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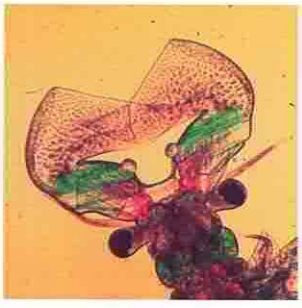

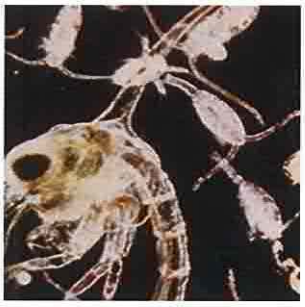

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4.2. Use of cysts

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4.2.1. Cyst biology

4.2.1.1. Cyst morphology

A schematic diagram of the ultrastructure of an *Artemia* cyst is given in Fig. 4.2.1.

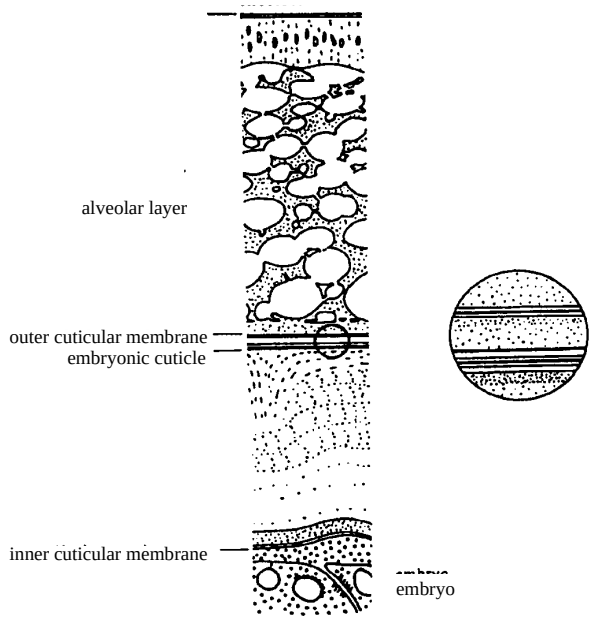

Figure 4.2.1. Schematic diagram of the ultrastructure of an *Artemia* cyst. (modified from Morris and Afzelius, 1967)

The cyst shell consists of three layers:

- alveolar layer: a hard layer consisting of lipoproteins impregnated with chitin and haematin; the haematin concentration determines the colour of the shell, *i.e.* from pale to dark brown. Its main function is to provide protection for the embryo against mechanical disruption and UV radiation. This layer can be completely removed (dissolved) by oxidation treatment with hypochlorite (= cyst decapsulation, see 4.2.3.).

- outer cuticular membrane: protects the embryo from penetration by molecules larger than the CO_2 molecule (= multilayer membrane with very special filter function; acts as a permeability barrier).

- embryonic cuticle: a transparent and highly elastic layer separated from the embryo by the inner cuticular membrane (develops into the hatching membrane during hatching incubation).

The embryo is an indifferentiated gastrula which is ametabolic at water levels below 10% and which can be stored for long periods without losing its viability. The viability is affected when cysts are stored at water levels higher than 10% (start of metabolic activity) and when cysts are exposed to oxygen; *i.e.* in the presence of oxygen cosmic radiation results in the formation of free radicals which destroy specific enzymatic systems in the ametabolic *Artemia* cysts.

4.2.1.2. Physiology of the hatching process

The development of an *Artemia* cyst from incubation in the hatching medium till nauplius release is shown in Fig. 4.2.2.

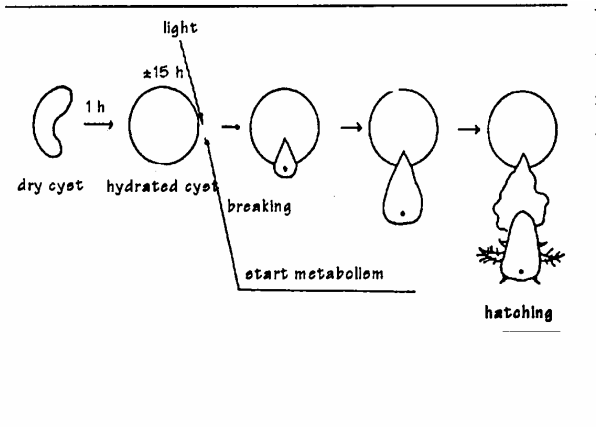

Figure 4.2.2. Development of an *Artemia* cyst from incubation in seawater until nauplius release.

