae. Apart from their high nutritional value, some other advantages can be obtained from the microalgae as food for rotifers:

- Algae act as bacteriostatic, controlling bacterial development
- Algae act as water conditioner, controlling the water quality of the medium and oxygenating the water through the photosynthesis process.

However, huge amounts of labour, time and facilities are needed for continuous mass culture of algae. Moreover, a stable algae supply is difficult to obtain in terms of quantity and punctuality, especially under mass culture conditions (Fukusho, 1983).

Freshwater *Chlorella* which has been condensed and enriched with vitamin B₁₂ can eliminate the drawback of using normal algae. Owing to the advancement of phytoplankton technology, freshwater *Chlorella regularis* and *Nannochloropsis oculata* become commercially available in condensed and refrigerated form (Yoshimura *et al.*, 1996b). By the introduction of these preserved diets to aquaculture facilities, rotifers are now cultivated at higher densities (Fu *et al.*, 1997, Yoshimura *et al.*, 1997a) with more stability. By using those products, Japanese scientists have developed an ultra-high density culture technology with fully automated systems.

Yeast

Besides the zootechnical aspects, e.g. water management, food appears to be one of the key elements in the successful mass production of rotifers (Sorgeloos and Leger, 1992). As mentioned before, a stable micro-algae supply for mass production of rotifers is difficult to obtain. Therefore, alternatively, baker's yeast is commonly being used. In 1967, Hirata and Mori conducted experiments on the use of baker's yeast as food for rotifers (Hirata, 1980). They reported that the rotifers could grow on a mixed food (50% *Chlorella* and 50% baker's yeast) as well as with 100% *Chlorella*.

There are several yeasts that can be used as rotifer feed, that is baker's yeast (fresh and instant) (*Saccharomyces cerevisiae*), caked yeast (*Rhodotorula*) and marine yeast (*Zygosaccharomyces marina*, *Torulopsis candida* var. *marina*, *T. larvae*, and *Saccharomyces acidophilus*). Baker's yeast has been used as a suitable algal substitute for *Brachionus* (Hirayama, 1987), because of its small particle size of 5-7 μm in diameter, high content of protein and also the presence of bacteria growing on the yeast surface.

Although yeasts have been accepted as food for rotifer cultures, they contain very low concentration of long chain highly unsaturated fatty acids (HUFA) of the n-3 series, mainly 20:5n-3 (Fukusho, 1983) and vitamin B₁₂ (Hirayama and Funamoto, 1983). Yoshimura *et al.* (1996b) stated that the supplementary feeding of baker's yeast makes the rotifer cultures less stable. The reason why baker's yeast has been used for rotifers is attributable to its supplemental nutritional effects to other micro-algae and bacteria (Fukusho, 1989). In order to improve the nutritional value of rotifers the administration of baker's yeast for mass production of rotifers needs to be combined with algae.

Formulated Diets

The bottlenecks in the optimal use of rotifers are mainly related to reliable and cost effective techniques for continuous mass production. A recent break-through in production technology has been the development of an artificial diet which completely eliminates the need of an extra enrichment period for enhancement of the rotifers' dietary value (Lavens *et al.*, 1995). The most frequently used formulated diet in rotifer cultures is Culture Selco[®] (CS) (INVE N.V., Belgium) available under a dry form. Candreva *et al.* (1996) reported that Culture Selco[®] is widely used by hatcheries in Europe.

The dry product needs to be suspended in water prior to feeding. Provided it is continuously aerated and cold-stored, the food suspension of Culture Selco[®] can be used in automatic feeding for up

to 30 hours. A standard feeding protocol using Culture Selco[®] has been developed and tested on several rotifer strains in 100 l tanks (Sorgeloos and Lavens, 1996).

Culture techniques

Much progress has been made in the area of fry production technology for marine finfish. Today, Japan, for instance, is the biggest producer of marine fish fry with about 200 million fry produced per year (Sorgeloos, 1994). It is not too much to say that the current increase in finfish fry production is based upon the successful introduction of the rotifer *B. plicatilis* as a food organism and the development of mass production technology for rotifers (Fukusho, 1989). Therefore, several culture designs, rotifer diets and feeding schemes for mass culture have been developed.

