A training manual on *Artemia* cyst hatching and decapsulation

Introducing circularity through climate-smart aquaculture in Bangladesh

Funded by the European Union

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Introducing circulatory through climate-smart aquaculture in Bangladesh (Artemia4Bangladesh)

A training manual on *Artemia* cyst hatching and decapsulation

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List of acronyms

%	Percentage
°C	Degree Centigrade
µl	Micro Litre
µm	Micro Meter
AAS	ascorbic acid 2-sulfate
am	Ante Meridiem
CFU	Colony Forming Unit
cm	Centimeter
DHA	Docosahexaenoic Acid
DO	Dissolved Oxygen
e.g.	Exempli Gratia
EPA	Eicosapentaenoic Acid
etc	et cetera
EU	European Union
FAA	Free amino acid
FAO	Food and Agriculture Organization of the United Nations
g	Gram
g/L	Gram Per Litre
h	Hour
HE	Hatching efficiency
HR	Hatching rate
i.e.	In Extensu
IU	International Unit
Kg	kilogram
L	Litre
mg	Milligram
mg/L	Milligram Per Litre
ml	Milliliter
mm	Millimeter
MT	Metric tonnes
pm	Post Meridiem
ppm	Parts Per Million
TCBS	Thiosulfate Citrate Bile Salts Sucrose Agar
UV	Ultraviolet
W	Watt
w/w	Weight Per Weight
ww	Wet Weight

1. Introduction

Artemia nauplii is an excellent live food source to fulfill the nutritional requirement in larviculture of crustaceans and marine fish due to its high digestible protein content, essential fatty acid and small in size (approximately 450 µm) (Sorgeloos et al., 1998, 2001). *Artemia* is mainly marketed worldwide as encapsulated cysts which allow it for long term storage, maintain the quality and are ready to hatch whenever necessary. Globally, annual *Artemia* cyst consumption increased from less than 100 metric tonnes (MT) in the 1980s to over 3,000 MT in recent years harvested mainly from natural salt lakes. This tremendous increase in demand followed by price hike of *Artemia* cyst in the international market caused limited application in quantity during larviculture in crustacean and marine fish hatcheries. In many countries, hatchery workers lack the sufficient knowledge and skill in standardization of the application of *Artemia* nauplii during larval rearing. This resulted in increased cost, disease incidence, reduced survival in larval rearing.

Several countries including Thailand, Vietnam are successful in commercial *Artemia* biomass production in the coastal salt farms (Anh et al., 1997, Montakin personal communication) to promote socio economics of thousands of salt farmers. In recent years, Vietnamese aquaculture activities are expanding with new aquaculture species such as mud crab, mud skipper, marine snails with the support of locally produced *Artemia* cyst and biomass (Hoa and Hong, 2019).

In Bangladesh, about hundred shrimp *(Penaeus monodon)* and prawn *(Macrobra chiumrosenbergii)* hatcheries produce more than 10 billion post larvae per year (DoF, 2019) with annual consumption of approximately 40 MT imported *Artemia* cyst. The objectives of the training manual on *Artemia* hatching and decapsulation are to enhance knowledge and skill of the aquaculture professionals to optimize the use of *Artemia* nauplii in the larval rearing. In addition, the standardized hatching procedure will be used to inoculate *Artemia* nauplii for the culture in the salt farms in Bangladesh.

2. Standard hatching procedure of *Artemia* cyst

2.1 Hatching conditions and equipment

The parameters need to be taken into consideration are aeration, water temperature, salinity, pH, cyst and illumination (Lavens and Sorgeloos, 1996) for the successful hatching of *Artemia* cysts.

2.1.1 Hatching tanks and water

Use tanks with a conical base to improves mixing of the tank and separation of the hatched nauplii. Transparent or translucent containers will facilitate inspection of the hatching suspension, especially when harvesting. Sterilise tanks, airlines and airstones with a 200m g/L chlorine solution and rinse thoroughly. Use sterilized sea water to fill *Artemia* hatching tank to the desired volume (Figure 1).

Figure 1: Treated seawater filling in the *Artemia* hatching tank

2.1.2 Aeration

The aeration intensity must be sufficient to maintain oxygen levels above 2 mg/L, preferentially 5 mg/L. The optimal aeration rate is a function of the tank size and the density of cysts incubated. Excessive foaming can be reduced by disinfection of the cysts prior to hatching incubation and/or by the addition of a few drops of a non-toxic antifoaming agent (e.g. silicone antifoam).

2.1.3 Water temperature

The temperature of the seawater is preferentially kept in the range of 25-28°C; below 25°C cysts hatch more slowly and above 33°C the cyst metabolism is irreversibly stopped. However, the effect of more extreme temperatures on the hatching output is largely strain specific. For routine operation, it is most efficient to work in standardized conditions (i.e. heaters with thermostat or climatized room to ensure constant temperature) to allow maximal production of a homogeneous instar I population after a fixed incubation time (Figure 2).

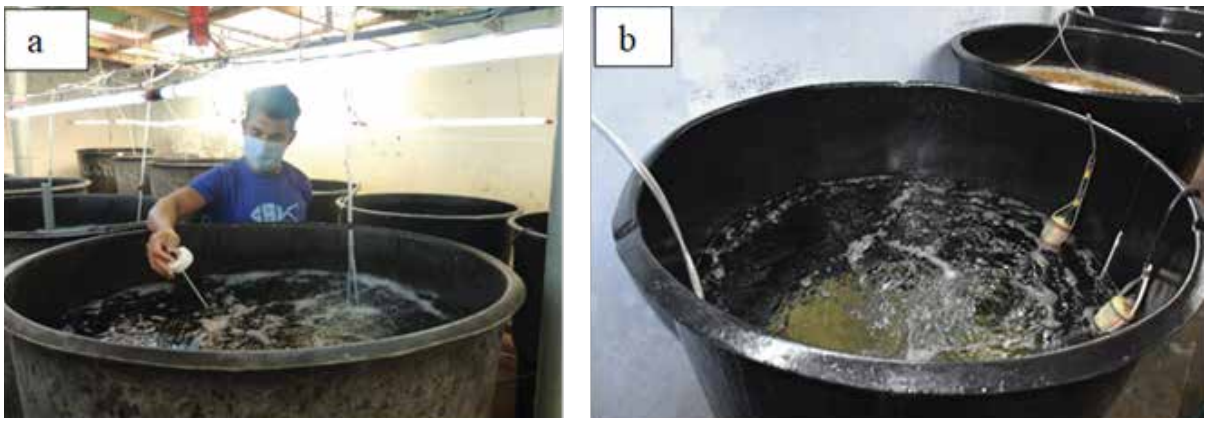

Figure 2: Maintain optimum water temperature in the hatching tank (a) check water temperature (b) theromostat to stable water temperature

2.1.4 Salinity

The fastest hatching rates will be at the lowest salinity levels since it will take less time to reach breaking point of cysts. Optimal hatching can be obtained in the salinity range 5-35 g/L. For reasons of practical convenience natural seawater is mostly used to hatch cysts. However, at 5 g/L salinity, the nauplii hatch faster, as less glycerol has to be built up. For some sources of cysts hatching the cysts at low salinity results in higher hatching efficiencies, and the nauplii have a higher energy content. The salinity can easily be measured by means of a refractometer.

