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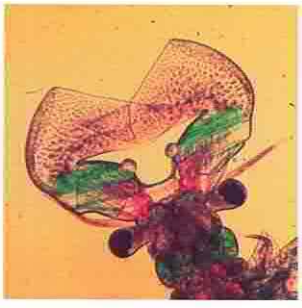

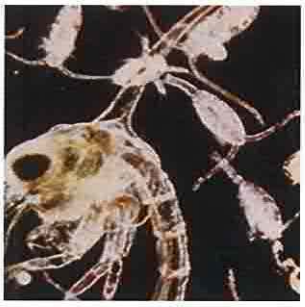

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3. ROTIFERS

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3.1. Introduction

Although *Brachionus plicatilis* was first identified as a pest in the pond culture of eels in the fifties and sixties, Japanese researchers soon realized that this rotifer could be used as a suitable live food organism for the early larval stages of marine fish. The successful use of rotifers in the commercial hatchery operations of the red sea bream (*Pagrus major*) encouraged investigations in the development of mass culture techniques of rotifers. Twenty five years after the first use of rotifers in larviculture feeding several culture techniques for the intensive production of rotifers are being applied worldwide. The availability of large quantities of this live food source has contributed to the successful hatchery production of more than 60 marine finfish species and 18 species of crustaceans. To our knowledge, wild populations of rotifers are only harvested in one region in the P.R. China, (*i.e.* the Bohai Bay saltworks) where *Brachionus plicatilis* is used as food in local shrimp and crab hatcheries. The success of rotifers as a culture organism are manifold, including their. planktonic nature, tolerance to a wide range of environmental conditions, high reproduction rate (0.7-1.4 off-spring.female^{-1}.day^{-1}). Moreoever, their small size and slow swimming velocity make them a suitable prey for fish larvae that have just resorbed their yolk sac but cannot yet ingest the larger *Artemia* nauplii. However, the greatest potential for rotifer culture resides, however, resides in the possibility of rearing these animals at very high densities (i.e. densities of 2000 animals.ml^{-1} have been reported by Hirata (1979). Even at high densities, the animals reproduce rapidly and can thus contribute to the build up of large quantities of live food in a very short period of time. Last, but not least, the filter-feeding nature of the rotifers facilitiates the inclusion into their body tissues of specific nutrients essential for the larval predators (i.e. through bioencapsulation; see further).

3.2. Morphology

Rotatoria (=Rotifera) belong to the smallest metazoa of which over 1000 species have been described, 90 % of which inhabit freshwater habitats. They seldom reach 2 mm in body length. Males have reduced sizes and are less developed than females; some measuring only 60 µm. The body of all species consists of a constant number of cells, the different *Brachionus* species containing approximately 1000 cells which should not be considered as single identities but as a plasma area. The growth of the animal is assured by plasma increase and not by cell division.

The epidermis contains a densely packed layer of keratin-like proteins and is called the lorica. The shape of the lorica and the profile of the spines and ornaments allow the determination of the different species and morphotypes (see 3.4.). The rotifer's body is differentiated into three distinct parts consisting of the head, trunk and foot (Fig. 3.1.). The head carries the rotatory organ or corona which is easily recognized by its annular ciliation and which is at the origin of the name of the Rotatoria (bearing wheels). The retractable corona assures locomotion and a whirling water movement which facilitates the uptake of small food particles (mainly algae and detritus). The trunk contains the digestive tract, the excretory system and the genital organs. A characteristic organ for the rotifers is the mastax (*i.e*. a calcified apparatus in the mouth region), that is very effective in grinding ingested particles. The foot is a ring-type retractable structure without segmentation ending in one or four toes.