When incubated in seawater the biconcave cyst swells up and becomes spherical within 1 to 2 h. After 1 2 to 20 h hydration, the cyst shell (including the outer cuticular membrane) bursts (= breaking stage) and the embryo surrounded by the hatching membrane becomes visible. The embryo then leaves the shell completely and hangs underneath the empty shell (the hatching membrane may still be attached to the shell). Through the transparent hatching membrane one can follow the differentiation of the pre-nauplius into the instar I nauplius which starts to move its appendages. Shortly thereafter the hatching membrane breaks open (= hatching) and the free-swimming larva (head first) is born.

Dry cysts are very hygroscopic and take up water at a fast rate (*i.e*. within the first hours the volume of the hydrated embryo increases to a maximum of 40% water content; Fig.4.2.3. However, the active metabolism starts from a 60% water content onwards, provided environmental conditions are favourable (see further).

The aerobic metabolism in the cyst embryo assures the conversion of the carbohydrate reserve trehalose into glycogen (as an energy source) and glycerol.

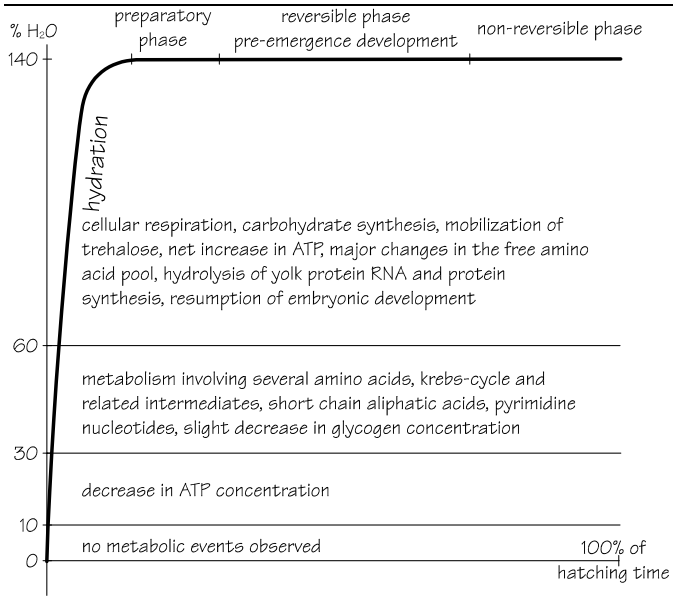

Figure 4.2.3. Cellular metabolism in *Artemia* cysts in function of hydration level.

Increased levels of the latter hygroscopic compound result in further water uptake by the embryo. Consequently, the osmotic pressure inside the outer cuticular membrane builds up continuously until a critical level is reached, which results in the breaking of the cyst envelope, at which moment all the glycerol produced is released in the hatching medium. In other words the metabolism in *Artemia* cysts prior to the breaking is a trehalose-glycerol hyperosmotic regulatory system. This means that as salinity levels in the incubation medium increase, higher concentrations of glycerol need to be built up in order to reach the critical difference in osmotic pressure which will result in the shell bursting, and less energy reserves will thus be left in the nauplius.

After breaking the embryo is in direct contact with the external medium through the hatching membrane. An efficient ionic osmoregulatory system is now in effect, which can cope with a big range of salinities, and the embryo differentiates into a moving nauplius larva. A hatching enzyme, secreted in the head region of the nauplius, weakens the hatching membrane and enables the nauplius to liberate itself into the hatching medium.

4.2.1.3. Effect of environmental conditions on cyst metabolism

Dry cysts (water content from 2 to 5 % ; see worksheet 4.2.1. for determination of water content and Table 4.2.6. for practical example) are very resistant to extreme temperatures; hatching viability not being affected in the temperature range -273C and above 60°C and up t 90°C only short exposures being tolerated.

Hydrated cysts have far more specific tolerances with mortalities occurring below -18°C and above +40°C; a reversible interruption of the metabolism (= viability not affected) occurring between -18°C and +4°C and between ± 33°C and ± 40°C, with the upper and lower temperature limits varying slightly from strain to strain. The active cyst metabolism is situated between +4°C and ± 33°C; the hatching percentage remains constant but the nauplii hatch earlier as the temperature is higher.

As for other environmental conditions, optimal hatching outputs are reached in the pH range 8-8.5. As a consequence, the addition of $NaHC_3$, up to 2 $g.l^{-1}$, to artificial or diluted seawater or to dense suspensions of cysts results in improved hatching. This might be related to the optimal pH activity range for the hatching enzyme.

An increased hatching has been reported with increasing oxygen level in the range 0. 6 and 2 ppm, and maximal hatching obtained above this concentration. To avoid oxygen gradients during hatching it is obvious that a good homogeneous mixing of the cysts in the incubation medium is required.

As stated above, hatching in a higher salinity medium will consume more of the energy reserves of the embryo. Above a threshold salinity (varying from strain to strain, ± 90 $g.l^{-1}$ for most strains) insufficient quantities of water can be taken up to support the embryo's metabolism. Optimal salinity for hatching is equally strain-specific, but generally situated in the range 15-70 $g.l^{-1}$.

Although the physiological role of light during the hatching process is poorly understood, brine shrimp cysts, when hydrated and in aerobic conditions, need a minimal light triggering for the onset of the hatching process, related to light intensity and/or exposure time.

As a result of the metabolic characteristics of hydrated cysts, a number of recommendations can be formulated with regard to their use. When cysts (both decapsulated and non-decapsulated) are stored for a long time, some precautions have to be taken in order to maintain maximal energy content and hatchability. Hatchability of cysts is largely determined by the conditions and techniques applied for harvesting, cleaning, drying and storing of the cyst material. The impact of most of these processes can be related to effects of dehydration or combined dehydration/hydration. For diapausing cysts, these factors may also interfere with the diapause induction/termination process, but for quiescent cysts, uncontrolled dehydration and hydration result in a significant drop of the viability of the embryos.

Hatching quality in stored cysts is slowly decreasing when cysts contain water levels from 10 to 35 % H_2O. This process may however be retarded when the cysts are stored at freezing temperatures. The exact optimal water level within the cyst (around 5 %) is not known, although there are indications that a too severe dehydration (down to 1-2 %) results in a drop in viability.

Water levels in the range 30-65 % initiate metabolic activities, eventually reducing the energy contents down to levels insufficient to reach the state of emergence under optimal hatching conditions. A depletion of the energy reserves is furthermore attained when the cysts undergo subsequent dehydration/hydration cycles. Long-term storage of such material may result in a substantial decrease of the hatching outcome. Cysts exposed for too long a period to water levels exceeding 65 % will have completed their pre-emergence embryonic development; subsequent dehydration of these cysts will in the worst case result in the killing of the differentiated embryos.

Sufficiently dehydrated cysts only keep their viability when stored under vacuum or in nitrogen; the presence of oxygen results in a substantial depletion of the hatching output through the formation of highly detrimental free radicals. Even properly packed cysts should be preferentially kept at low temperatures. However, when frozen, the cysts should be acclimated for one week at room temperature before hatching.

4.2.1.4. Diapause

As *Artemia* is an inhabitant of biotopes characterized by unstable environmental conditions, its survival during periods of extreme conditions (i.e. desiccation, extreme temperatures, high salinities) is ensured by the production of dormant embryos. *Artemia* females can indeed easily switch from live nauplii production (ovoviviparity) to cyst formation (oviparity) as a fast response to fluctuating circumstances. Although the basic mechanisms involved in this switch are not yet fully understood, sudden fluctuations seem to trigger oviparity (oxygen stress, salinity changes...). The triggering mechanism for the induction of the state of diapause is however not yet known. In principle, *Artemia* embryos released as cysts in the medium are in diapause and will not resume their development, even under favourable conditions, until they undergo some diapause deactivating environmental process; at this stage, the metabolic standstill is regulated by internal mechanisms and it can not be distinguished from a non-living embryo. Upon the interruption of diapause, cysts enter the stage of quiescence, meaning that metabolic activity can be resumed at the moment they are brought in favourable hatching conditions, eventually resulting in hatching: in this phase the metabolic arrest is uniquely dependent of external factors (Fig. 4.2.4.). As a result, synchronous hatching occurs, resulting in a fast start and consequent development of the population shortly after the re-establishment of favourable environmental conditions. This allows effective colonization in temporal biotopes.

For the user of *Artemia* cysts several techniques have proven successful in terminating diapause. It is important to note here that the sensitivity of *Artemia* cysts to these techniques shows strain- or even batch-specificity, hence the difficulty to predict the effect on hatching outcome. When working with new or relatively unknown strains, the relative success or failure of certain methods has to be found out empirically.

In many cases the removal of cyst water is an efficient way to terminate the state of diapause. This can be achieved by drying the cysts at temperatures not exceeding 35-4°C or by suspending the cysts in a saturated NaCl brine solution (300 $g.l^{-1}$). As some form of dehydration is part of most processing and/or storage procedures, diapause termination does not require any particular extra manipulation. Nevertheless, with some strains of *Artemia* cysts the usual cyst processing techniques does not yield a sufficiently high hatching quality, indicating that a more specific diapause deactivation method is necessary.

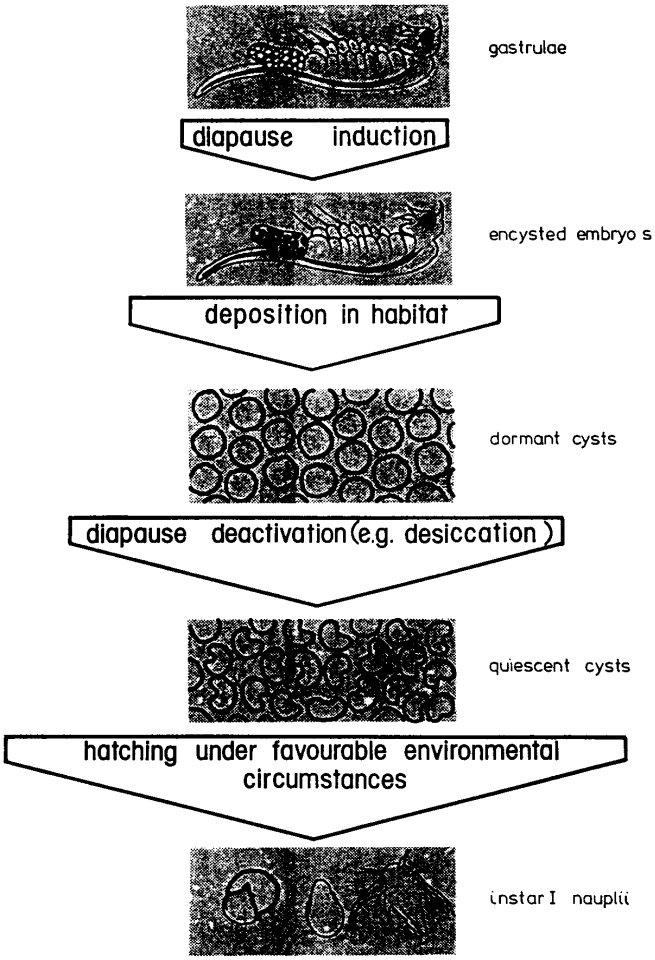

Figure 4.2.4. Schematic diagram explaining the specific terminology used in relation with dormancy of *Artemia* embryos.

Table 4.2.1. Effect of cold storage at different temperatures on the hatchability of shelf dried *Artemia* cysts from Kazakhstan

	storage temperature		
storage time	+4°C	-25°C	-80°C
0 days	7	7	7
2 weeks	-	-	4
1 month	7	16	12
2 months	27	44	50

Hatchability is expressed as hatching percentage

The following procedures have proven to be successful when applied with specific sources of *Artemia* cysts (see worksheet 4.2.2.):

- freezing: "imitates" the natural hibernation period of cysts originating from continental biotopes with low winter temperatures (Great Salt Lake, Utah, USA; continental Asia; Table 4.2.1.);

- incubation in a hydrogen peroxide (H_2O_2) solution. In most cases, the sensitivity of the strain (or batch) to this product is difficult to predict, and preliminary tests are needed to provide information about the optimal dose/period to be applied, and about the maximal effect that can be obtained (Table 4.2.2.). Overdosing results in reduced hatching or even complete mortality as a result of the toxicity of the chemical. However, in some cases no effect at all is observed.

In general other diapause termination techniques (cyclic dehydration/hydration, decapsulation, other chemicals...) give rather erratic results and/or are not user-friendly. One should, however, keep in mind that the increase in hatching percentage after any procedure might (even partially) be the result of a shift in hatching rate (earlier hatching).

Table 4.2.2. Dose-time effect of H_2O_2 preincubation treatment on the hatchability of *Artemia* cysts from Vung Tau (Viet Nam)

Time	Doses(%)					
(min.)	0.5	1	2	3	5	10
1					46	10
2					94	5
5			54	69	102	
10	47		90	81	88	32
15		46	100	76		
20			91	94	52	
30		91	95			
60	56	85		6	1	
120		15				
180	47					

Data are expressed as percentage of hatching results obtained at 2%/15 min. treatment (74% hatch)

4.2.2. Disinfection procedures

A major problem in the early rearing of marine fish and shrimp is the susceptibility of the larvae to microbial infections. It is believed that the live food can be an important source of potentially pathogenic bacteria, which are easily transferred through the food chain to the predator larvae. *Vibrio sp*. constitute the main bacterial flora in *Artemia* cyst hatching solutions. Most *Vibrio* are opportunistic bacteria which can cause disease/mortality outbreaks in larval rearing, especially when fish are stressed or not reared under optimal conditions. As shown on Fig. 4.2.5., *Artemia* cyst shells may be loaded with bacteria, fungi, and even contaminated with organic impurities;
bacterial contamination in the hatching medium can reach numbers of more than 10^7 $CFU.ml^{-1}$ (= colony forming units). At high cyst densities and high incubation temperatures during hatching, bacterial development (*e.g.* on the released glycerol) can be considerable and hatching solutions may become turbid, which may also result in reduced hatching yields. Therefore, if no commercially disinfected cysts are used, it is recommended to apply routinely a disinfection procedure by using hypochlorite (see worksheet 4.2.3.). This treatment, however, may not kill all germs present in the alveolar and cortical layer of the outer shell. Complete sterilization can be achieved through cyst decapsulation, described in the following chapter.