In 1964, the mass culture of the marine *Chlorella* and rotifers were initiated by the Yashima Station, Japanese Sea-Farming Fisheries Association (JSFFA) (Hirata, 1980). Since the demand of rotifers continuously increased, several culture techniques have been developed. Most of the rearing techniques can be described as batch or semi-continuous systems. Recently, more sophisticated methods have been developed, such as continuous systems with or without high level of mechanization and automation (Morizane, 1991), and the ultra-high density mass culture of rotifers (Yoshimura *et al.*, 1996b).

Batch culture

Batch culture systems seem to be the most common type of rotifer production used in hatcheries. The size of the rearing tanks varies from 500 to 1000 l plastic tanks up to 10000 l for concrete tanks. In these systems the rotifers are inoculated at a density of 50 to 200 rotifers.ml⁻¹. The density at harvest time is about 600 rotifers.ml⁻¹ after 4 days culture (Sorgeloos and Lavens, 1996). In the batch culture technique rotifers are harvested completely. A part of the harvested rotifers are administered as food for fish larvae or crustaceans and the remaining is used as inoculum for the next culture with a density of 250 rotifers.ml⁻¹.

Depending on the culture volume and rotifer density during the rearing period, two strategies can be applied: a constant culture volume can be maintained with increasing rotifer density or the volume of the culture can be adjusted in order to maintain a constant rotifer density (Hirata, 1980; Lubzens, 1987)

Semi-continuous culture

In high density cultures of rotifers, a large amount of suspended organic matter accumulates in the culture medium. Such wastes are mainly composed of rotifer feces, amictic egg shells, microbes (bacteria, protozoa, fungi, etc.), the food organism *Chlorella* and flocks of various sizes are formed and coagulate (Yoshimura, 1997a). In order to avoid this phenomenon of self-pollution, the semi-continuous culture system has been developed.

In the semi-continuous culture systems the rotifer density is kept constant by harvesting periodically. Girin and Devauchele (1974) removed about 25% of the culture volume every day and replaced it by the same amount of new water. The system is therefore also known as the thinning culture method. If all requirements of this system are satisfied the method allows the maintenance of a stock of a constant number of individuals (Coves *et al.*, 1990).

Semi-continuous culture systems are usually performed in larger tanks (50-200 m³) than the ones used in the batch culture. The culture period is longer than that in the batch culture system. Morizane (1991) reported that they could continue culturing rotifers without changing tanks, harvesting a large number rotifers, for an entire year. The inoculated density of rotifers is from 50 to 200 rotifers.ml⁻¹ and can reach up to 300 to over 1000 rotifers.ml⁻¹ in 3 to 7 days at harvesting time, using micro-algae and baker's yeast.

Continuous Culture

Continuous culture systems are a logic process in the intensification of rotifer production. The aim of this system is nearly the same as for the semi-continuous culture system, to maintain good water quality by improvement of water management through high water exchange rate and the use of chemostats. The new water is always supplied into culture tanks, so that the water quality is kept in good quality or nontoxic without the need for any procedure such as pH control for reducing unionized ammonia. In this system, a constant rotifer density with high quality is reached, and it is also possible to maintain the culture without any decline of rotifer productivity for a long period. Abu-Rezeq (1997) reported that the continuous culture systems have higher productivity than batch and semi-continuous culture systems. The initial density of rotifers varies, and during the culture period the rotifer density is maintained constant and the production is dependent on other factors such as feeding regime and water quality.

Although the continuous culture systems have a lot of advantages they are only applied on an experimental scale and are not

applied in the hatcheries. Since this system is very costly and a lot of variables need to be controlled the risk for technical failure is considerably increased.

Ultra-high density culture

The intensive ultra-high density rotifer culture techniques have been firstly developed by Japanese scientists. Yoshimura *et al.*, (1995) reported that very high rotifer productions could be achieved in a 1 m³ tank in a batch culture method in 2-day intervals with a initial density of 10,000 individuals.ml⁻¹. The latest, ultra-high density (maximum density from 20,000 up to 40,000 rotifers.ml⁻¹) rotifer mass culture has been developed based on concentrated freshwater *Chlorella* as food (Yoshimura *et al.*, 1994, 1997a, Fu *et al.*, 1997).

