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 offspring.female\(^{-1}\).day\(^{-1}\)). Moreover, 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 facilitates 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 \(\mu\)m. 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.
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° 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

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**Figure 3.1.** *Brachionus plicatilis*, female and male (modified from Koste, 1980).
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 \( \mu \text{m} \), 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 \( \mu \text{m} \) (average 239 \( \mu \text{m} \)), and of the S-type ranging from 100 to 210 \( \mu \text{m} \) (average 160 \( \mu \text{m} \)). Moreover, the lorica of the S-type shows pointed spines, while of the L-type has obtuse angled spines (Fig. 3.3.).

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 \( \mu \text{m} \)). 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>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Temperature (°C).</strong></td>
</tr>
<tr>
<td>Time for embryonic development (days).</td>
</tr>
<tr>
<td>Time for young female to spawn for the first time (days).</td>
</tr>
<tr>
<td>Interval between two spawnings (hours).</td>
</tr>
<tr>
<td>Length of life (days).</td>
</tr>
<tr>
<td>Number of eggs spawned by a female during her life.</td>
</tr>
</tbody>
</table>

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 (NH₃)

The NH₃/NH₄⁺ 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 NH₃-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 B₁₂ 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 *Vibrio* (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 generally 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 *Vibrioaceae* 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 10⁶-10⁷ 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 2.5.10⁴ 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$_2$O, 60 mg MgSO$_4$ and 4 mg KCl in 1 l 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 convolus* 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 oxolate 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 should 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.

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 Na₂S₂O₃ (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 1-2.10⁸ 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.](image)

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* 1.6.10⁶ 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 10⁶ 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 m³) 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 10⁹) are produced daily with algae (4-40 m³) 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:
**Batch culture system**

The tanks (1 200 l capacity) are half filled with algae at a density of 13-14 x 10^6 cells.ml^-1 and inoculated with rotifers at a density of 100 individuals.ml^-1. The salinity of the water is 23 ppt and the temperature maintained at 30°C. The first day active baker's yeast is administered two times a day at a quantity of 0.25 g/10^6 rotifers. The next day the tanks are completely filled with algae at the same algal density and 0.375 g baker's yeast per million rotifers is added twice a day. The next day the rotifers are harvested and new tanks are inoculated (i.e. two-day batch culture system).

**Semi-continuous culture**

In this culture technique the rotifers are kept in the same tank for five days. During the first two days the culture volume is doubled each day to dilute the rotifer density in half. During the next following days, half the tank volume is harvested and refilled again to decrease the density by half. On the fifth day the tank is harvested and the procedure started all over again (i.e. five-day semi-continuous culture system).

The nutritional composition of algae-fed rotifers does not automatically meet the requirements of many predator fish and sometimes implies an extra enrichment step to boost the rotifers with additional nutritional components such as fatty acids, vitamins or proteins (see 3.6.). Also, the addition of vitamins, and in particular vitamin B_{12}, has been reported as being essential for the culture of rotifers (Yu et al., 1989).

**3.5.3.5. Mass culture on yeast**

Baker's yeast has a small particle size (5-7 µm) and a high protein content and is an acceptable diet for *Brachionus*. The first trials to replace the complete natural rotifer diet by baker's yeast were characterized by varying success and the occurrence of sudden collapses of the cultures (Hirayama, 1987). Most probably the reason for these crashes was explained by the poor digestibility of the yeast, which requires the presence of bacteria for digestion. Moreover, the yeast usually needs to be supplemented with essential fatty acids and vitamins to suit the larval requirements of the predator organisms. Commercial boosters, but also home-made emulsions (fish oils emulgated with commercial emulgators or with egg-yolk lecithin), may be added to the yeast or administered directly to the rotifer tank (see 3.6.1.3.). Better success was obtained with so called ω-yeast-fed rotifers (rotifers fed on a yeast preparation produced by adding cuttlefish liver oil at a 15% level to the culture medium of baker's yeast) which ensured a high level of (n-3) essential fatty acids in the rotifers (Watanabe et al., 1983). The necessity of adding the component in the food of the rotifer or to the rotifers' culture medium was later confirmed by using microparticulate and emulsified formulations (Watanabe et al., 1983; Léger et al., 1989). Apart from fresh baker's yeast, instant baker's yeast, marine yeast (*Candida*) or caked yeast (*Rhodotorula*) may also be used.