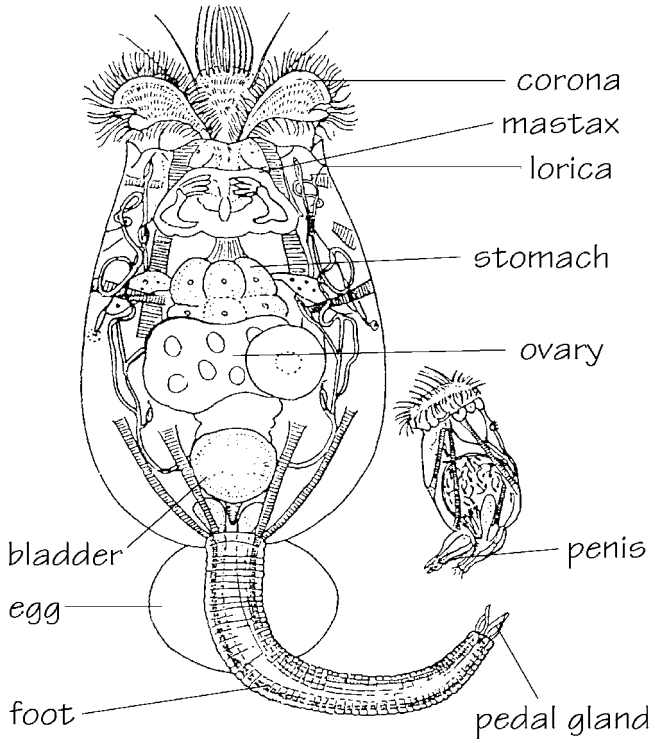

Figure 3.1. *Brachionus plicatilis*, female and male (modified from Koste, 1980).

3.3. Biology and life history

The life span of rotifers has been estimated to be between 3.4 to 4.4 days at 25^{o} C. Generally, the larvae become adult after 0.5 to 1.5 days and females thereafter start to lay eggs approximately every four hours. It is believed that females can produce ten generations of offspring before they eventually die. The reproduction activity of *Brachionus* depends on the temperature of the environment as illustrated in Table 3.1.

The life cycle of *Brachionus plicatilis* can be closed by two modes of reproduction (Fig. 3.2.). During female parthenogenesis the amictic females produce amictic (diploid, 2n chromosomes) eggs which develop and hatch into amictic females. Under specific environmental conditions the females switch to a more complicated sexual reproduction resulting in mictic and amictic females. Although both are not distinguishable morphologically, the mictic females produce haploid (n chromosomes) eggs. Larvae hatching out of these unfertilized mictic eggs develop into haploid males. These males

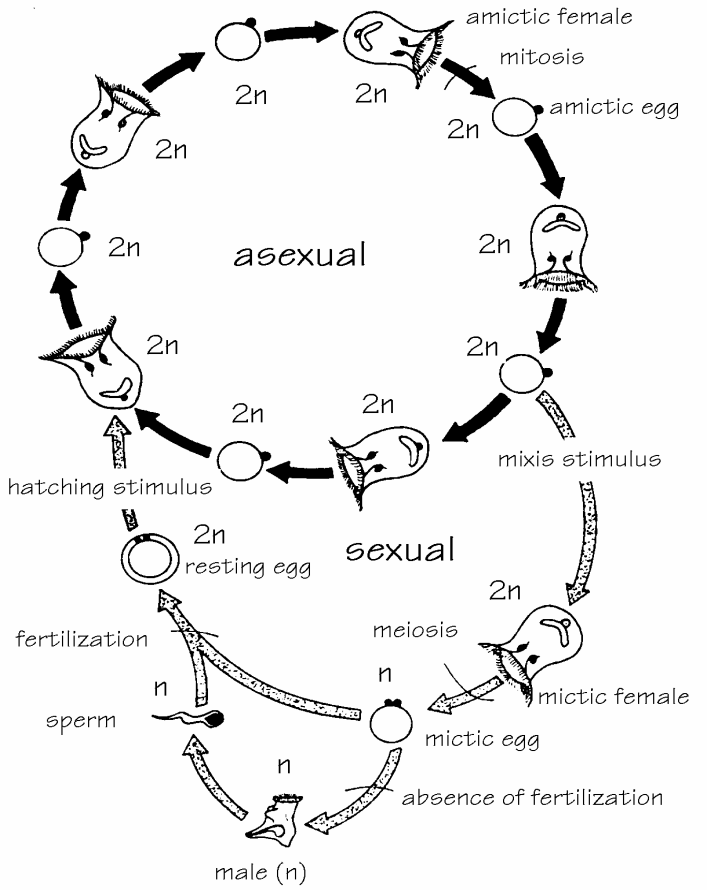

Figure 3.2. Parthenogenetical and sexual reproduction in *Brachionus plicatilis* (modified from Hoff and Snell, 1987).

are about one quarter of the size of the female; they have no digestive tract and no bladder but have an over-proportionated single testis which is filled with sperm. Mictic eggs which will hatch into males are significantly smaller in size, while the mictic fertilized eggs are larger and have a thick, faintly granulated outer layer.