The ultra-high density culture systems are an effective way to produce rotifers without expanding the culture space. These systems have several advantages:

- much lower labor and space needed
- high production of rotifers
- consistent or year-round production

In the rotifer mass production system the most labour-intensive step is the harvesting of the culture tanks before feeding or enrichment (Dehasque *et al.*, 1997). Several advantages can be obtained from the automated system over the manual system are as follows:

- production techniques are simplified
- more intensification is made possible which means less tanks are needed and required space/infrastructure is reduced
- less labour is required
- manipulations are reduced and higher outputs per units of volume are reached
- the extra cost to install automated procedures are minimal (Concentrator/Rinser; pumps) and compensated by reduction in tanks and labour

The schematic overview of this system is shown in **Figure 4**.

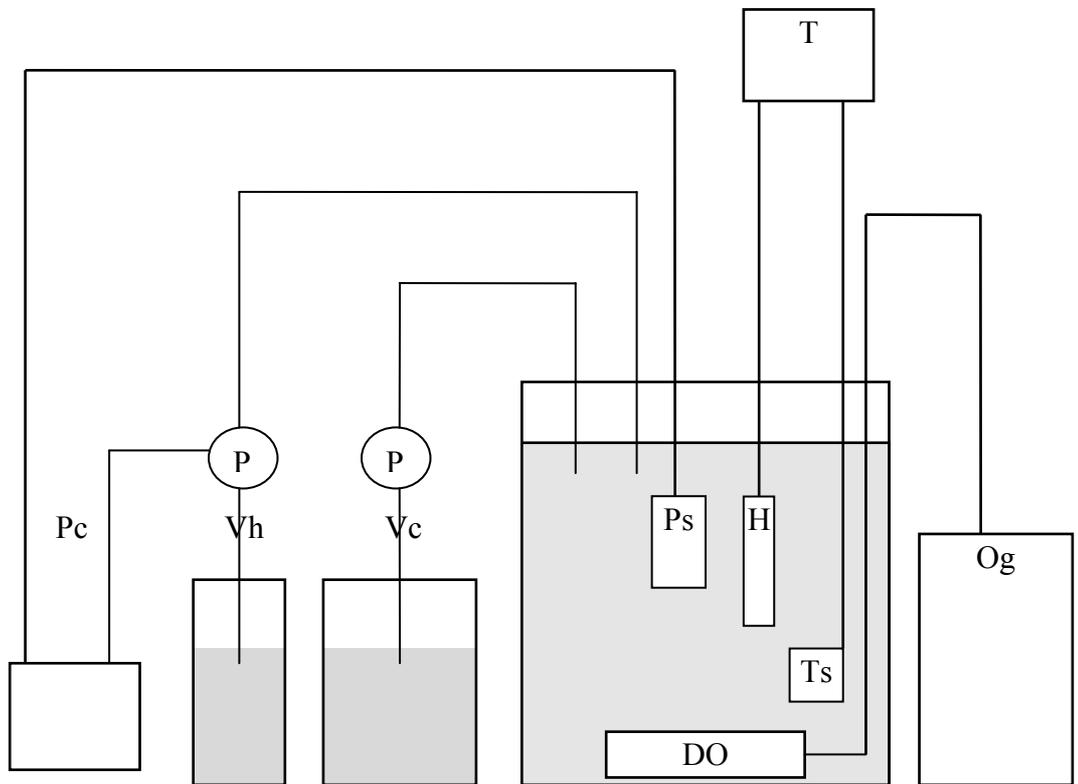


Figure 4. Schematic overview of the high density rotifer mass culture system in a 1 m³ tank (Yoshimura *et al.*, 1995). Pc: pH control; P: petri pump; Vh: Hydrochloric acid; Vc: Condensed freshwater *Chlorella*; Ps: pH sensor; Do: Dispenser of oxygen gas; Ts: Temperatur sensor; H: Titanium heater; T:thermostat; Og: Electric oxygen generator.