2.1.5 pH

The pH must remain above 8 during the hatching process (Figure 3) so as to ensure optimal functioning of the hatching enzyme. If needed, (i.e. when low salinity water is used), the buffer capacity of the water should be increased by adding up to 1 g $NaHCO_3$/L. Increased buffer capacities can also become essential when high densities of cysts are hatched (= high CO_2 production).

Figure 3: Measuring water pH

2.1.6 Cyst surface sterilisation

Add the required amount of dry cysts to 1 - 2 litres of seawater and allow to hydrate for 30 minutes (Figure 4). Add 2-4 g of calcium hypochlorite, or 4-8ml of liquid bleach and aerate strongly for 15 minutes (worksheet 1). Collect cysts in a sieve (approximately 120µm) and rinse thoroughly in clean seawater or freshwater.

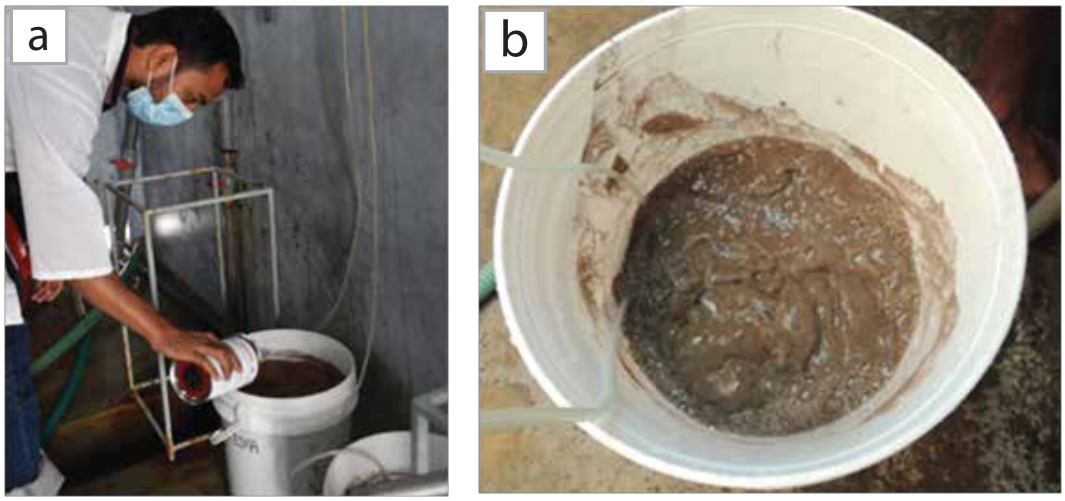

Figure 4: (a) Hydration of cyst (b) cyst surface disinfection with bleach

2.1.7 Cyst density

Cyst density may also interfere with the other abiotic factors that are essential for hatching, such as pH, oxygen, and illumination. The density may be as high as 5 g/L for small volumes (<20 L) but should be decreased to maximum 2 g/L for larger volumes, so as to minimize the mechanical injury of the nauplii and to avoid suboptimal water conditions (Figure 5).

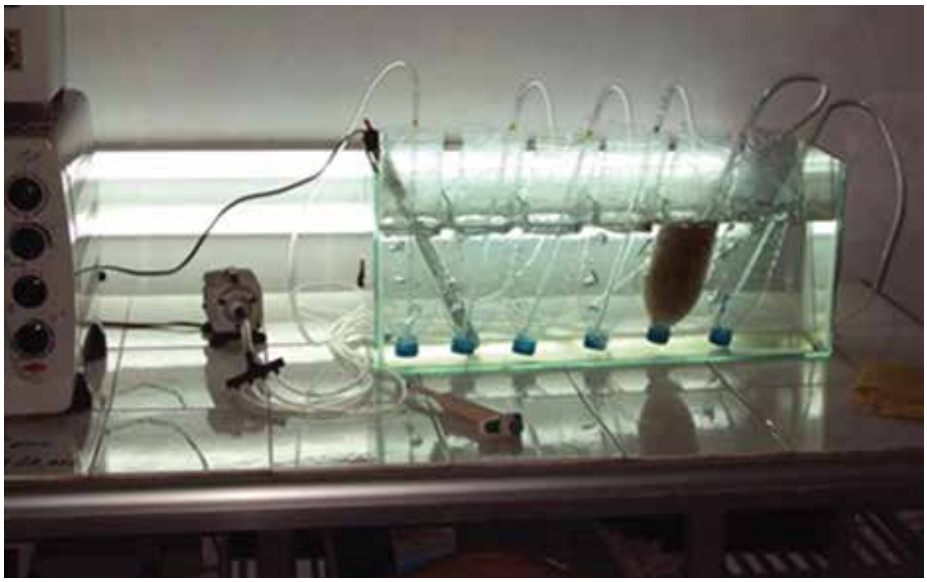

Figure 5: *Artemia* cyst incubation in the laboratory

2.1.8 Illumination

Strong illumination (about 2000 lux at the water surface) is essential (Figure 6), at least during the first hours after complete hydration, in order to trigger/start embryonic development. Although this level of illumination is mostly attained during daytime in transparent tanks that are set up outdoors in the shade, it is advisory to keep the hatching tanks indoors and to provide artificial illumination so as to ensure good standardisation of the hatching process.