These are the resting eggs that will only develop and hatch into amictic females after exposure to specific environmental conditions. These can be the result of changes in environmental conditions eventually creating alternations in temperature or salinity or changing food conditions. It should be emphasized that the rotifer density of the population also plays an important role in the determination of the mode of reproduction. Although the mechanism is not completely understood, it is generally believed that the production of resting eggs is a survival strategy of the population through unfavourable environmental conditions such as drought or cold.

3.4. Strain differences

Only a few rotifer species belonging to the genus *Brachionus* are used in aquaculture. As outlined in the introduction the most widely used species is *Brachionus plicatilis,* a cosmopolitan inhabitant of inland saline and coastal brackish waters. It has a lorica

length of 100 to 340 µm, with the lorica ending with 6 occipital spines (Fukusho, 1989).

However, for use in aquaculture, a simple classification is used which is based on two different morphotypes, namely *Brachionus rotundiformis* or small (S-type) rotifers and *Brachionus plicatilis* or large (L-type) rotifers. The differences among the two types can be clearly distinguished by their morphological characteristics: the lorica length of the L-type ranging from 130 to 340 µm (average 239 µm), and of the S-type ranging from 100 to 210 µm (average 160 µm). Moreover, the lorica of the S-type shows pointed spines, while of the L-type has obtuse angled spines (Fig. 3.3.).

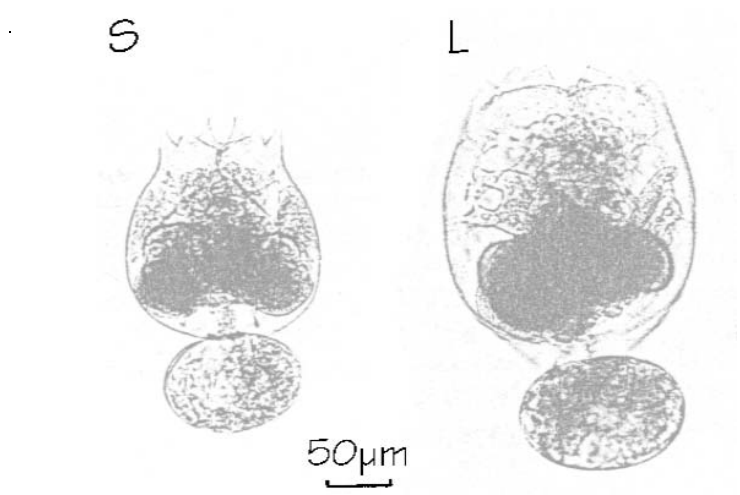

Figure 3.3. *Brachionus rotundiformis* (S-type) and *Brachionus plicatilis* (L-type) (modified from Fu *et al*., 1991).

In tropical aquaculture the SS-type rotifers (Super small rotifers) are preferred for the first feeding of fish larvae with small mouth openings (rabbitfish, groupers, and other fish with mouth openings at start feeding of less than 100 µm). Those rotifers, however, are genetically not isolated from S-strains, but are smaller than common S-strains.

The S- and L- morphotypes also differ in their optimal growth temperature. The S-type has an optimal growth at 28-35°C, while the L-type reaches its optimal growth at 18-25°C. Since contamination with both types of rotifers occurs frequently, lowering or increasing culture temperatures can be used to obtain pure cultures: rotifers at their upper or lower tolerance limit do not multiply as fast and can in this way be out-competed in favour of the desired morphotype.

It should be emphasized that, besides intraspecific size variations, important interspecific variation in size can occur as a function of salinity level or dietary regime. This polymorphism can result in a difference of maximal 15% (Fukusho and Iwamoto, 1981). Rotifers fed on baker's yeast are usually larger than those fed on live algae.