2. Nutrition

Cost-effective biomass production of rotifers relies on the use of a cheap food source and explains why baker's yeast was (is) used as an important diet. When applied as a sole diet it may support the mass production of rotifers in non-axenic culture conditions where micro-organisms provide essential nutrients (Hirayama, 1987). However, it is well known that yeast-fed rotifers lack the essential fatty acids required for the proper development and survival of several species of marine fish (see reviews of

Watanabe, 1979, 1993; Watanabe et al., 1983; Lubzens et al., 1989). Therefore a number of other feed materials and enrichment techniques are applied to produce rotifers with higher levels of essential fatty acids and

Enrichment with algae

When good quality algae are available in large numbers they may be used as an excellent live food diet for boosting the fatty acid content in rotifers. The specific content of the essential fatty acid eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3) in some microalgae (e.g. 20:5n-3 in *Nannochloropsis oculata*; Watanabe, 1979; Watanabe et al., 1983; Koven et al., 1990; Seto et al., 1992; Sukenik et al., 1993 and 22:6n-3 in *Isochrysis galbana* and *Rhodomonas* sp.; Lubzens et al., 1985; Ben-Amotz et al., 1987; Whyte and Nagata, 1990; Sukenik and Wahnon, 1991; Mourente et al., 1993) and their relatively easy mass culture make them very attractive in commercial hatcheries. Rotifers incubated in these algae cultures (at approximately $5-25 \cdot 10^6$ algae.ml⁻¹) are incorporating the essential fatty acids in a few hours time and attain a DHA/EPA level above 2 in *Isochrysis* and below 0.5 for *Tetraselmis*. However, most of the time, algae of good quality are not available in large enough quantities, are too labour intensive to be produced or too expensive for rotifer enrichment (Coutteau and Sorgeloos, 1997). For this reason rotifers are generally boosted in oil emulsions before they are fed to the predators. The latter may be kept in clear water or in “green water”. This “green water”, consists of $\pm 0.2 \cdot 10^6$ algal cells.ml⁻¹ (*Tetraselmis*, *Nannochloropsis*, or *Isochrysis*) and is used as a “water conditioner” and as a nutritional factor to maintain an appropriate HUFA content in the live prey before they are eventually ingested by the predator (Dhert et al., 1998).

The use of storable algal products (algal pastes and frozen algae) has found some new interest for rotifer cultures (Hamada et al., 1993; Lubzens et al., 1995). Lubzens et al. (1995) accredit this new interest in the new products to the fact that :

- (1) the algal products can be transported and stored for longer periods (app. 2 weeks for pastes) relieving the hatcheries from their direct dependence
- (2) algae can be cultured under conditions that ensure the highest quality
- (3) the chemical composition and quality can be determined in advance
- (4) high density rotifer cultures can be obtained (see further)

Enrichment with oil emulsions

For the enrichment or boosting of rotifers several approaches can be followed : 1) the adjustment of the lipid and vitamin content of the rotifers just before feeding them to other organisms is referred to as short term enrichment (generally less than 8 h exposure) and 2) the feeding of rotifers on a complete diet or long term enrichment (rearing of the rotifers on the enrichment diet for more than 24 h).

Many authors have elaborated on both techniques and each of them has its benefits and disadvantages. The short term enrichment technique has the advantage of being fast and flexible, but very often produces lower quality rotifers with a too high lipid content (Dhert et al., 1990; Støttrup and Attramadal, 1992) and poor hygienic quality. The biggest problem in this enrichment technique resides in the fact that a lot of rotifers are lost when they are concentrated (sticking of the rotifers) at high density. Also transfer of oil to larval rearing tanks with consequent loss of water quality and associated problems of larval viability have been reported (Foscarini, 1988). On top of that, the retention time of the nutrients, which are mainly accumulated in the digestive tract of the rotifers, is very short and can create problems when the rotifers are not eaten immediately.

Since rotifers are not selective for the uptake or catabolism of highly unsaturated fatty acids, high HUFA levels can be accumulated without problem. Especially DHA, an essential fatty acid that is accumulating in the brain of fish during early development where it increases neural functions (Bell et al., 1995), is easily incorporated in rotifers unlike *Artemia* that is catabolizing this fatty acid (Dhert et al., 1993). Especially for this last reason the feeding with DHA-enriched rotifers is often prolonged in flatfish cultures, where the enrichment at an early stage has been successful in improving pigmentation (Miki et al., 1990; Kanazawa, 1993; Reitan et al., 1994).

In contrast to n-3 PUFA, n-6 PUFAs have been largely neglected in studies on marine fish nutrition. Especially arachidonic acid (20:4n-6, ARA) as the preferred substrate for producing eicosanoids (Tocher and Sargent, 1987; Sargent et al., 1995) can be blended in an optimal ratio with DHA and EPA and retrieved in approximately the same ratio in rotifers without the same risk of preferential catabolism as in *Artemia* (Estévez et al., 1999). However, the exact balance of DHA, EPA and ARA in the

nutrition of larval fish still needs further investigation (Estévez et al., 1999; Sargent et al., 1999).

Emulsions with phospholipids have also been used as a more efficient FA source for fish (McEvoy et al., 1996, Coutteau et al., 1997) but they are immediately broken down in rotifers.

Enrichment with vitamins

The vitamin C content of rotifers reflects the dietary ascorbic acid (AA) levels both after culture and enrichment. Rotifers cultured on e.g. instant baker's yeast contain low AA levels ($150 \text{ mg.g}^{-1} \text{ DW}$), while the AA content in *Chlorella*-fed rotifers may vary (from 1000 up to $2300 \text{ mg.g}^{-1} \text{ DW}$) depending on the quality of the algae. In commercial marine fish hatcheries a wide range of products is used for the culture and subsequent boosting of rotifers with vitamins. Oil-soluble vitamins or derivatives from water soluble vitamins (ascorbyl palmitate (AP) for ascorbic acid) have been formulated in the commercial lipid enrichment products. The non-bioactive ascorbyl palmitate is accumulated by the rotifers together with the oil emulsion and converted to free AA by the enzymes of the rotifers. Merchie et al. (1995) demonstrated that this process was very effective since 5% AP (w/w) in the emulsion produced rotifers with an active AA concentration of $1700 \text{ mg.g}^{-1} \text{ DW}$ after 24 h enrichment and this high concentration remained in the rotifers after storage in seawater during the next 24 h. Since the technique has been introduced in Mediterranean bream hatcheries, problems related to stress and operculum deformities have been reduced which might indicate that vitamin C concentrations in live food may also be critical (Merchie et al., 1997).

Enrichment with proteins

Only a few reports treat protein content of rotifers and requirements for fish larvae (Watanabe et al., 1983; Øie and Olsen, 1997). The protein content of rotifers is reported to vary between 28-67% of dry weight, whereas the amino acid profiles of rotifers fed different diets appear to be fairly constant and independent of food quality (Lubzens et al., 1989; Øie and Olsen, 1997). The changes in protein content in rotifers are attributed to their nutritional status, this is for a large part reflected by the applied feeding strategy, and the general rotifer condition, more specifically the reproduction rate of the rotifer. The range of

variation is large enough to affect larval rearing success especially during first feeding of marine fish larvae. Øie and Olsen (1997) emphasize on the modified protein/lipid ratio after short term enrichment of rotifers with lipid rich diets which significantly increase the lipid content whereas the protein content remains constant resulting in a low protein/lipid ratio. Also during the rearing of rotifers the protein/lipid content is subject to variation and is positively correlated with the specific growth rate of the rotifers. Since rotifers are generally cultured in batch systems in which the specific growth rate tends to decrease significantly towards the end of the culture period this means that the protein/lipid balance in rotifers may show variations as high as 150-200% depending if the rotifers are harvested shortly after restocking or towards the end of the culture period. Especially in first feeding marine fish larvae, it is important to provide high nutritional quality rotifers since the nutritional content of the rotifers tends to decrease after transfer to the fish tanks in clear water systems (Øie et al., 1995; Olsen et al., 1993; Reitan et al., 1993). For turbot, for instance a positive effect was seen on growth and survival when fast reproducing rotifers (i.e. high protein content with high protein/lipid ratio) were fed (Øie et al., 1997).

Use of formulated diets

The long term enrichment is based on the continuous administration of the essential nutritional compounds during the rearing of the rotifers. This ensures that not only the digestive tract of the animals but also their complete body has been modified to a composition close to that of the diet on which the rotifers were grown. Rotifers fed following this feeding/enrichment strategy are nutritionally more stable and lose their reserves very slowly. This feeding strategy is more popular in continuous cultures or recirculation systems. The Japanese model is making use of condensed *Chlorella* paste supplemented with vitamins and HUFA (Fu et al., 1997; Yoshimura et al., 1997a), while the European model is working with a completely formulated diet.

Culture Selco[®] (CS), is the most widely used diet for rotifers. It has an excellent HUFA composition: respectively 5.4, 4.4 and 15.6 mg.g⁻¹ dry matter of EPA, DHA and (n-3) HUFA. This HUFA composition results in significantly higher DHA and EPA concentrations in rotifers, than for cultures grown on mixtures of algae and baker's yeast (Léger et al., 1989). The level of total lipids is approximately 18% and thus less fat than the oil emulsions.

Since the use of CS allows direct enrichment of the rotifers without the need of a cumbersome bioencapsulation treatment, complementary diets as Protein Selco[®] (PS) and DHA Culture Selco[®] (DHA-CS) have been launched in order to incorporate higher levels of protein and DHA. The advantage of direct (or long term) enrichment are multiple; the fatty acid profile obtained is stable and reproducible, the lipid content is comparable to that obtained in wild zooplankton, rotifer losses are lower and labour costs can be reduced. Also for the high density culture of rotifers new diet formulations are being proposed (Suantika et al., 2000b; De Wolf et al., 1998).

3. Culture conditions

In 1964, the Japanese Sea-Farming Fisheries Association (JSFFA, Yashima Station) initiated the mass culture of marine *Chlorella* and rotifers using a “daily tank-transfer” method (Hirata, 1980). These rotifers were used as a commercial diet for red sea bream (Fukusho, 1989). Since these early pioneering days the demand for rotifers has continuously increased and several culture techniques have been developed for rotifer mass production (Fukusho, 1983; 1989). Till the late eighties, the most popular techniques in laboratory and commercial hatcheries were classified as batch cultures (Walz et al., 1997) and semi-continuous cultures (Snell, 1991). More sophisticated methods have been developed, such as continuous systems with several degrees of mechanization and automation (Morizane, 1991; Fu et al., 1997; Abu-Rezq et al., 1997), and the ultra-high density mass culture of rotifers on algae (Yoshimura et al., 1996) and on artificial diets (Suantika et al., 2000a).

Batch cultures

Batch cultivation, due to its simplicity, is probably the most common type of rotifer production in marine fish hatcheries (Fukusho, 1983; Nagata and Hirata, 1986; Snell, 1991). The culture strategy consists in either the maintenance of a constant culture volume with an increasing rotifer density or the maintenance of a constant rotifer density by increasing the culture volume. In the batch culture a total harvest of the rotifers is applied with part of the rotifers used as food for fish larvae and part used as inoculum for the next culture (Hirata, 1980; Lubzens, 1987). Using an artificial diet (e.g. Culture Selco[®]), the density at harvest

time is about 600 rotifers.ml⁻¹ after 4 days culture starting from 200-250 rotifers.ml⁻¹ (Dhert, 1996; Suantika et al., 2000a). Generally, the size of the tanks for batch culture is flexible, 500 to 1000 l plastic tanks and up to 10 ton concrete tanks are used. Many disadvantages are, however, attributed to the batch culture system : the cultures are subjected to highly variable conditions both in growth performance as in biochemical composition of the rotifer population, unstable physico-chemical water parameters, low efficiency in terms of labour and utilization of infrastructure. These problems contribute to unstable/unpredictable culture conditions and a relatively low production yield (Walz et al., 1997) at high cost. A lot of improvements have been made to create more stable culture and rotifer production in batch cultures. Boraas & Benneth (1988) and Walz et al. (1997) have developed a turbidostat system. In this system the rotifer production is stabilized by maintaining constant algal densities by turbidity regulation. Increased interest is also seen in the use of artificial diets (see nutrition). These diets are becoming more performant and allow reliable production cycles (Candrea et al., 1996; De Wolf et al., 1998).