**3.5.3.6. Mass culture on formulated diets**

The most frequently used formulated diet in rotifer culture in Europe is Culture Selco® (CS) available under a dry form. It has been formulated as a complete substitute for live microalgae and at the same time guarantees the incorporation of high levels of EFA and vitamins in the rotifers. The biochemical composition of the artificial diet Culture Selco® consists of 45% proteins, 30% carbohydrates, 15% lipids (33% of which are (n-3) HUFA), and 7% ash. Its physical characteristics are optimal for uptake by rotifers: the particle, having a 7 µm particle size, remaining in suspension in the water column with a relatively strong
aeration, and not leaching. However, the diet needs to be suspended in water prior to feeding, which facilitates on one hand the possibilities for automatic feeding but on the other hand requires the use of aeration and cold storage. The following standard culture procedure has been developed and tested on several rotifer strains in 100 l tanks.

Cylindro-conical tanks of 100 l with dark smooth walls (polyethylene) are set up in shaded conditions. The culture medium consists of diluted seawater of 25 ppt kept at 25°C. No water renewal takes place during the 4-day culture period. Air stones are installed a few cm above the cone bottom of the tank to allow sedimentation and possible flushing of waste particles. Food flocculates are trapped in pieces of cloth which are suspended in the water column (Fig. 3.6a.), or in an air-water-lift trap filled with sponges (Fig. 3.6b.).

![Figure 3.6. Piece of cloth (a) and air-water-lift filled with sponges (b) to trap the floccules in the rotifer tank.](image)

<table>
<thead>
<tr>
<th>Rotifer density.ml⁻¹ (L-strain)</th>
<th>Culture Selco® per 10⁶ rotifers.day⁻¹ (in g)</th>
<th>Culture Selco® per m³.day⁻¹ (in g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 - 150</td>
<td>0.53</td>
<td>53 - 80</td>
</tr>
<tr>
<td>150 - 200</td>
<td>0.47</td>
<td>70 - 93</td>
</tr>
<tr>
<td>200 - 250</td>
<td>0.40</td>
<td>80 - 100</td>
</tr>
<tr>
<td>250 - 300</td>
<td>0.37</td>
<td>92 - 110</td>
</tr>
<tr>
<td>300 - 350</td>
<td>0.33</td>
<td>100 - 117</td>
</tr>
<tr>
<td>350 - 400</td>
<td>0.30</td>
<td>105 - 120</td>
</tr>
</tbody>
</table>
Table 3.2. (contd.) Feeding regime for optimal rotifer culture in function of the rotifer density using the formulated diet Culture Selco®.

<table>
<thead>
<tr>
<th>Density (rotifers/ml)</th>
<th>Feeding Rate (g/L)</th>
<th>pH</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 - 450</td>
<td>0.27</td>
<td></td>
<td>107 - 120</td>
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<tr>
<td>450 - 500</td>
<td>0.23</td>
<td></td>
<td>105 - 117</td>
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<tr>
<td>&gt; 500</td>
<td>0.25</td>
<td></td>
<td>125</td>
</tr>
<tr>
<td>&gt; 1200</td>
<td>0.20</td>
<td></td>
<td>240</td>
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</table>

Furthermore, all efforts are made to maintain a good water quality with minimal accumulations of wasted food by assuring short retention times of the food particles. This is achieved by using high starting densities of 200 rotifer/ml and the distribution of small amounts of feed at hourly intervals; the latter can easily be automated by pumping the feed suspension from a gently aerated stock kept in a refrigerator at 4°C for up to 30 h (Fig. 3.7.). Applying this feeding strategy, an optimized feeding regime is developed in function of the rotifer density and the culture performance (Table 3.2.). It should be indicated that this protocol is developed for the L-rotifer strain and should be slightly adapted (less feed) when a S-rotifer strain is used.