3.5. General culture conditions

3.5.1. Marine rotifers

3.5.1.1. Salinity

Although *Brachionus plicatilis* can withstand a wide salinity range from 1 to 97 ppt, optimal reproduction can only take place at salinities below 35 ppt (Lubzens, 1987). However, if rotifers have to be fed to predators which are reared at a different salinity (± 5 ppt), it is safe to acclimatize them as abrupt salinity shocks might inhibit the rotifers' swimming or even cause their death.

3.5.1.2. Temperature

The choice of the optimal culture temperature for rearing rotifers depends on the rotifer-morphotype; L-strain rotifers being reared at lower temperatures than S-type rotifers. In general, increasing the temperature within the optimal range usually results in an increased reproductive activity. However, rearing rotifers at high temperature enhances the cost for food. Apart from the increased cost for food, particular care has also to be paid to more frequent and smaller feeding distributions. This is essential for the maintenance of good water quality, and to avoid periods of overfeeding or starvation which are not tolerated at suboptimal temperature levels. For example, at high temperatures starving animals consume their lipid and carbohydrate reserves very fast.
Rearing rotifers below their optimal temperature slows down the population growth considerably. Table 3.1 shows the effect of temperature on the population dynamics of rotifers.

Table 3.1. Effect of temperature on the reproduction activity of *Brachionus plicatilis*. (After Ruttner-Kolisko, 1972).

Temperature (°C).	15 °C	20 °C	25 °C
Time for embryonic development (days).	1.3	1.0	0.6
Time for young female to spawn for the first time (days).	3.0	1.9	1.3
Interval between two spawnings (hours).	7.0	5.3	4.0
Length of life (days).	15	10	7
Number of eggs spawned by a female during her life.	23	23	20

3.5.1.3. Dissolved oxygen

Rotifers can survive in water containing as low as 2 $mg.l^{-1}$ of dissolved oxygen. The level of dissolved oxygen in the culture water depends on temperature, salinity, rotifer density, and the type of the food. The aeration should not be too strong as to avoid physical damage to the population.

3.5.1.4. pH

Rotifers live at pH-levels above 6.6, although in their natural environment under culture conditions the best results are obtained at a pH above 7.5.

3.5.1.5. Ammonia (NH_3)

The NH_3/NH_4^+ ratio is influenced by the temperature and the pH of the water. High levels of un-ionized ammonia are toxic for rotifers but rearing conditions with NH_3-concentrations below 1 $mg.l^{-1}$ appear to be safe.

3.5.1.6. Bacteria

Pseudomonas and *Acinetobacter* are common opportunistic bacteria which may be important additional food sources for rotifers. Some *Pseudomonas* species, for instance, synthesize vitamin B_{12} which can be a limiting factor under culture conditions (Yu *et al.,* 1988).

Although most bacteria are not pathogenic for rotifers their proliferation should be avoided since the real risk of accumulation and transfer via the food chain can cause detrimental effects on the predator.

A sampling campaign performed in various hatcheries showed that the dominant bacterial flora in rotifer cultures was of V*ibrio* (Verdonck *et al*., 1994). The same study showed that the microflora of the live food was considerably different among hatcheries; especially after enrichment, high numbers of associated bacteria were found. The enrichment of the cultures generaly induces a shift in the bacterial composition from *Cytophaga*/*Flavobacterium* dominance to *Pseudomonas*/ *Alcaligenes* dominance. This change is partly due to a bloom of fast growing opportunistic bacteria, favoured by high substrate levels (Skjermo and Vadstein, 1993).

The bacterial numbers after enrichment can be decreased to their initial levels by appropriate storage (6°C) and adjustment of the rotifer density (Skjermo and Vadstein, 1993). A more effective way to decrease the bacterial counts, especially the counts of the dominant *Vibrionaceae* in rotifers, consists of feeding the rotifers with *Lactobacillus plantarum* (Gatesoupe, 1991). The supplementation of these probiotic bacteria not only has a regulating effect on the microflora but also increases the production rate of the rotifers.