Semi-continuous cultures

The semi-continuous culture is also known as "thinning culture" since the rotifer density is kept constant by periodic harvesting (Coves et al., 1990; Girin and Devauchele, 1974). Contrary to the batch culture, this long-term culture is maintained at low densities for a period of 7-14 days without water renewal (Lubzens, 1987). The size of the culture tank is usually larger than that used in the batch cultures. The inoculated density varies between 50 and 200 individuals.ml⁻¹. This rotifer density might reach 300 to over 1000 individuals.ml⁻¹ in 3 to 7 days, using microalgae and/or baker's yeast as food.

High density cultures

Japanese scientists have developed intensive ultra-high density rotifer culture techniques. The latest, ultra-high density rotifer mass culture (maximum density from 20,000 up to 35,000 rotifers.ml⁻¹) has been developed based on concentrated freshwater *Chlorella* as food (Yoshimura et al., 1994, 1995, 1997a; Fu et al., 1997). Although this technique enables higher productions of rotifers compared to the batch culture system, the high food supply necessary to support the cultures causes accumulation of organic

wastes in the culture water (Yoshimura et al., 1997b, 1997c). Especially, the high ammonium concentrations and resulting free ammonia toxicity is reduced by lowering the pH of the culture water by regular monitoring (Yoshimura et al., 1995). The excessive foam formation is controlled by the use of antifoam, but this is toxic for rotifers (Yoshimura et al., 1996). Also, the viscosity of the culture water tends to increase during the rearing process resulting in a lower oxygen exchange and reduced rotifer feeding (Suantika, G., 2003).

In order to solve the problem of water quality and to improve culture stability, as well as to reduce labour and utilized tank capacity, new methods have been developed for the high-density rearing of rotifers in continuous culture. In a chemostat culture system for rotifer production Abu-Rezq et al. (1997) achieved a daily rotifer production of 2×10^9 individuals in 1 m³ tank. In this system, the pH level could be stabilized at pH 7.5-8.1 while the dissolved oxygen remained high (6.8 to 5.6 ppm) during a 3-months culture period. Fu et al. (1997) developed an automatic continuous culture system with a filtration unit, a culture unit and a harvest unit to improve the stability in the mass production of rotifers. In this system, filtered water and food (*Chlorella vulgaris*) are continuously supplied to a rotifer tank and the same amount of culture water is transferred into a harvesting tank where on a daily basis a rotifer biomass of 2.1×10^9 S-type rotifers or 1.7×10^8 L-type rotifers can be harvested per m³. Another high density mass culture system for rotifers was developed by Yoshimura et al. (1997a). In this system, filtering equipment was used to prevent particulate organic matter, debris and bacteria from clogging the collection net during harvest. The last progress in the area of high density rotifer production has been achieved in a closed recirculation system. A protein skimmer equipped with an ozone generator is removing most of the suspended matter and part of the soluble components before the water is treated in a submerged biofilter. This biofilter has been seeded with a single inoculum of nitrifying bacteria at the start of the culture. The combination of improved diet formulation and the new culture design enables rotifer densities of 23,000 individuals.ml⁻¹ (Dhert et al., 2000). The rotifer production unit has been tested on a commercial scale in 1 m³ tanks yielding 3.4×10^{10} rotifers in 21 days with a daily rotifer harvest of 2.1×10^9 rotifers (approximately 30% of the standing crop). Using this system, low ammonium (0-0.8 mg.l⁻¹) and nitrite (0.2-3.5 mg.l⁻¹) concentrations could be maintained during the entire culture period. Thanks to the natural carbonate

pebbles in the biofilter the pH is kept at 7.3 without adjustments during the culture period. The microbial counts also remained stable during the whole culture period and were about 1-2 logs lower than in batch systems.

The advantages of this rearing procedure reside in a considerable reduction in the maintenance of the culture, high productivity and the production of cleaner rotifers with the possibility for further automation of the system

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