Figure 6: Sufficient illumination in the hatching tank

2.2 Hatching quality and evaluation

An acceptable cyst product should contain minimal quantities of impurities, such as sand, cracked shells, feathers, and salt crystals, etc. Hatching synchrony must be high; when incubated in 33 g/L seawater at 25°C, the first nauplii should appear after 12 to 16 h incubation (T0; see further) and the last nauplii should have hatched within 8 h thereafter (T100). When hatching synchrony is low (T100-T0 > 10 h), first-hatched nauplii will have consumed much of their energy reserves by the time that the last nauplii will have hatched and harvesting is completed (Figure 7). Moreover, since the total incubation period exceeds 24 the aquaculturist will not be able to restock the same hatching containers for the next day'sharvest, which in turn implies higher infrastructural costs. The hatching efficiency (the number of nauplii hatched per g of cysts) and hatching percentage (the total percentage of the cysts that actually hatch) often varies considerably between different commercial batches and obviously account for much of the price differences. In this respect, hatching efficiency

may be a better criterion than hatching percentage as it also takes into account the content of impurities (i.e.. empty cyst shells). Hatching values may be as low as 100,000 nauplii g/L of commercial cyst product, while premium quality cysts from Great Salt Lake yield 270,000 nauplii per g of cysts (with an equivalent hatching percentage of >90 %); batches of small (=lighter) cysts (e.g. SFB type) may yield even higher numbers of nauplii, (i.e. 320,000 nauplii/g cysts).

Figure 7: Microscopic observation of *Artemia* nauplii to determine hatching quality

To evaluate the hatching quality of a cyst product, the following criteria are being used (see worksheet 2 and 3, for practical examples, see Tables 1 and 2):

2.2.1 Hatching percentage

It refers number of nauplii that can be produced under standard hatching conditions from 100 full cysts; this criterion does not take into account cyst impurities, (i.e. cracked shells,s and, salt, etc.), and refers only to the hatching capacity of the full cysts, which in turn depends upon:

a) degree of diapause termination: cysts that are still in diapause do not hatch, even under favourable hatching conditions
b) energy content of cysts: may be too low to build up sufficient levels of glycerol to enable breaking and hatching, as a consequence of, for example, improper processing and/or storage, environmental or genotypical conditions affecting parental generation...
c) amount of dead/non-viable/abortic embryos, due to improper processing and/orstorage.

2.2.2 Hatching efficiency

It specify the number of nauplii that can be produced from 1 g dry cyst product under standard hatching conditions. This criterion reflects:

a) the hatching percentage (see above)
b) the presence of other components apart from full cysts in the cyst product (i.e. empty shells, salt, sand, water content of the cysts)
c) the individual cyst weight (i.e. more cysts/g for smaller strains)

As this criterion can refer to the ready-to-use commercial product, it has very practical implications, since the price of the product can be directly related to its output.

2.2.3 Hatching rate

This criterion refers to the time period for full hatching from the start of incubation (= hydration of cysts) until nauplius release (hatching), and considers a number of time intervals, including:

T_0 = incubation time until appearance of first free-swimming nauplii

T_{10}= incubation time until appearance of 10% of total hatchablenauplii, etc. (Figure 8).

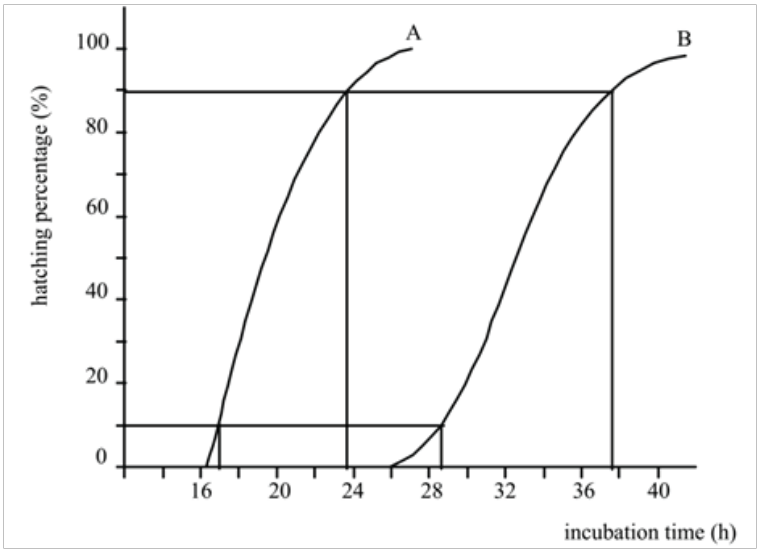

Figure 8: Hatching rate curves from different cyst batches. Curve A: T10= 17 h, T90 = 23.5 h, Ts = 6.5 h; Curve B: T10 = 28.5 h, T90 = 37.5 h, Ts = 9 h

Data on the hatching rate allow the calculation of the optimal incubation period so as to harvest nauplii containing the highest energy content (Figure 9). It is important that the T90 is reached within 24 h; if not more hatching tanks will be needed so as to ensure a daily supply of a maximal number of instar I nauplii.

Instar I

Instar II

Instar I

Ash free dry weight - 24%
Individual calorie content - 27%
Lipid content - 27%

Instar II

Figure 9: Changes in nutritional content of *Artemia* nauplii molt from Instar I to Instar II

2.2.4 Hatching synchrony

The time lapse during which most nauplii hatch, i.e. Ts = T90-T10

A high hatching synchrony ensures a maximal number of instar I nauplii available within ashort time span; in case of poor synchrony the same hatching tank needs to beharvested several times in order to avoid a mixed instar I-II-III population whenharvesting at T90.

2.2.5 Hatching output

= dry weight biomass of nauplii that can be produced from 1 g dry cyst product incubated under standard hatching conditions; best products yield about 600 mg nauplii g/L cysts.The calculation is made as follows:

= hatching efficiency x individual dry weight of instar I nauplius. The hatching efficiency only accounts for the number of nauplii that are produced, and not for the size of these nauplii (strain dependent); by contrast the hatching output criterion is related to the total amount of food available for the predator per g of cyst product (cf. calculation of food conversion)

2.3 Harvesting of nauplii

As a consequence of specific characteristics, the interactions of the hatching parameters might be slightly different from strain to strain, resulting in variable hatching results.

After hatching and prior to feeding the nauplii to fish/crustacean larvae, they should be separated from the hatching wastes (empty cyst shells, unhatched cysts, debris, microorganisms and hatching metabolites). Five to ten minutes after switching off the aeration, cyst shells will float and can be removed from the surface, while nauplii and unhatched cysts will concentrate at the bottom (Figure 10).

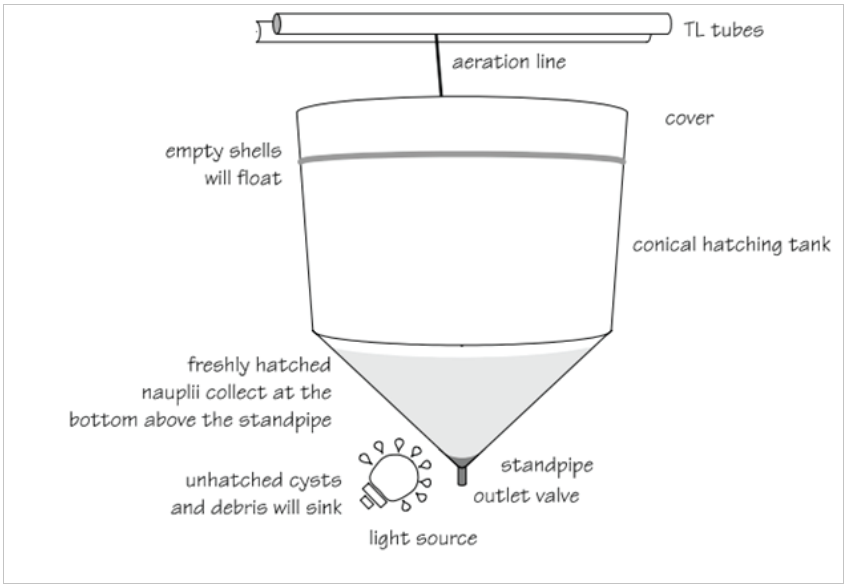

Figure 10: *Artemia* cyst hatching container

Since nauplii are positively phototactic, their concentration can be improved by shading the upper part of the hatching tank (use of cover) and focusing a light source on the transparent conical part of the bottom. Nauplii should not be allowed to settle for too long (i.e., maximum 5 to 10 minutes) in the point of the conical container, to prevent dying off due to oxygen depletion. Firstly, unhatched cysts and other debris that have accumulated underneath the nauplii are siphoned or drained off when necessary (i.e. when using cysts of a lower hatching quality). Then the nauplii are collected on a filter using a fine mesh screen (<150 µm), which should be submerged all the time so as to prevent physical damage of the nauplii (Figure 11).

Figure 11: (a) Harvesting *Artemia* nauplii, (b) washing nauplii with sterilized sea water

They are then rinsed thoroughly with water in order to remove possible contaminants and hatching metabolites like glycerol. Installation of automated systems simplify production techniques in commercial operations, (i.e. by the use of a concentrator/rinser equipped with a stainless steel cross-flow sieve; Figure 12) that enables fast harvesting of large volumes of *Artemia* nauplii and allows complete removal of debris from the hatching medium. This technique results in a significant reduction of labour and productioncosts.

Figure 12: Nauplii concentrator/rinser in use

As live food is suspected to be a source of bacterial infections eventually causing disease problems in larval rearing, microbial contamination should be kept to a minimum.

At the moment of cyst breaking glycerol is released in the hatching water. This carbohydrate is a suitable substrate for bacterial development, and as a result bacterial numbers, especially Vibrio spp. increase by 103 to 105 compared to the initial population before the breaking of the cysts.

Harvesting *Artemia* nauplii at Instar I are preferable considering most nutritious and can withstand sudden salinity shocks when transferred into the salt ponds for inoculation purposes. When nauplii molt into instar II stage their mouth is open and they start ingesting bacteria that are present in the hatching medium and thus can become carriers of opportunistic Vibrio pathogens. When harvesting instar I nauplii this bacterial contamination can be removed by thorough washing.

Bacterial development during the hatching incubation can be suppressed by the addition of commercial disinfection products. Still one should aim to harvest *Artemia* in its most nutritious and biosecure stage as instar I nauplii. When stored at room temperature second-instar meta-nauplii have already consumed 25 to 30% of their energy reserves within 24 h after hatching (Figure 13). Moreover, instar II *Artemia* are less visible as they are transparent, are larger and swim faster than first instar larvae, and as a result consequently are less accessible as a prey. Furthermore, they contain lower amounts of free amino acids, and their lower individual organic dry weight and energy content will reduce the energy uptake by the predator per hunting effort. All this may be reflected in a reduced growth of the larvae, and an increased *Artemia* cyst bill as about 20 to 30% more cysts will be needed to be hatched to feed the same weight of starved meta-nauplii to the predator.When harvested instar I nauplii cannot be offered immediately as live food they should be stored at low temperature as to slow down their metabolic activity and consequent loss in nutritional value.

Molting of the *Artemia* nauplii to the second instar stage may be avoided and their energy metabolism greatly reduced (Figure 13 and 14) by storage of the freshly-hatched nauplii at a temperature below 10°C in densities of up to 8 million per liter. Only a slight aeration is needed in order to prevent the nauplii from accumulating at the bottom of the tank where they would suffocate. In this way nauplii can be stored for periods up to more than 24 h without significant mortalities and a reduction of energy of less than 5%. Applying 24 h cold storage using styrofoam insulated tanks and blue ice packs or ice packed in closed plastic bags for cooling, commercial hatcheries are able to economize their *Artemia* cyst hatching efforts (i.e., reduction of the number of hatchings and harvests daily, fewer tanks, bigger volumes). Furthermore, cold storage allows the farmer to consider more frequent and even automated food distributions of an optimal live food. This appeared to be beneficial for fish and shrimp larvae as food retention times in the larviculture tanks can be reduced and hence growth of the *Artemia* in the culture tank can be minimized. For example, applying one or maximum two feedings per day, shrimp farmers often experienced juvenile *Artemia* in their larviculture tanks competing with the shrimp postlarvae for the algae.

With poor hunters such as the larvae of turbot *Scophthalmus maximus* and tiger shrimp *P. monodon*, feeding cold-stored, less active *Artemia* furthermore results in much more efficient food uptake.

An important factor affecting the nutritional value of *Artemia* as a food source for marine larval organisms is the content of essential fatty acids, eicosapentaenoic acid (EPA: 20:5n-3) and even more importantly docosahexaenoic acid (DHA: 22:6n-3). In contrast to freshwater species, most marine organisms do not have the capacity to biosynthesize these EFA from lower chain unsaturated fatty acids, such as linolenic acid (18:3n-3). In view of the fatty acid deficiency of *Artemia*, research has been conducted to improve its lipid composition by prefeeding with (n-3) highly unsaturated fatty acid (HUFA) rich diets. It is fortunate in this respect that *Artemia*, because of its primitive feeding characteristics, allows a very convenient way to manipulate its biochemical composition. Thus, since *Artemia* on molting to the second larval stage (i.e. about 8 h following hatching), is non-selective in taking up particulate matter, simple methods have been developed to incorporate lipid products into the brine shrimp nauplii prior to offering them as a prey to the predator larvae. This method of bioencapsulation, also called *Artemia* enrichment or boosting (Figure 15), is widely applied at marine fish and crustacean hatcheries all over the world for enhancing the nutritional value of *Artemia* with essential fattyacids. Different commercial self-emulsifying products are available for application with specific species.

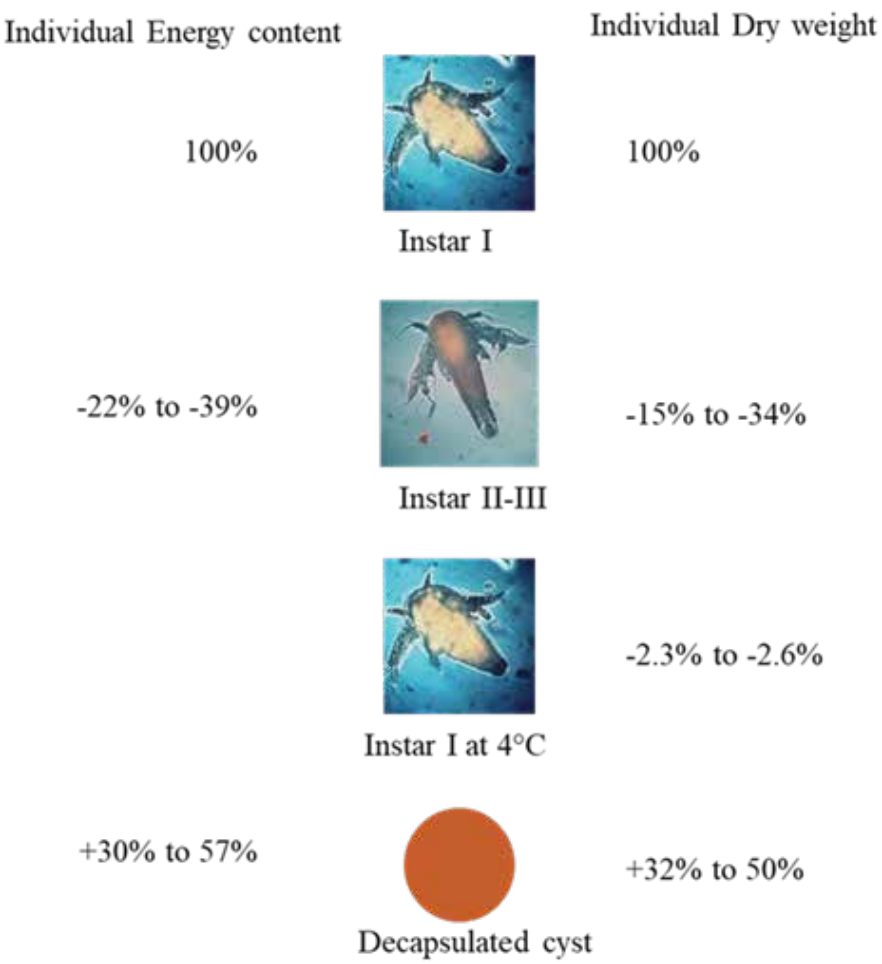

Figure 13: Change in energy and dry weight of different forms of *Artemia* (newly hatched instar I nauplii are considered to have 100 % values for those variables). The % decrease or increase is shown for Instar I, Instar II-III meta-nauplii, Instar I naupli in store

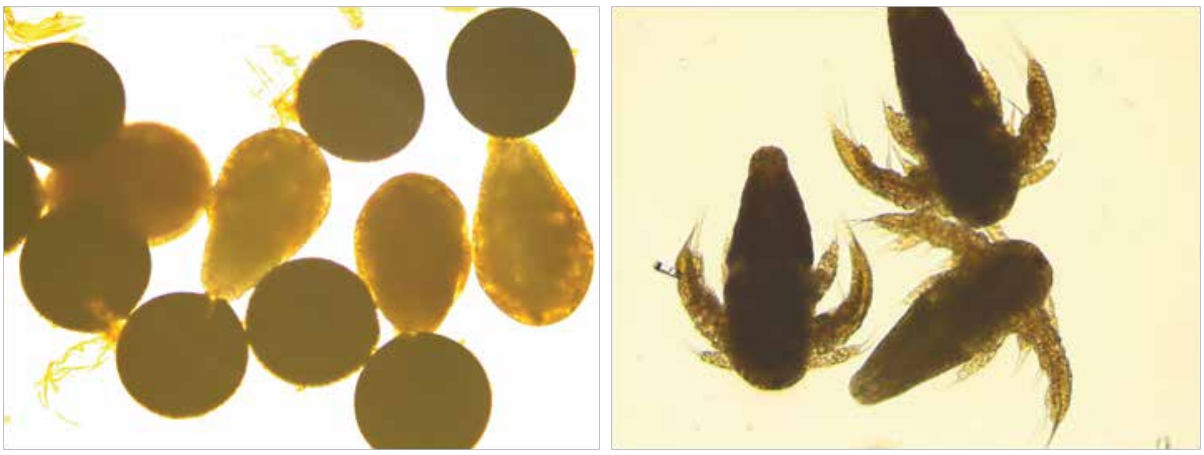

Figure 14: (a) *Artemia* nauplii at umbrella stage (b) Instar I *Artemia* nauplii under microscope

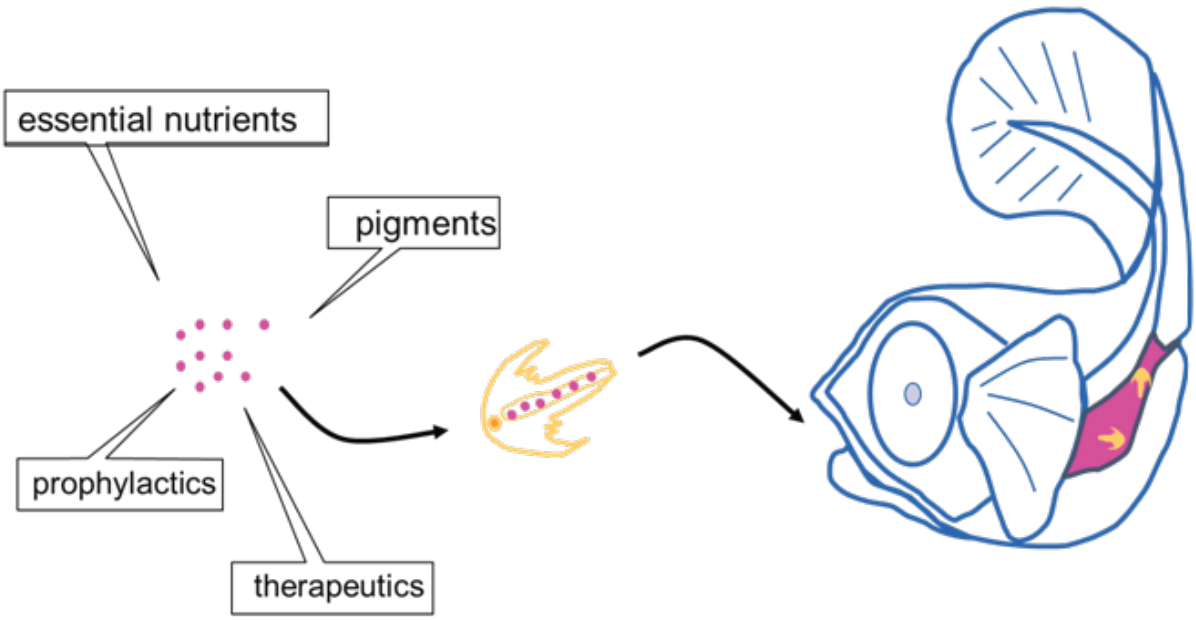

Figure 15: Schematic diagram of the use of *Artemia* as vector for transfer of specific components into the cultured larvae

3. *Artemia* cyst decapsulation

The hard shell that encysts the dormant *Artemia* embryo can be completely removed by short-term exposure to a hypochlorite solution. This procedure is called decapsulation. Decapsulated cysts offer a number of advantages compared to the non-decapsulated ones:

- Cyst shells are not introduced into the culture tanks. When hatching normal cysts, the complete separation of *Artemia* nauplii from their shells is not always possible. Unhatched cysts and empty shells can cause deleterious effects in the larval tanks when they are ingested by the predator: they cannot be digested and may obstruct thegut.
- Nauplii that are hatched out of decapsulated cysts have a higher energy content and individual weight (30-55 % depending on strain) than regular instar I nauplii, because they do not spend energy necessary to break out of the shell .
- Decapsulated cysts can be used as a direct energy-rich food source for fish and shrimp (Table 3)

The decapsulation procedure involves the hydration of the cysts (as complete removal of the envelope can only be performed when the cysts are spherical), removal of the brown shell in a hypochlorite solution (Figure 16), and washing and deactivation of the remaining hypochlorite (see worksheets 4 and 5). These decapsulated cysts can be directly hatched into nauplii, or dehydrated in saturated brine and stored for later hatching or for direct feeding. They can be stored for a few days in the refrigerator at 0-4°C without a decrease in hatching. If storage for prolonged periods is needed (weeks or few months), the decapsulated cysts can be transferred into a saturated brine solution. During overnight dehydration (with aeration to maintain a homogeneous suspension) cysts usually release over 80% of their cellular water, and upon interruption of the aeration, the now coffee-bean shaped decapsulated cysts settle out. After harvesting of these cysts on a mesh screen they should be stored cooled in fresh brine. Moreover, since they lose their hatchability when exposed to UV light it is advised to store them protected from direct sunlight.

The direct use of *Artemia* cysts, in its decapsulated form, is much more limited in larviculture of fish and shrimp, compared to the use of *Artemia* nauplii. Nevertheless, dried decapsulated *Artemia* cysts have proven to be an appropriate feed for larval rearing of various species like the freshwater catfish (*Clarias gariepinus*) and carp (*Cyprinus carpio*), marine shrimp and milkfish larvae. The use of decapsulated cysts in larval rearing presents some distinct advantages, both from a practical and nutritional point of view.

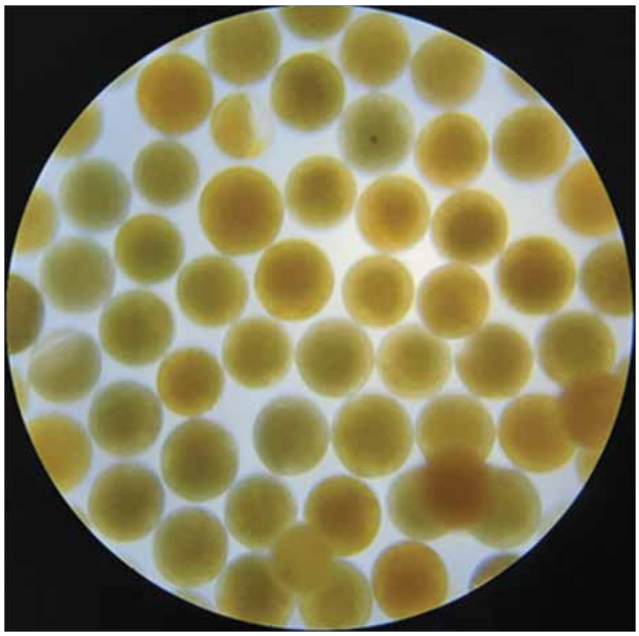

Figure 16: Decapsulated *Artemia* cyst under microscope

The daily production of nauplii is labour intensive and requires additional facilities. Furthermore, *Artemia* cysts of a high hatching quality are often expensive, and decapsulation of non-hatching cysts means valorization of an otherwise inferior product. The cysts have the appearance and the practical advantages of a dry feed and, in contrast to *Artemia* nauplii (470-550 µm), their small particle size (200-250 µm) is more suitable for small predator stages. If they have been dried before application, they have a high floating capacity, and sink only slowly to the bottom of the culture tank. Leaching of nutritional components (for example, with artificial diets) does not occur, since the outer cuticular membrane acts as a barrier for larger molecules).

On the other hand, a possible major drawback of decapsulated cysts is their immobility, and thus low visual attractivity for the predator. Moreover, decapsulated cysts dehydrated in brine sink rapidly to the bottom, thus reducing their availability for fish larvae feeding in the water column. Extra aeration or drying is therefore needed to keep these particles better in suspension. However, on the contrary, older penaeid larvae are mainly bottom feeders and so do not encounter this problem.

From the nutritional point of view, the gross chemical composition of decapsulated cysts is comparable to freshly-hatched nauplii (Table 3). In addition, their individual dry weight and energy content is on the average 30 to 40 % higher than for instar I nauplii. For example, for the culture of carp larvae during the first two weeks, the use of decapsulated cysts constitutes a saving of over one third in the amount of *Artemia* cysts used, compared to the use of live nauplii.

Furthermore, some differences are found for specific nutrients/components which may have an effect on their nutritional quality.

- Fatty acids: the fatty acid spectrum of cysts and nauplii is nearly identical, although differences can be found in lipid levels, FAME levels, fatty acid composition and energy content of different strains.
- Free amino acids: the ratio of free amino acids (FAA) to protein content is generally higher for instar I nauplii, compared to cysts, although large variations may exist from strain to strain. This may have dietary consequences when decapsulated cysts are used, since marine fish larvae use their large pool of free amino acids as an energy substrate during the first days after hatching.
- Vitamin C (ascorbic acid) is considered as an essential nutrient during larviculture. It is found as ascorbic acid 2-sulfate (AAS) in cysts of brine shrimp, a very stable form but with low bio-availability. During the hatching process the AAS is hydrolyzed into free ascorbic acid, a more unstable form, but directly available in the nauplii for the predator. Decapsulation of cysts does not lead to ascorbic sulfate hydrolysis. Resorption and biological activity of AAS in the predator's tissue is still subject of research, and although several freshwater fish have been grown successfully with decapsulated cysts in the larval phase (see above), one can state that feeding decapsulated cysts to larval fish for a prolonged time might lead to vitamin C deficiency in the case that the predator is lacking the sulfatase enzyme needed to break down AAS. 118
- Carotenoids: the carotenoid pattern, and more specifically the canthaxanthin contents, show qualitative differences between cysts and nauplii. In *Artemia* cysts the unusual cisconfiguration is found, whereas in developing nauplii it is converted into the more stable trans-canthaxanthin.

4. Worksheets

Worksheet 1: Disinfection of *Artemia* cysts with liquid bleach

- Prepare 200 ppm hypochlorite solution: ± 20 ml liquid bleach (NaOCl))/10 L (see cyst decapsulation)
- Soak cysts for 30 min. at a density of ± 50 g cysts/L
- Wash cysts thoroughly with tap water on a 125 µm screen
- Cysts are ready for hatching incubation

Worksheet 2: Counting of *Artemia* nauplii

- Count Artemia nauplii by taking sub samples, preferably with a micropipette, from a well mixed batch of nauplii.
- Take at least 5 samples of 100µl (depending on the density of the sample, take a smaller sample volume from a dense collection of larvae, and a larger sample from a dilute collection to count at least 40 nauplii per sample)
- Add iodine or formalin to stop swimming activity and count under low power magnification.
- The formula to calculate the number of Artemia nauplii per litre = Average number of Artemia nauplii per 100 µl x 10 x 1000

Worksheet 3: Determination of hatching percentage, hatching efficiency and hatching rate

- Incubate exactly 1.6 g of cysts in exactly 800 ml 33 g/L seawater under continuous illumination (2000 lux) at 28°C in a cylindroconical tube (preferentially) or in a graduated cylinder; provide aeration from bottom as to keep all cysts in suspension (aeration not too strong as to prevent foaming); run test in triplicate.
- After 24 h incubation take 6 subsamples of 250 µl out of each cone.
- Pipet each subsample into a small vial and fixate nauplii by adding a few drops of lugol solution.
- Per cone (i = 6 subsamples), count nauplii (ni) under a dissection microscope and calculate the mean value (N), count umbrellas (ui) and calculate mean value (U).
- Decapsulate unhatched cysts and dissolve empty cyst shells by adding one drop of NaOH solution (40 g/100 ml distilled water) and 5 drops of domestic bleach solution (5.25% NaOCl) to each vial.
- Per cone (i = 6), count unhatched (orange colored) embryos (ei) and calculate mean value (E).
- Hatching percentage H% = (N x 100).(N + U + E)-1
- calculate H% value per cone and calculate mean value and standard deviation of 3 cones = final value
- Hatching efficiency HE = (N x 4 x 800).(1.6)-1 or HE = N x 2000* (*conversion factor to calculate for number of nauplii per g of incubated cysts) calculate HE value per cone and calculate mean value and standard deviation of 3 cones = final value

- Eventually leave hatching tubes for another 24 h, take subsamples again and calculate H% and HE for 48 h incubation.
- Hatching rate (HR): start taking subsamples and calculating HE from 12 h incubation in seawater onwards (follow procedure above). Continue sampling/counting procedures until mean value for HE remains constant for 3 consecutive h. The mean values per hour are then expressed as percentage of this maximal HE. A hatching curve can be plotted and T10, T90 etc. extrapolated from the graph. A simplified procedure consists in sample taking e.g. every 3 or more h.

Worksheet 4: Procedures for the decapsulation of *Artemia* cysts

Hydration step

- Hydrate cysts by placing them for 1 h in water (< 100 g/L), with aeration, at 25°C.

Decapsulation step

- Collect cysts on a 125 µm mesh sieve, rinse, and transfer to the hypochlorite solution.
- The hypochlorite solution can be made up (in advance) of either liquid bleach sodium hypochlorite (NaOCl) (fresh product; activity normally =11-13 % w/w) or bleaching powder calcium hypochlorite {$Ca(OCl)_2$} (activity normally ± 70 %) in the following proportions:
  - 0.5 g active hypochlorite product (activity normally labeled on the package, otherwise to be determined by titration) per g of cysts; for procedure see further); an alkaline product to keep the pH>10; per g of cysts use:
  - 0.15 g technical grade sodium hydroxide (NaOH) when using liquid bleach;
  - either 0.67g sodium carbonate ($NaCO_3$) or 0.4 g calcium oxide (CaO) for bleaching powder; dissolve the bleaching powder before adding the alkaline product; use only the supernatants of this solution;
  - seawater to make up the final solution to 14 ml per g of cysts.
- Cool the solution to 15-20°C (i.e. by placing the decapsulation container in a bath filled with ice water). Add the hydrated cysts and keep them in suspension (i.e. with an aeration tube) for 5-15 minutes. Check the temperature regularly, since the reaction is exothermic; never exceed 40°C (if needed add ice to decapsulation solution). Check evolution of decapsulation process regularly under binocular.

Washing step

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

Deactivation step

- Deactivate all traces of hypochlorite by dipping the cysts (< 1 min.) either in 0.1 N hydrochloric acid (HCl) or in 0.1 % sodium thiosulphate ($Na_2S_2O_3$) solution, then rinse again with water. Hypochlorite residues can be detected by putting some decapsulated cysts in a small amount of starch-iodine indicator (= starch, KI, H_2SO_4 and water). When the reagent turns blue, washing and deactivation has to be continued.

Use

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

Worksheet 5: Titrimetric method for the determination of active chlorine in hypochlorite solutions

- **Principle:** active chlorine will liberate free iodine from KI solution at pH 8 or less. The liberated iodine is titrated with a standard solution using $Na_2S_2O_3$, with starch as the indicator.
- **Reagents:** acetic acid (glacial, concentrated) KI crystals $Na_2S_2O_3$, 0.1 N standard solution starch indicator solution: mix 5 g starch with a little cold water and grind in a mortar. Pour into 1 L of boiling distilled water, stir, and let settle overnight. Use the clear supernatants. Preserve with 1.25 g salicylic acid.
- **Procedure:** dissolve 0.5 to 1 g KI in 50 ml distilled water, add 5 ml acetic acid, or enough to reduce the pH to between 3.0 and 4.0; add 1 ml sample; titrate protected from direct sunlight. Add 0.1 N $Na_2S_2O_3$ from a buret until the yellow colour of the liberated iodine is almost disappearing. Add 1 ml starch solution and titrate until the blue colour disappears.
- **Calculation:** 1 ml 0.1 N $Na_2S_2O_3$ equals 3.54 mg active chlorine.

Worksheet 6: Estimation of *Vibrio sp*. Prevalence in *Artemia* tank

Total count of bacteria

Needed:

- 2 plates of Marine Agar (MB) and Thiosulfate citrate bile salts sucrose (TCBS) per dilution
- Sterile filtered artificial seawater (32g/l)
- Sterile tubes of minimum 10 ml
- Drigalsky spatula
- TCBS from commercial company Sigma-Aldrich, MB from Carl Roth and add Agar 15g/l
- TCBS is not autoclaved but boiled up

Method:

- Make serial dilutions of your sample.
  0 = no dilution (sample as such)
  -1 = 10 times diluted
  -2 = 100 times diluted…..
- Fill tube with 9 ml of seawater; add 1 ml of sample into the tube (-1). Vortex very well and make next dilution starting from -1…..
- Plate your samples, take 100 µl and spread with drigalsky spatula
- Incubate for 48 hrs/28°C and count the colonies. Take only the dilutions into account that are between 30 and 300 colonies.
- Calculate the colony forming units (CFU/ml)

Table 1: Practical example of hatching percentage and hatching efficiency

nauplii (n)	umbrellas (u)	embryos (e)	H% = n*100 (n+u+e)$^{-}$ 1
replicate 1			
110	3	17	84.62
129	4	14	87.76
122	3	13	88.41
108	2	15	86.40
117	2	16	86.67
101	3	10	88.60
average nauplii = 115			average H% =87.08
replicate 2			
124	1	14	89.21
122	1	21	84.72
138	0	18	88.46
103	3	7	91.45
142	0	12	92.21
130	4	13	88.44
average nauplii = 127			average H% =89.03
replicate 3			
127	3	14	88.19
107	4	10	88.43
133	2	18	86.93
135	5	13	88.24
125	1	15	88.65
128	1	15	88.89
average nauplii = 126			average H% =88.23
average H% = (87.08+89.03+88.23).3-1 x 100 = 88.11 (standard deviation = 0.98)			
average HE = (115+127+126).3-1 x 2000 = 245 300 (standard deviation = 13000)			

Table 2: Practical example to calculate hatching rate

incubation time (in h)	HE (N/g)	% of maximal HE
12	0	0
13	800	0.4
14	9 000	5
15	29 400	15
16	79 800	42
17	144 400	76
18	158 200	83
19	184 600	97
20	185 000	97
21	191 000	100
Characteristic time-intervals	T10 = 14.5 h	
	T50 = 16.2 h	
	T90 = 18.5 h	
	Ts = T90-T 10 = 4.0 h	

Table 3: The proximate composition (in % of dry matter) of decapsulated *Artemia* cysts and instar I nauplii

	GSL		SFB	
	cysts	nauplii	cysts	nauplii
protein	±50	41-47	±57	47-59
lipid	±14	21-23	±13	16-27
carbohydrate	-	11	-	11
ash	±9	10	±5	6-14

5. References

Anh, N.T.N., Quynh, V.D., Hoa, N.V., Baert, P., 1997. Present situation of *Artemia* and salt production in the coastal salinas from SocTrang and Bac Lieu provinces. Scientific Magazine. Can Tho University, Vietnam, pp. 18–29 (Abstract in English).

DoF. 2019. Yearbook of Fisheries Statistics of Bangladesh, 2018-19. Fisheries Resources Survey System (FRSS), Department of Fisheries, Bangladesh: Ministry of Fisheries and Livestock, 2019. Volume 36: 135p.

Hoa NV and Hong Van NT. 2019. Principle of *Artemia* culture in solar saltworks. Agriculture Publishing House, 219 pp.

Lavens, P., &Sorgeloos, P. (1996). Manual on the production and use of live food for aquaculture (No. 361). Food and Agriculture Organization (FAO).

Rahman, M. M., Hoa, N. V., Sorgeloos, P. (2020). Guidelines for *Artemia* production in artisanal solar salt farms in Cox's Bazar, Bangladesh. Unpublished Book.

Sorgeloos, P., Coutteau, P., Dhert, P., Merchie, G., Lavens, P., 1998. Use of brine shrimp, *Artemia* spp., in larval crustacean nutrition: a review. Rev. Fish. Sci. 6, 55–68.

Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture 200, 147–159.

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