For stable rotifer cultures, the microflora as well as the physiological condition of the rotifers, has to be considered. For example, it has been demonstrated that the dietary condition of the rotifer *Brachionus plicatilis* can be measured by its physiological performance and reaction to a selected pathogenic bacterial strain *(Vibrio anguillarum* TR27); the *V. anguillarum* strain administered at 10^6-10^7 colony forming units (CFU).ml^{-1} causing a negative effect on rotifers cultured on a sub-optimal diet while the rotifers grown on an optimal diet were not affected by the bacterial strain. Comparable results were also reported by Yu *et al*. (1990) with a *Vibrio alginolyticus* strain Y5 supplied at a concentration of $2.5.10^4 CFU.ml^{-1}$.

3.5.1.7. Ciliates

Halotricha and Hypotricha ciliates, such as *Uronema* sp. and *Euplotes* sp., are not desired in intensive cultures since they compete for feed with the rotifers. The appearance of these ciliates is generally due to sub-optimal rearing conditions, leading to less performing rotifers and increased chances for competition. Ciliates produce metabolic wastes which increase the NO_2^--N level in the water and cause a decrease in pH. However, they have a positive effect in clearing the culture tank from bacteria and detritus. The addition of a low formalin concentration of 20 $mg.l^{-1}$ to the algal culture tank, 24 h before rotifer inoculation can significantly reduce protozoan contamination. Screening and cleaning of the rotifers through the use of phytoplankton filters (< 50 µm) so as to reduce the number of ciliates or other small contaminants is an easy precaution which can be taken when setting up starter cultures.

3.5.2. Freshwater rotifers

Brachionus calyciflorus and *Brachionus rubens* are the most commonly cultured rotifers in freshwater mass cultures. They tolerate temperatures between 15 to 31°C. In their natural environment they thrive in waters of various ionic composition. *Brachionus calyciflorus* can be cultured in a synthetic medium consisting of 96 mg $NaHCO_3$, 60 mg $CaSO_4.2H_2O$, 60 mg $MgSO_4$ and 4 mg KCl in 1 1 of deionized water. The optimal pH is 6-8 at 25 °C, minimum oxygen levels are 1.2 $mg.l^{-1}$. Free ammonia levels of 3 to 5 $mg.l^{-1}$ inhibit reproduction.

Brachionus calyciflorus and *Brachionus rubens* have been successfully reared on the microalgae *Scenedesmus costato-granulatus*, *Kirchneriella contorta*, *Phacus pyrum*, *Ankistrodesmus convoluus* and *Chlorella*, as well as yeast and the artificial diets Culture Selco® (Inve Aquaculture, Belgium) and Roti-Rich (Florida Aqua Farms Inc., USA). The feeding scheme for *Brachionus rubens* needs to be adjusted as its feeding rate is somewhat higher than that of *B. plicatilis*.

3.5.3. Culture procedures

Intensive production of rotifers is usually performed in batch culture within indoor facilities; the latter being more reliable than outdoor extensive production in countries where climatological constraints do not allow the outdoor production of microalgae. Basically, the production strategy is the same for indoor or outdoor facilities, but higher starting and harvesting densities enable the use of smaller production tanks (generally 1 to 2 m^3) within intensive indoor facilities. In some cases, the algal food can be completely substituted by formulated diets (see 3.5.3.6.).

3.5.3.1. Stock culture of rotifers

Culturing large volumes of rotifers on algae, baker's yeast or artificial diets always involves some risks for sudden mortality of the population. Technical or human failures but also contamination with pathogens or competitive filter feeders are the main causes for lower reproduction which can eventually result in a complete crash of the population. Relying only on mass cultures of rotifers for reinoculating new tanks is too risky an approach. In order to minimize this risk, small stock cultures are generally kept in closed vials in an isolated room to prevent contamination with bacteria and/or ciliates.

These stock cultures which need to generate large populations of rotifers as fast as possible are generally maintained on algae.