Figure 3.7. Refrigerated feed suspension distributed to the individual rotifer tanks by means of a peristaltic pump.
Applying this standard culture strategy a doubling of the population is achieved every two days, reaching a harvest density of 600 rotifiers.ml⁻¹ after four days only (Table 3.3.), which is better than for the traditional technique using live algae (and baker's yeast). There is no high variation in production characteristics among the various culture tests and crashes are rarely observed, which most probably is due to the non-introduction of microbial contaminants and the overall good water quality over the culture period. In this respect, it should be emphasized that hygienic precautions should be taken to avoid contacts among different rearing units. All material used during the production (i.e. glass ware) can be disinfected in water baths with NaOCl, HCl or other disinfectants. After each production cycle (4 days) the tanks, airstones and tubing need to be disinfected thoroughly. In order to avoid crashes it is recommended that after approximately one month of culture that the complete system be disinfected and the cultures started again using rotifers from starter cultures.

In commercial hatcheries, peristaltic pumps are not always available. In this case the artificial diet can be fed on a daily basis at a concentration of 400-600 mg/10⁻⁶ rotifers, and administered in 4 to 6 rations with a minimum quantity of 50 - 100 mg.l⁻¹ culture medium. Analogous production outputs are achieved under upscaling conditions in commercial hatcheries (Table 3.3.).

<table>
<thead>
<tr>
<th><strong>Table 3.3. Growth and reproduction characteristics of rotifers reared on CS under experimental and upscaled conditions.</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental</strong></td>
</tr>
<tr>
<td>Age of the population</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 2</td>
</tr>
<tr>
<td>Day 3</td>
</tr>
<tr>
<td>Day 4</td>
</tr>
<tr>
<td>Growth rate.day⁻¹</td>
</tr>
<tr>
<td>Doubling time</td>
</tr>
<tr>
<td><strong>Commercial</strong></td>
</tr>
<tr>
<td>Age of the population</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 2</td>
</tr>
<tr>
<td>Day 3</td>
</tr>
<tr>
<td>Day 4</td>
</tr>
<tr>
<td>Day 5</td>
</tr>
</tbody>
</table>
In order to avoid several manual feedings per day, a simple drip-feeding technique can be used as illustrated in Fig. 3.8. A concentrated food suspension is placed in the tank and water is dripped in the food suspension that is gradually diluted and allowed to over-flow into the rotifer tank. Since the overhead tank only contains water the flow rate can be adjusted without danger of clogging. The dimensions of the tank should be made as such that the complete content of the food tank is diluted in 24 h.

![Figure 3.8. Illustration of the drip-feeding technique which can be applied when no sophisticated pumping devices are available.](image)

### 3.5.3.7. High density rearing

Although high density rearing of rotifers increases the risk for more stressful rearing conditions, and an increased risk of reduced growth rates due to the start of sexual reproduction, promising results have been obtained in controlled cultures. The technique is the same as the one used for the mass culture on Culture Selco® but after each cycle of 4 days the rotifer density is not readjusted. The feeding scheme is adjusted to 0.25-0.3 g/10^6 of rotifers for densities between 500 and 1500 rotifers.ml⁻¹ and to 0.2 g for densities above 1500 rotifers.ml⁻¹. Rearing rotifers at high stocking densities has a direct repercussion on the egg ratio (Fig. 3.9.). This latter is dropping from an average of 30% at a density of 150 rotifers.ml⁻¹ to 10% at a density of 2000 rotifers.ml⁻¹ and less than 5% at densities of 5000 rotifers.ml⁻¹. Maintaining cultures with this low egg ratio is more risky and thus the system should only be used under well controlled conditions.
High density cultivation of *Brachionus* is also being performed in Japan. In this technique *Nannochloropsis* is being supplemented with concentrated fresh water *Chlorella*, baker’s yeast and yeast containing fish oil. Freshwater *Chlorella* is being used for vitamin B\textsubscript{12} supplementation (± 12 mg.l\textsuperscript{-1} at a cell concentration of 1.5.10\textsuperscript{10} cells.ml\textsuperscript{-1}). In continuous cultures the rotifer population doubles every day. Half the culture is removed daily and replaced by new water. Using this system average densities of 1000 rotifers.ml\textsuperscript{-1} are achieved with peaks of more than 3000 animals.ml\textsuperscript{-1}.

### 3.5.4. Harvesting/concentration of rotifers

Small-scale harvesting of rotifers is usually performed by siphoning the content of the culture tank into filter bags with a mesh size of 50-70 µm. If this is not performed in submerged filters the rotifers may be damaged and result in mortality. It is therefore recommended to harvest the rotifers under water; concentrator rinsers are very convenient for this purpose (Fig. 3.10.). Aeration during the concentration of rotifers will not harm the animals, but should not be too strong so as to avoid clogging of the rotifers, this can be very critical, specially after enrichment (see Fig. 3.6.4.).

![Figure 3.9. Effect of high density rotifer culture on the egg ratio.](image)
3.6. Nutritional value of cultured rotifers

3.6.1. Techniques for (n-3) HUFA enrichment

3.6.1.1. Algae

The high 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 occulata* and 22:6n-3 in *Isochrysis galbana*) have made them excellent live food diets for boosting the fatty acid content of the rotifers. Rotifers submerged in these algae (approximately $5 \times 10^6$ algae ml$^{-1}$) are incorporating the essential fatty acids in a few hours time and come to an equilibrium with a DHA/EPA level above 2 for rotifers submerged in *Isochrysis* and below 0.5 for *Tetraselmis* (Fig. 3.11.). However, the culture of microalgae as a sole diet for rotifer feeding is costly due to the labour intensive character of microalgae production. Most of the time the rotifers are boosted in oil emulsions (see 3.6.1.3.) and fed to the predators which are kept in “green water”. This “green water”, consisting of $\pm 0.2 \times 10^6$ algal cells ml$^{-1}$ (*Tetra-selmis, Nannochloropsis*, or *Isochrysis*) is applied to maintain an appropriate HUFA (but also other components) content in the live prey before they are eventually ingested by the predator (see also 2.5.3.).
3.6.1.2. Formulated feeds

Rotifers grown on the CS® replacement diet have already an excellent HUFA composition: 5.4, 4.4 and 15.6 mg.g⁻¹ dry matter of EPA, DHA and (n-3) HUFA respectively (Fig. 3.12.), which is significantly higher than for cultures grown on algae/baker’s yeast but comparable in case the latter cultures are subjected to an additional enrichment treatment (Léger et al., 1989). The level of total lipids is approximately 18%. Since the use of CS® allows direct enrichment of the rotifers without the need of a cumbersome bioencapsulation treatment, complementary diets such as Protein Selco® (PS) and DHA Culture Selco® (DHA-CS) have been developed in order to incorporate higher levels of protein and DHA (Table 3.4.). The advantage of direct (or long term) enrichment are multiple; in that. 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.

Table 3.4. Characteristics of some diets and emulsions containing high DHA levels (in mg.g⁻¹ DW).

<table>
<thead>
<tr>
<th>Diets</th>
<th>EPA</th>
<th>DHA</th>
<th>DHA/EPA</th>
<th>Σ(n-3)HUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt; 20:3n-3</td>
</tr>
<tr>
<td>CS</td>
<td>18.9</td>
<td>15.3</td>
<td>0.8</td>
<td>36.4</td>
</tr>
<tr>
<td>DHA-CS</td>
<td>16.9</td>
<td>26.7</td>
<td>1.6</td>
<td>45.4</td>
</tr>
<tr>
<td>DHA-PS</td>
<td>24.4</td>
<td>70.6</td>
<td>2.9</td>
<td>99.3</td>
</tr>
</tbody>
</table>

Emulsions

| DHA7    |       | 452.3 | 6.7     | 550.6       |
| DHA20   | 0.8   | 15.6  | 19.5    | 16.4        |

Figure 3.11. Changes in DHA/EPA ratio of rotifers in different algal media.
However, for some marine larval fishes that require still higher (n-3) HUFA levels an additional enrichment with boosters may be necessary (Table 3.4.).

3.6.1.3. Oil emulsions

One of the cheapest ways to enrich rotifers is by using oil emulsions. Although home-made emulsions can be prepared with egg lecithin and fish oils (Watanabe et al., 1982). Commercial emulsions are generally more stable and have a selected HUFA composition.

- Home-made emulsions

The first emulsions were made from (n-3) HUFA rich fish oils (i.e. cuttlefish oil, pollack liver oil, cod liver oil, menhaden oil, etc.) and emulsified with egg yolk and seawater (Watanabe et al., 1982, 1983). Recently, more purified oils containing specifically high levels of the essential fatty acids 20:5n-3 and 22:6n-3 have been used. Since the stability and storage possibility of these products is relatively low they are usually made on the spot and used immediately.

For very specific applications, or when the requirements of the fish can not be fulfilled with commercial emulsions, this technique may also be used to incorporate lipid extracts from zooplankton, fish, fish roe, or other sources. A comparison of two commercially formulated (Super Selco® and DHA-Super...
Selco®) and two self home-made emulsified enrichment diets are given in Figs. 3.13. and 3.14.

• Commercial emulsions

Several emulsified diets are commercially available and based on well-defined formulations. Very popular are the self-emulsifying concentrates (Selco®, Inve Aquaculture NV, Belgium) which can boost the HUFA content of the rotifers in a few hours. In this technique a rotifer suspension containing 200-300 individuals.ml⁻¹ is immersed in a diluted oil-emulsion for 6 h, harvested, rinsed and concentrated before being fed to the predators.

In view of the importance of DHA in marine larviculture, considerable efforts have recently been made to incorporate high levels of DHA and/or high ratios of DHA/EPA in rotifers. To date the best results have been obtained using the self-emulsifying product DHA-Super Selco®. Compared to the results obtained with Super Selco®, the boosting of CS-rotifers with this product under standard enrichment practices results in a threefold increase of DHA and total (n-3) HUFA.
Furthermore, the evolution of the concentrations of EFA within enriched rotifers after being administered to the predator tanks has been investigated. Results reveal that EFA levels remain rather constant for at least 7 h under clear water culture conditions at 20°C; with only a 30% drop in DHA being noted after 12 h (Table 3.5.).

Most commercial emulsions are rich in triacylglycerols and/or methyl esters and no emulsions have been formulated with phospholipids and/or wax esters. In Fig. 3.13, the most commonly used commercial emulsions are compared with home-made emulsions obtained from halibut roe and copepod extracts. Although the content of DHA and EPA is much lower in the latter emulsions, their relative concentration to total FA is much higher.

It is interesting to note that after enrichment the composition of the rotifers did not differ more than a fraction of 30 to 45% in (n-3) HUFA (Fig. 3.13). Moreover, the lipid composition of the rotifers was also little affected by the composition of the diet. However, when the efficiency of DHA and (n-3) HUFA incorporation in rotifers is analyzed it is obvious that better results are obtained with the extraction products. Since all diets are consumed with approximately equal efficiency it means that
phospholipids (present in the extraction products) were more easily assimilated and metabolized by the rotifers.

Table 3.5. Fatty acid concentration in enriched rotifers (in mg.g⁻¹ DW).

<table>
<thead>
<tr>
<th>Type of enrichment</th>
<th>EPA</th>
<th>DHA</th>
<th>DHA/EPA</th>
<th>(n-3) HUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>5.4</td>
<td>4.4</td>
<td>0.8</td>
<td>15.6</td>
</tr>
<tr>
<td>Nannochloropsis sp.</td>
<td>7.3</td>
<td>2.2</td>
<td>0.3</td>
<td>11.4</td>
</tr>
<tr>
<td>DHA-Super Selco</td>
<td>41.4</td>
<td>68.0</td>
<td>1.6</td>
<td>116.8</td>
</tr>
<tr>
<td></td>
<td>40.6*</td>
<td>73.0*</td>
<td>1.8*</td>
<td>123.1*</td>
</tr>
<tr>
<td></td>
<td>43.1**</td>
<td>46.0**</td>
<td>1.1**</td>
<td>95.0**</td>
</tr>
</tbody>
</table>

* Concentration after 7 h storage at 20°C
** Concentration after 12 h storage at 20°C

3.6.2. Techniques for vitamin C enrichment

The vitamin C content of rotifers reflects the dietary ascorbic acid (AA) levels both after culture and enrichment (Table 3.6.). For example, rotifers cultured on instant baker’s yeast contain 150 mg vitamin C/g⁻¹ DW, while for Chlorella-fed rotifers contain 2300 mg vitamin C/g⁻¹ DW. Within commercial marine fish hatcheries a wide range of products are used for the culture and subsequent boosting of rotifers (Table 3.6.). In general commercial-scale enrichment is scoring lower than laboratory enrichment. Problems of operculum deformities currently occurring in Mediterranean gilthead seabream hatcheries might be related to the changes in live food production management and reduced vitamin C levels.

Enrichment of rotifers with AA is carried out using ascorbyl palmitate (AP) as a source of vitamin C to supplement the boosters. AP is converted by the rotifers into active AA up to 1700 mg.g⁻¹ DW after 24 h enrichment using a 5 % AP (w/w) emulsion (Fig. 3.15.). The storage of rotifers in seawater after culture or enrichment has no effect on the AA content during the first 24 h (Fig. 3.15.), indicating that the rotifers maintain their nutritional value when fed to the larval fish during the culture run.
Table 3.6. Ascorbic acid content (mg.g⁻¹ DW) of rotifers cultured on a laboratory and hatchery scale (modified from Merchie et al., 1995).

<table>
<thead>
<tr>
<th>diet / culture</th>
<th>culture (3 d)</th>
<th>enrichment (6 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella / Isochrysis</td>
<td>2289</td>
<td>2155</td>
</tr>
<tr>
<td>baker’s yeast / Isochrysis</td>
<td>148</td>
<td>1599</td>
</tr>
<tr>
<td>Culture Selco™ / Protein Selco™</td>
<td>322</td>
<td>1247</td>
</tr>
<tr>
<td>baker’s yeast + Chlorella / Chlorella</td>
<td>928</td>
<td>1255</td>
</tr>
<tr>
<td>baker’s yeast + Nannochloris / Nannochloris</td>
<td>220</td>
<td>410</td>
</tr>
<tr>
<td>Culture Selco™ / Protein Selco™</td>
<td>136</td>
<td>941</td>
</tr>
<tr>
<td>Culture Selco™ / Isochrysis</td>
<td>327</td>
<td>1559</td>
</tr>
</tbody>
</table>

¹vit C -boosted, Inve Aquaculture N.V.
3.6.3. Techniques for protein enrichment

To our knowledge Protein Selco® is the only enrichment diet especially designed for protein enrichment in rotifers. The high levels of proteins allow the cultures to continue to grow and to develop during the enrichment period. Normally it is used in the same way as an oil emulsion (blended in a kitchen blender) and distributed in the tank at a concentration of 125 mg.l⁻¹ seawater at two time intervals of 3 to 4 hours.

Table 3.7. gives a comparison of the protein content of rotifers enriched with three different enrichment strategies (A: long term enrichment during the culture with baker’s yeast + 10% Super Selco®; B: short term enrichment with DHA-Selco®; C: short term enrichment with Protein Selco®). Dry weight is significantly higher in rotifers enriched with Protein Selco® and similar for A and B. The protein level is significantly higher for C than B rotifers, but no significant difference can be observed between the protein level of A and C rotifers. Lipid levels are significantly higher for C than for A rotifers, but no difference can be found between C and B rotifers. A rotifers have the highest protein/lipid ratio and B the lowest ratio (Zie et al., 1996).
<table>
<thead>
<tr>
<th></th>
<th>Long term Selco® enrichment</th>
<th>Short term DHA-Selco® enrichment</th>
<th>Short term Protein Selco® enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng protein.ind⁻¹*</td>
<td>200 ± 31</td>
<td>163 ± 13</td>
<td>238 ± 44</td>
</tr>
<tr>
<td>ng protein.ind⁻¹**</td>
<td>117</td>
<td>100</td>
<td>165</td>
</tr>
<tr>
<td>protein/lipid*</td>
<td>3.7</td>
<td>2.3</td>
<td>2.6</td>
</tr>
<tr>
<td>protein/lipid**</td>
<td>2.2</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>ng DW.ind⁻¹</td>
<td>376 ± 20</td>
<td>331 ± 13</td>
<td>502 ± 33</td>
</tr>
</tbody>
</table>

*protein expressed as N x 6.25  **protein expressed as sum amino acids

Fig. 3.16. illustrates the range in amino acid content in individual rotifers. It is clear from this figure that for most amino acids rotifers are quite conservative even when they are exposed to starvation conditions.

Figure 3.16. Ranges in amino acid concentration for starved (lower value) and well-fed (higher value) rotifers (Makridis and Olsen, pers. comm.).
3.6.4. Harvesting/concentration and cold storage of rotifers

As explained earlier, the harvesting and concentrating of non-enriched rotifers should be performed in submerged filters (see 3.5.4.). Harvesting of enriched rotifers should be carried out with extreme care in order to prevent them sticking together in clumps. Especially when the enriched animals are concentrated before the washing, aeration can easily result in clumping. Instead of pouring enriched rotifers in a bucket it is therefore recommended to siphon them so as to avoid the interference of the air bubbles.

Rotifers that can not be fed immediately need to be stored at a cold temperature (4°C) in order to prevent the reduction of their nutritional quality. During a starvation period of one day at 25°C, rotifers can lose up to 26% of their body weight as a result of metabolic activity. Different culture and enrichment procedures also influence the effect of starvation. For example, the starvation of gut-enriched rotifers (i.e., rotifers boosted with oil emulsions, microparticulated diets or microalgae) immediately before feeding to the predator (indirect enrichment procedure, short term enrichment) results in a very fast loss of their fatty acid content, as the animals start to empty their guts after 20 to 30 min! After about 6 hours in the larval rearing tanks, the rotifer HUFA content may have dropped to 1/3 of its original level. Tissue enrichment (direct enrichment procedure, long term enrichment), on the other hand takes place during the rotifer culture, and allows a slow but steady increase in the fatty acid content of the rotifers. This reserve in fatty acids is thus more stable and less exposed to fast decrease by starvation.

3.7. Production and use of resting eggs

For the mass rearing of rotifers as larval food the amictic way of reproduction (see 3.3.) should be favored. However, when the interest is in production of resting eggs for use as a storable off-the-shelf product mixis needs to be induced. These resting eggs, also called cysts, are relatively large (their volume is almost 60% of that of a normal adult female, Fig. 3.17.), are ideal for storage and transport and can be used as inocula for mass cultures. Mass production of rotifers for cyst production is performed in batch cultures in concrete tanks (Hagiwara et al., 1995; Dhert et al., 1995) or resting eggs are collected from sediments in earthen ponds. Resting egg production can be induced by limiting the food supply or changing the temperature and/or salinity. Resting eggs will sink and need to be harvested from the bottom. In case a lot of waste is trapped at the bottom it is advised to replace the water by brine so that resting eggs will float and can be collected from the water surface. If the sediment on the bottom is too important, to collect the resting eggs the water needs to be replaced by brine and the resting eggs will come to the surface from where they can be harvested. Dry resting eggs can be stored for more than one year. When placed in seawater, rotifer cysts hatch in about 24 hours at 25°C under light conditions. Newly-hatched rotifers undergo asexual reproduction.
There are several advantages of using rotifer cysts to initiate mass cultures. The use of stock cultures is not required which considerably reduces labor cost and algal production costs. Moreover, the upscaling from stock culture to production unit can be considerably reduced by the use of larger numbers of cysts. The use of cysts is also highly recommended to prevent contamination. Cysts can easily be treated before hatching in order to ensure start cultures free from bacteria and ciliates. The resting eggs could be disinfected with heavy doses of antibiotics, so that the emerging rotifers are essentially bacteria free. The resting eggs can also resist short exposure to disinfectants such as NaOCl or glutaraldehyde.

Figure 3.17. Microscopic view of resting eggs (length 100-170 µm; a. at same magnification as two amictic females; b. at high magnification
3.8. Literature of interest


## Worksheets

### WORKSHEET 3.1. PREPARATION OF AN INDICATOR SOLUTION FOR DETERMINATION OF RESIDUAL CHLORINE

- make in two separate bottles, a KI and a starch solution of 3 g in 100 ml deionised water
- heat the starch solution until it becomes clear
- dissolve in the mean time the KI
- stock the two labelled bottles in the refrigerator
- to check the presence of chlorine, put a few drops of each solution in a small sample
- if your sample turns blue, chlorine is still present