The rotifers for stock cultures can be obtained from the wild, or from research institutes or commercial hatcheries. However, before being used in the production cycle the inoculum should first be disinfected. The most drastic disinfection consists of killing the free-swimming rotifers but not the eggs with a cocktail of antibiotics (*e.g.* erythromycin 10 $mg.l^{-1}$, chloramphenicol 10 $mg.l^{-1}$, sodium oxolinate 10 $mg.l^{-1}$, penicillin 100 $mg.l^{-1}$, streptomycin 20 $mg.l^{-1}$) or a disinfectant. The eggs are then separated from the dead bodies on a 50 µm sieve and incubated for hatching and the offspring used for starting the stock cultures. However, if the rotifers do not contain many eggs (as can be the case after a long shipment) the risk of loosing the complete initial stock is too big and in these instances the rotifer shouldl be disinfected at sublethal doses; the water of the rotifers being completely renewed and the rotifers treated with either antibiotics or disinfectants. The treatment is repeated after 24 h in order to be sure that any pathogens which might have survived the passage of the intestinal tract of the rotifers are killed as well. The concentration of the disinfection products differs according to their toxicity and the initial condition of the rotifers. Orientating concentrations for this type of disinfection are 7.5 $mg.l^{-1}$ furazolidone, 10 $mg.l^{-1}$ oxytetracycline, 30 $mg.l^{-1}$ sarafloxacin, or 30 $mg.l^{-1}$ linco-spectin.

Figure 3.4. Stock cultures of rotifers kept in 50 ml centrifuge tubes. The tubes are fixed on a rotor. At each rotation the medium is mixed with the enclosed air.

At the Laboratory of Aquaculture & Artemia Reference Center the stock cultures for rotifers are kept in a thermo-climatised room (28°C ± 1°C). The vials (50 ml conical centrifuge tubes) are previously autoclaved and disposed on a rotating shaft (4 rpm). At each rotation the water is mixed with the enclosed air (± 8 ml), providing enough oxygen for the rotifers (Fig. 3.4.). The vials on the rotor are exposed to the light of two fluorescent light tubes at a distance of 20 cm (light intensity of 3000 lux on the tubes).

The culture water (seawater diluted with tap water to a salinity of 25 ppt) is aerated, prefiltrated over a 1 µm filter bag and disinfected overnight with 5 $mg.l^{-1}$ NaOCl. The next day the excess of NaOCl is neutralized with $Na_2S_2O_3$ (for neutralization and color reaction see worksheet 3.1.) and the water is filtered over a 0.45 µm filter.

Inoculation of the tubes is carried out with an initial density of 2 $rotifers.ml^{-1}$. The food consists of marine *Chlorella* cultured according to the procedure described in 2.3. The algae are centrifuged and concentrated to 1-2.10^8 $cells.ml^{-1}$. The algal concentrate is stored at 4°C in a refrigerator for a maximum period of 7 days, coinciding with one rotifer rearing cycle. Every day the algal concentrate is homogenized by shaking and

200 µl is given to each of the tubes. If fresh algae are given instead of the algal concentrate 4 ml of a good culture is added daily.

After one week the rotifer density should have increased from 2 to 200 individuals.ml^{-1} (Fig. 3.5.). The rotifers are rinsed, a small part is used for maintenance of the stock, and the remaining rotifers can be used for upscaling. Furthermore, after some months of regular culture the stock cultures will be disinfected as described earlier in order to keep healthy and clean stock material. However, the continuous maintenance of live stock cultures of *Brachionus* does not eliminate the risk of bacterial contamination.

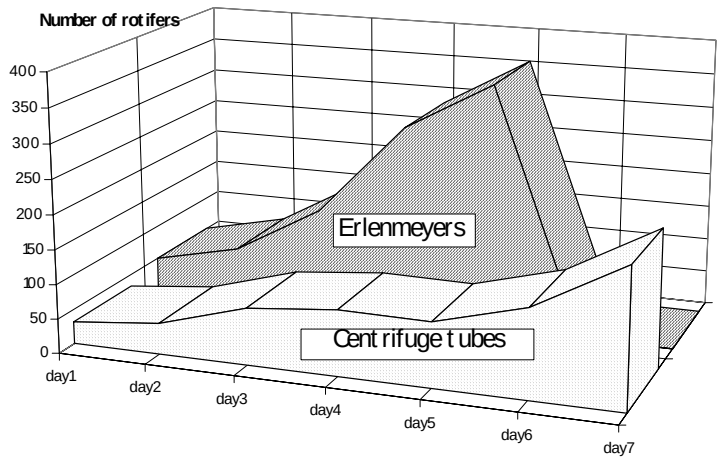

Figure 3.5. Growth rate of the rotifer population in the stock cultures (centrifuge tubes) and during the upscaling in erlenmeyers.

Treatment with anti-biotics might lower the bacterial load, but also implies the risk for selection of antibiotic-resistant bacteria. However, the commercial availability of resting eggs could be an alternative to maintaining stock cultures and reducing the chances for contamination with ciliates or pathogenetic bacteria (see Fig. 3.7.).

3.5.3.2. Upscaling of stock cultures to starter cultures

The upscaling of rotifers is carried out in static systems consisting of erlenmeyers of 500 ml placed 2 cm from fluorescent light tubes (5000 lux). The temperature in the erlenmeyers should not be more than 30°C. The rotifers are stocked at a density of 50 individuals.ml^{-1} and fed 400 ml freshly-harvested algae *(Chlorella* 1.6.10^{6} cells.ml^{-1}); approximately 50 ml of algae being added every day to supply enough food. Within 3 days the rotifer concentration can increase to 200 rotifers.ml^{-1} (Fig. 3.5.). During this short rearing period no aeration is applied.

Once the rotifers have 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) retains large waste particles, while the lower sieve (50 µm) collects the rotifers. If only single strainers are available this handling can be carried out with two separate filters.

Moreover, if rinsing is performed under water the rotifers will not clog and losses will be limited to less than 1%.

The concentrated rotifers are then distributed in several 15 l bottles filled with 2 l water at a density of 50 individuals.ml^{-1} and a mild tube aeration provided. In order to avoid contamination with ciliates the air should be filtered by a cartridge or activated carbon filters. Fresh algae (*Chlorella* 1.6 x 10^6 cells.ml^{-1}) are supplied daily. Every other day the cultures are cleaned (double-screen filtration) and restocked at densities of 200 rotifers.ml^{-1}. After adding algae for approximately one week the 15 l bottles are completely full and the cultures can be used for inoculation of mass cultures.

3.5.3.3. Mass production on algae

Undoubtedly, marine microalgae are the best diet for rotifers and very high yields can be obtained if sufficient algae are available and an appropriate management is followed. Unfortunately in most places it is not possible to cope with the fast filtration capacity of the rotifers which require continuous algal blooms. If the infrastructure and labor is not limiting, a procedure of continuous (daily) harvest and transfer to algal tanks can be considered. In most places, however, pure algae are only given for starting up rotifer cultures or to enrich rotifers (see 3.5.3.1. and 3.6.1.1.).

Batch cultivation is probably the most common method of rotifer production in marine fish hatcheries. The culture strategy consists of 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 (see 3.5.3.4.). Extensive culture techniques (using large tanks of more than 50 m^3) as well as intensive methods (using tanks with a volume of 200-2000 l) are applied. In both cases large amounts of cultured microalgae, usually the marine alga *Nannochloropsis*, are usually inoculated in the tanks together with a starter population containing 50 to 150 rotifers.ml^{-1}.

3.5.3.4. Mass production on algae and yeast

Depending on the strategy and the quality of the algal blooms baker's yeast may be supplemented. The amount of yeast fed on a daily basis is about 1 g.million^{-1} of rotifers, although this figure varies depending on the rotifer type (S,L) and culture conditions. Since algae have a high nutritional value, an excellent buoyancy and do not pollute the water, they are used as much as possible, not only as a rotifer food, but also as water conditioners and bacteriostatic agents.

In contrast to most European rearing systems, Japanese developed large culture systems of 10 to 200 metric tons. The initial stocking density is relatively high (80-200 rotifers.ml^{-1}) and large amounts of rotifers (2-6 x 10^9) are produced daily with algae (4-40 m^3) supplemented with yeast (1-6 kg).

The mass production on algae and yeast is performed in a batch or semi-continuous culture system. Several alterations to both systems have been developed, and as an example the rearing models used at The Oceanic Institute in Hawaii are described here: