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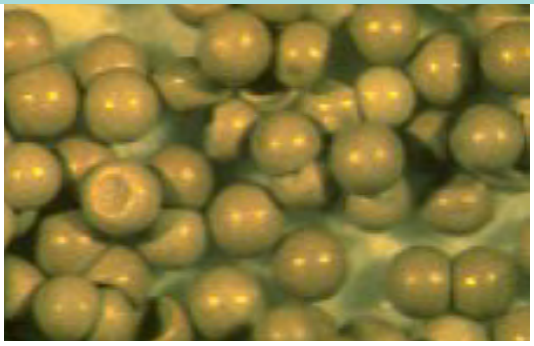
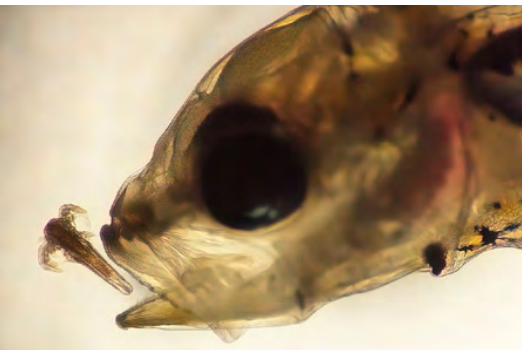
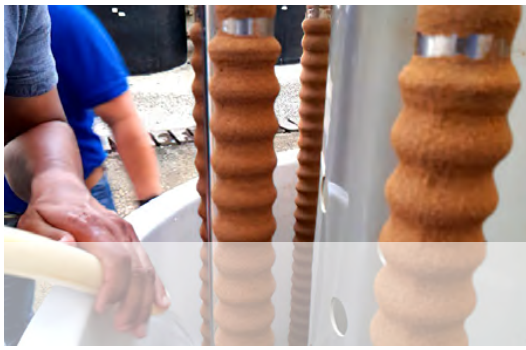
Manual on *Artemia* production and use





***Cover photographs*:**

Cysts floating on the water surface, driven by the wind to the pond corner (top left – ©Van Nguyen Hoa); Dry cysts of Artemia (top centre – ©ARARC); Adult female Artemia (top right – ©LAARC); Fish larva feeding on Artemia larvae (middle left – ©Charles Weirich); Embryo in umbrella stage (left) and instar I nauplius (middle centre – ©LAARC); Shrimp larva feeding on Artemia larvae (middle right – © INVE); Hatching tank with sufficient aeration (bottom left – ©Viet Uc Shrimp hatchery); Set–up for hatching quality control (bottom centre – ©LAARC); Application of SEP–Art® technology (Bottom right – ©INVE)

Manual on *Artemia* production and use

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Preparation of this document

This manual provides a first point of entry for anyone interested in *Artemia* and is designed to be a clear, concise, practical and all-in-one textbook on the production and use of this most widely used live food organism for food and larviculture globally. Given the rapid development of aquaculture and the responsible utilization of natural aquatic resources, this compilation and presentation of the latest available knowledge and best practices are especially important and relevant to the aquaculture community worldwide.

In the preparation process of this manual, the Food and Agriculture Organization of the United Nations (FAO) partnered with the Laboratory of Aquaculture and *Artemia* Reference Centre of Ghent University, as the leading institution, which established a task force and acted as coordinator with the other two co-leading institutions, the Asian Regional *Artemia* Reference Centre at Tianjin University of Science and Technology, China, and the College of Fisheries and Aquaculture at Can Tho University, Viet Nam. Other identified stakeholders included those active in *Artemia* study, production and application (i.e. research institutes, private companies, consultants) who were invited to provide the needed inputs towards a new update of the *Artemia* manual.

Prepared by a group of experts with diverse backgrounds and from all over the world, this manual covers *Artemia* biology, production (in salt lakes and ponds separately) and application of its different forms, together with a series of illustration material, making it an invaluable addition to the reference library for anyone working with *Artemia*. Spanning nearly 200 pages and three major chapters, this new edition builds on, updates and complements the material in the last edition of the FAO *Artemia* manual – Manual on the Production and Use of Live Food for Aquaculture – which was published in 1996.

It is worth recalling some significant milestones of the document preparation process: The FAO Fisheries and Aquaculture Division (NFI) through the Aquaculture Technology and Production Team (NFIAT) organized several online workshops to discuss the progress, outline and contents of the documentation. Prof. Patrick Sorgeloos was invited to deliver a presentation at the NFI seminar on the International *Artemia* Aquaculture Consortium: New initiative to consolidate/extend the role of *Artemia* in fish and crustacean aquaculture. On 22 September 2021, a Sustainable Development Goal-aligned *Artemia* Aquaculture Workshop was held as a special event at the Global Conference on Aquaculture Millennium +20, which was moderated by Rodrigo Roubach and Prof. Sui Liying. Other workshops and events have also been organized on the resources and utilization of *Artemia* worldwide.

This manual has been prepared to align with the FAO Blue Transformation Roadmap 2022–2030 that sustainable aquaculture intensification and expansion are required to satisfy the global demand for aquatic food and to distribute benefits equitably. The manual offers clear and updated technical guidance on sustainable provision and use of *Artemia* for food and aquaculture practices, aiming to support sustainable and efficient aquaculture intensification and enhance the awareness of *Artemia* biodiversity issue and biodiversity of hypersaline ecosystems in general, among *Artemia* users and producers. The following supplements can be accessed through the link provided with this manual: methods for the disinfection of non-decapsulated *Artemia* cysts, decapsulation of *Artemia* cysts, determination of dry weight of one *Artemia*, determination of water content of *Artemia*, and determination of cyst count, hatching percentage, hatching efficiency and hatching rate of *Artemia* cysts.

Abstract

This FAO publication delves into the significance of *Artemia*, the brine shrimp, as a key component in the feeding of marine and freshwater crustacean and fish larvae. *Artemia*, which can be hatched from dormant cysts, has evolved into a fundamental resource within aquaculture, supporting a diverse range of species. There is increasing demand for *Artemia* cysts within the hatchery industry, as it has a pivotal role in the production of over 900 billion crustacean postlarvae and fish fry, contributing to a multi-billion-dollar aquaculture sector.

The manual represents a comprehensive update of the FAO *Artemia* Manual on the Production and Use of Live Food for Aquaculture, as certain aspects described in earlier documents may no longer apply, and newer techniques and knowledge have emerged. It starts with a brief overview of morphology and the stages of *Artemia*'s life cycle and introduces its ecological role and natural distribution on a global scale. This is followed by information on taxonomic classification of *Artemia*, description of characteristics specific to different *Artemia* strains, and the biology of its cysts. The next chapter provides insights into various habitats where *Artemia* is produced, covers the specifics of pond and tank-based production of brine shrimp cysts as well as biomass, and includes an exploration of techniques used in harvesting and processing *Artemia*. The final chapter details ideal conditions and equipment for using cyst products in hatcheries and elaborates methods for assessing the quality of *Artemia* cyst products. The manual ends with the provision of practical worksheets with illustrations which could be used as tools that help the reader gain a better understanding of how to effectively execute the proper procedures in daily practice.

This publication stresses the importance of optimizing *Artemia* use for both cost-efficiency and nutritional quality, reflecting recent findings in *Artemia* biology and worldwide *Artemia* use practices. It caters to both newcomers and experts, providing valuable insights into biology, production, and use of *Artemia*, along with guidelines and methodologies for its proper utilization in aquaculture. Therefore, it is an invaluable resource for anyone engaged with *Artemia*, developed by a team of leading global experts in *Artemia* biology and production.

Keywords: *Artemia*, brine shrimp, cysts, hatchery, production, biomass.

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1. The Excel spreadsheets are accessible from the preview card online at https://doi.org/10.4060/cd0313en

Acknowledgements

This manual is a knowledge product cooperated between FAO and its partners, in particular, the Laboratory of Aquaculture and *Artemia* Reference Centre of Ghent University, the Asian Regional *Artemia* Reference Centre at Tianjin University of Science and Technology, China, and the College of Fisheries and Aquaculture at Can Tho University, Viet Nam.

We would like to express our gratitude and appreciation to colleagues at FAO who initiated the project and guided the process – Matthias Halwart, Liu Zhongwei (South-South and Triangular cooperation Division) and Rodrigo Roubach.

We would also like to give special thanks to Patrick Sorgeloos, who mentored the whole process. Thanks also go to the three main contributors, Gilbert Van Stappen, Sui Liying and Nguyen Van Hoa, as well as the team from Ghent University, Christ Mahieu, Geert Van de Wiele, Annelies Declercq and Caroline Van Geeteruyen, for their support.

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Abbreviations

AAC	*Artemia* Association of China, Tianjin, China
ALA	alpha linolenic acid
ARA	arachidonic acid
ARARC	Asian Regional *Artemia* Reference Centre (China)
ATP	adenosine triphosphate
BFT	biofloc technology
BY	Bolshoe Yarovoe
CFU	colony forming unit
C:N	carbon:nitrogen
DAG	diacylglycerol
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
EFA	essential fatty acid
EPA	eicosapentaenoic acid
FAA	free amino acid
FAO	Food and Agriculture Organization of the United Nations
FBD	fluidized bed dryer
FFA	free fatty acids
FID	flame ionization detector
GART	gnotobiotic *Artemia* rearing system
GC	gas chromatography
GSL	Great Salt Lake
GSLBSC	Great Salt Lake Brine Shrimp Cooperative, Inc. (United States of America)
H%	hatching percentage
HEff	hatching efficiency
HPLC	high performance liquid chromatography
HPTLC	high performance thin-layer chromatography
HUFA	highly unsaturated fatty acids
IAAC	International *Artemia* Aquaculture Consortium
ICP–MS	inductively coupled plasma mass spectrometry
INVE	INVE Aquaculture (Belgium)
KBG	Kara Bogaz Gol
LA	linoleic acid
LAARC	Laboratory of Aquaculture and *Artemia* Reference Centre (Belgium)
LED	light-emitting diode
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
MAC	Macau

MEG	Megalon Embolon
NACA	Network of Aquaculture Centres in Asia–Pacific
NATO	North Atlantic Treaty Organization
N:P	nitrogen to phosphorus ratio
ω3	omega-3
ω6	omega-6
O_2	oxygen
OTU	operational taxonomic unit
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
PVC	polyvinyl chloride
rRNA	ribosomal ribonucleic acid
RAS	recirculating aquaculture system
SCP	single-cell protein
SEC	Spencer Entomological Collection (Canada)
SFB	San Francisco Bay
SOP	standard operating procedure
SWA	Swakopmund (Namibia)
TAA	total amino acid
TAG	triacylglycerol
TCBS	thiosulphate-citrate-bile salts-sucrose
TL	total lipids
UDWR	Utah Division of Wildlife Resources
UNESCO	United Nations Educational, Scientific and Cultural Organization
USD	United States dollar
UV	ultraviolet
VC	Vinh Chau (Viet Nam)
WW	wet weight
XARI	Xinjiang Aquaculture Research Institute (China)

Chemical elements and formulae

Br	bromine
$Ca(H_2PO_4)_2.H_2O$	calcium dihydrogen phosphate monohydrate
$Ca(OCl)_2$	calcium hypochlorite
$Ca(OH)_2$	calcium hydroxide
$CaCO_3$	calcium carbonate
$CaHPO_4.2H_2O$	dicalcium phosphate dihydrate
CaO	calcium oxide
$CaSO_4$	calcium sulphate
$CaSO_4 \cdot 2H_2O$	calcium sulphate dihydrate
CO_2	carbon dioxide

H_2O_2	hydrogen peroxide
H_2S	hydrogen sulphide
KCl	potassium chloride
$KMnO_4$	potassium permanganate
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
N	nitrogen
$Na_2S_2O_3$	sodium thiosulphate
$(NH_4)_2SO_4$	ammonium sulfate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
NH_3	ammonia
NO_2^-	nitrite
NO_3^-	nitrate
P_2O_5	phosphate

Units of measurement

min	minute
h	hour
mg	milligram
g	gram
kg	kilogramme
ppm	parts per million
pmol	picomole
mol	moles
µm	micrometre
cm	centimetre
m	metre
m^2	square metre
m^3	cubic metre
km	kilometre
°Bé	degrees Baumé
°C	degrees Celsius
ha	hectare
L	litre
ml	millilitre
µl	microlitre
mV	millivolt
>	more than
<	less than
≥	more/equal than
≤	less/equal than

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Foreword

Larvae (nauplii) of the brine shrimp *Artemia* that can easily be hatched from "dried cysts" (i.e. inactive embryos in late gastrula stage) have been used for some decades now as a suitable substitute for natural live zooplankton in the feeding of a wide variety of farmed marine and freshwater crustacean and fish larvae. Commercial use of *Artemia* started in the 1960s, initially in Japan with Japanese seabream (*Pagrus major*) and kuruma shrimp (*Marsupenaeus japonicus*), and soon thereafter in other parts of the world with many more species. With the expansion of hatchery production of more aquaculture species, the demand for *Artemia* cysts has continued to increase; annual consumption is now estimated to be at least 3 500 tonnes, equivalent to approximately USD 150 million, for the production of over 900 billion crustacean postlarvae and fish fry by a hatchery industry valued at more than USD 2 billion and responsible for the final production of over 10 million tonnes of high-value aquaculture species. In addition, more than 100 000 tonnes of *Artemia* biomass, valued at more than USD 50 million, is used for feeding aquaculture animals.

Although in the 1980s and 1990s several scientific papers, manuals and handbooks were published describing the biology of *Artemia* and its optimal use in aquaculture hatcheries, some of the aspects of good practices described in these documents might not be relevant for the current larviculture situation. On the other hand, other aspects that are still very much valid are nowadays sometimes not given sufficient attention or are even entirely ignored. Many of the techniques and protocols that are widely applied do not follow the good practices in *Artemia* use as recommended by FAO in the *Manual on the Production and Use of Live Food for Aquaculture*, published in 1996, an important part of which was dedicated to *Artemia*. Moreover, scientific knowledge on *Artemia* biology has increased in the interim, new techniques and products have been developed, which can result in more controlled and optimized use of *Artemia* cysts in hatchery practices.

This was for example illustrated in the webinar "Status of the use of *Artemia* cysts in fish/crustacean hatcheries around the world", which was organized by the Network of Aquaculture Centres in Asia–Pacific on 2 September 2021. The presentations on *Artemia* use, given by speakers from a range of countries all around the globe, where aquaculture is an important activity, and the poll, organized among its 359 attendants, clearly showed that there is extensive room for improvement in the use of *Artemia* cysts in aquaculture hatcheries. Improved practices should result not only in economizing its use but especially in offering a highly nutritional and biosecure live food to fish and crustaceans in the larval stages. One of the main conclusions of this webinar was the strong recommendation for an update of "good practices" to be made available, among other interventions, under the form of updated manuals and other types of documentation/demonstration material. Similar conclusions were also reported in an online international workshop on *Artemia* pond/tank production, organized by the WorldFish (Bangladesh) on 15 June 2021, where *Artemia* pond and tank production practices in different countries in Asia and Africa were reviewed by diverse participants, and the need for renewed, comprehensive guidelines was addressed.

The idea to produce an updated "*Artemia* manual" has been further triggered by the recent initiative to establish the International *Artemia* Aquaculture Consortium, which initially grew out of an informal collaboration network of different institutes, each having specific expertise in the field of *Artemia*. Together, they realized that the need for renewed information on proper *Artemia* use fits within a broader perspective to guarantee

a more sustainable provision of *Artemia*. This includes issues of bioconservation and effects of climate change, more in–depth studies on specific aspects of *Artemia* biology, and integration of *Artemia* production in other types of aquatic production.

This FAO publication on the brine shrimp *Artemia* is a manual for all those who are using *Artemia* or have an interest in this organism, either as a source of live food in the hatchery, as a model organism in research, or for whatever purpose. It is intended for those who wish to update their knowledge on *Artemia* biology, production or use, but also for those who want to learn about it for the first time. It follows the overall structure of the 1996 manual, in the sense that proper understanding of good practices in *Artemia* production and use requires prior basic knowledge on the biology and ecology of this organism. For the rest, the contents, structure and layout have been thoroughly updated to reflect the current status of knowledge, insights and consensus, but also to meet the demands of a new reading audience, which expects information to be provided not just through a narrative manual but also in a more pluriform way. The most important difference as compared to the 1996 edition, however, is that this work is the creation of a large group of experts with diverse backgrounds and expertise from all around the globe, working in research, education and the industry. Others also have played a vital role in coordinating, streamlining and shaping this piece of work. The editors especially wish to thank Christ Mahieu and Geert Van de Wiele of the Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University, Belgium, for their valuable assistance. The contribution, big or small, of everyone who contributed and the exchange of ideas, following from the interaction of all experts, gives unique value to this manual, which hopefully will be used as a work of reference for years to come.

The editors,

Gilbert Van Stappen
Patrick Sorgeloos
Geert Rombaut

1. Biology and ecology of *Artemia*

by *Liying Sui*

1.1 MORPHOLOGY AND LIFE CYCLE

In its natural environment, at certain times of the year, brine shrimp *Artemia* produce cysts that float at the water surface (Plate 1) and that are thrown ashore by wind and waves. These cysts are metabolically inactive and do not further develop as long as they are kept dehydrated (either dry or in saturated brine). Upon immersion in lower salinity water (e.g. seawater in a hatchery), the biconcave-shaped cysts hydrate, become spherical, and within the shell the embryo resumes its interrupted metabolism. After about 10 to 20 h (depending on cyst strain and temperature), the outer cuticular membrane of the cyst bursts (=breaking stage) and the embryo appears, surrounded by the embryonic membrane (=hatching membrane) (Plate 2). While the embryo hangs underneath the empty shell (= umbrella stage), the development of the nauplius is completed, and within a short period of time the hatching membrane is ruptured (=hatching) and the free-swimming nauplius is born (Plate 3).

PLATE 1
Harvesting of brine shrimp from salt ponds
(a, c, and d); brine shrimp *Artemia* cysts (b)

PLATE 2
Cyst in breaking stage
(1) nauplius eye

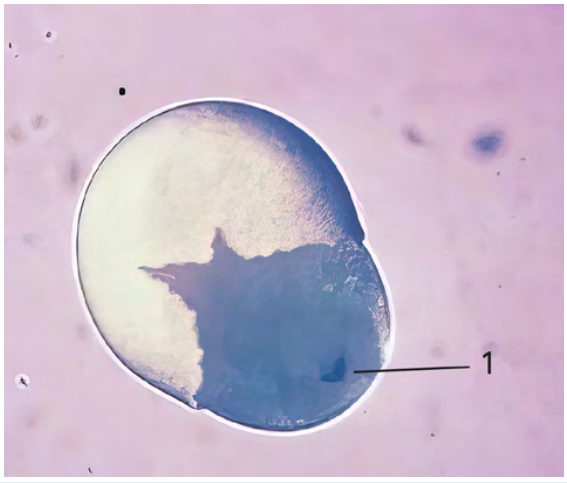
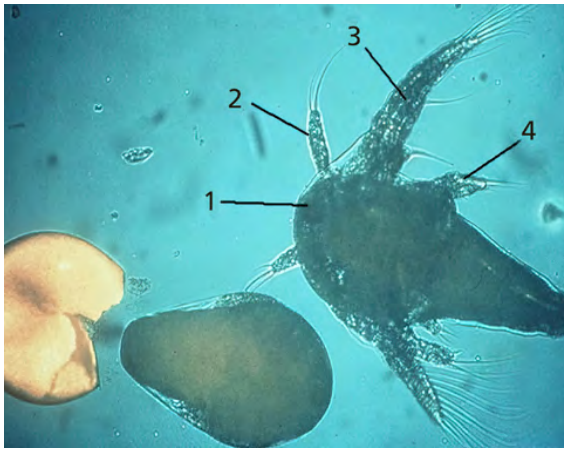


PLATE 3
Embryo in "umbrella" stage (left) and instar I nauplius (right)
(1) nauplius eye; (2) antennula; (3) antenna; (4) mandible

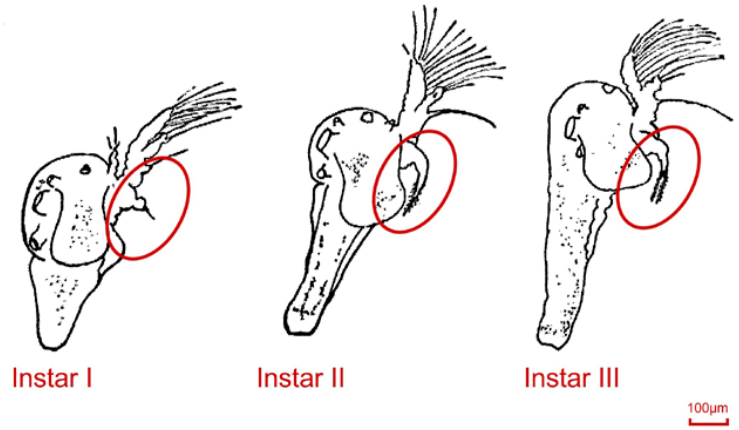


The first larval stage (instar I; 400 µm to 500 µm in length) has a brownish-orange colour, a red nauplius eye in the head region and three pairs of appendages: i.e. the first antennae (sensorial function), the second antennae (locomotory and filter-feeding function) and the mandibles (food uptake function). The ventral side is covered by a large labrum (food uptake: transfer of particles from the filtering setae into the mouth). The instar I larva does not take up food, as its digestive system is not functional yet; it thrives completely on its yolk reserves.

After about 6 to 8 h (strain and temperature dependent), the animal moults into the second larval stage (instar II). Small food particles (e.g. microalgal cells, bacteria/archaea, protozoa and small particulate matter) are filtered out by the second antennae and ingested via the mouth into the functional digestive tract. *Artemia* particle size preference for early developmental stages varies from 4-8 µm to 6.8-27.5 µm, with an optimum size of about 16.0 µm. Individual bacterial cells are generally thought to be taken up with low efficiency by *Artemia* due to their small size. The clearance rate of *Artemia* larvae fed bacterial particles with a size of 0.5 µm has been shown to be 69 times less than when fed microalgal particles (size 12 µm) (Seixas *et al.*, 2009). On the other hand, *Artemia* adults are able to ingest all particles < 50 µm in size but preferentially graze on particles < 30 µm (Gelabert-Fernandez and Solis, 1994; Makridis and Vadstein, 1999; Gelabert and Pacheco, 2011).

The morphology and size of instar I, instar II and instar III *Artemia* nauplii can be distinguished under the microscope. In addition to larval length and general morphology, the structure of the mandibular gnathobase setae, in particular, makes it possible to unambiguously distinguish between the successive instar stages (Figure 1).

FIGURE 1
Morphology and size of instar I, instar II and instar III nauplii. The red circle indicates the gnathobase and its setae



Source: Hentschel, E. 1968. Die postembryonalen Entwicklungsstadien von *Artemia salina* LEACH bei verscheidenen Temperaturen (Anostraca, Crustaceae). *Zool. Anz.*, 180: 372–384. (Modified from Hentschel, E. 1968).

The larva grows and while moulting *Artemia* goes through 17 developmental stages in its entire life cycle (Criel and MacRae, 2002). In the subsequent larval stages, paired lobular appendages appear in the trunk region and differentiate into thoracopods (Plate 4). On both sides of the nauplius, lateral complex eyes develop (Plate 5 and Plate 6). From the 10th instar stage on, important morphological as well as functional changes continue to take place, i.e. the antennae have lost their locomotory function and undergo sexual differentiation. In males (Plate 7 and Plate 8), the antennae develop into hooked graspers, while the female antennae degenerate into sensorial appendages (Plate 9). The thoracopods are now differentiated into three functional parts (Plate 10), namely, the telopodites and endopodites (locomotory and filter-feeding) and the membranous exopodites (gills).

PLATE 4
Instar V larva
(1) nauplius eye; (2) lateral complex eye; (3) antenna; (4) labrum; (5) budding of thoracopods; (6) digestive tract

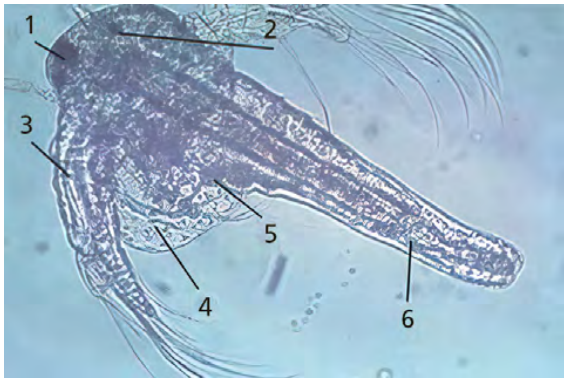


PLATE 5
Head and anterior thoracic region of instar XII
(1) nauplius eye; (2) lateral complex eye; (3) antennula; (4) antenna; (5) exopodite; (6) telopodite; (7) endopodite

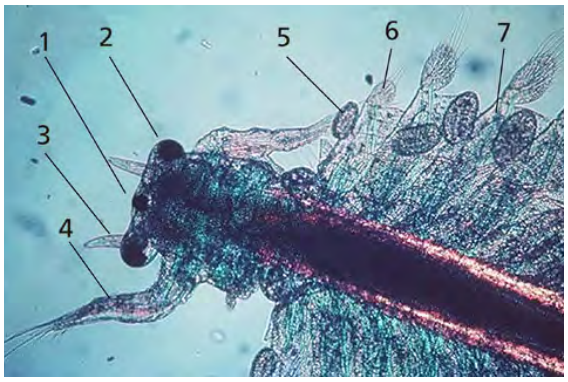


PLATE 6
Head and thoracic region of a young male
(1) antenna; (2) telopodite; (3) exopodite

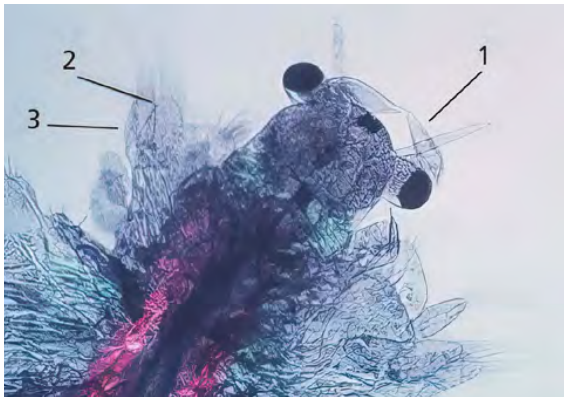


PLATE 7
Head of an adult male
(1) antenna; (2) antennula; (3) lateral complex eye; (4) mandible

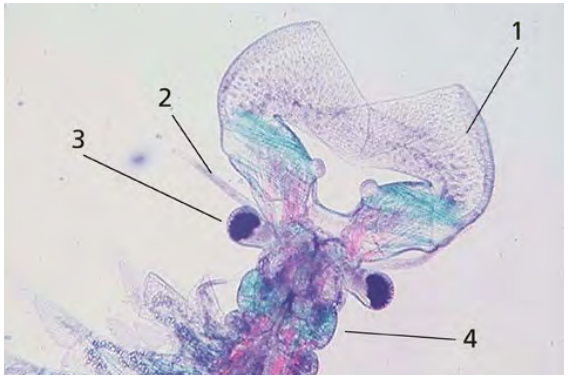


PLATE 8
Adult male

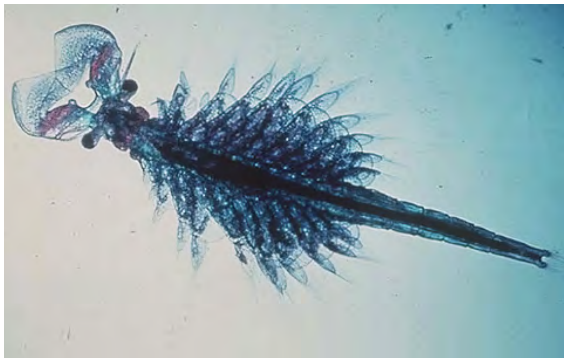
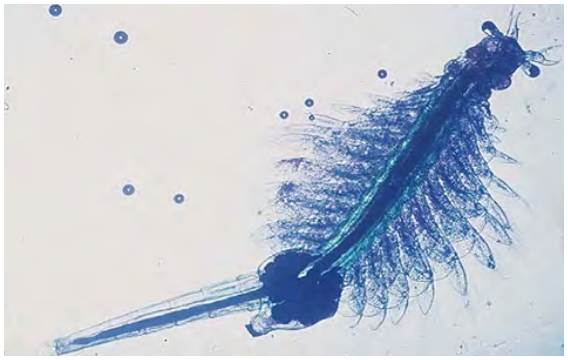


PLATE 9
Adult female



Adult *Artemia* (±1 cm in length) have an elongated body with two stalked complex eyes, a linear digestive tract, sensorial antennulae and 11 pairs of functional thoracopods (Plate 8 and Plate 9). The male (Plate 8) has a paired penis in the posterior part of the trunk region (Plate 11). Female *Artemia* can easily be recognized by the brood pouch or uterus situated just behind the 11th pair of thoracopods (Plate 9). During pre-copulation behaviour, animals can be seen in "riding position" (Plate 11),

whereas copulation itself shows a bending abdomen, with one penis into the uterus. Eggs develop in two tubular ovaries in the abdomen (Plate 12). Once ripe, the eggs become spherical and migrate via two oviducts in the unpaired uterus.

Fertilized eggs normally develop into free-swimming nauplii (= ovoviviparous reproduction) (Plate 13), which are released by the female. In extreme conditions (high salinity, low oxygen levels, food scarcity, etc.), the embryos only develop up to the gastrula stage. At this moment, they become surrounded by a thick shell (secreted by the brown shell glands located in the uterus), enter a state of metabolic standstill or diapause, and are then released by the female (= oviparous reproduction) (Plate 14). In principle, both oviparity and ovoviviparity are found in all *Artemia* strains, and females can switch in-between two reproduction cycles from one mode of reproduction to the other.

The cysts usually float in the high salinity waters and are blown ashore where they accumulate and dehydrate. As a result of this dehydration process, the diapause mechanism is generally inactivated; cysts are now in a state of quiescence and can resume their further embryonic development when hydrated in optimal hatching conditions.

Under optimal conditions, brine shrimp can live for several months, grow from nauplius to

PLATE 10
Detail of anterior thoracopods in adult *Artemia*
(1) exopodite; (2) telopodite; (3) endopodite

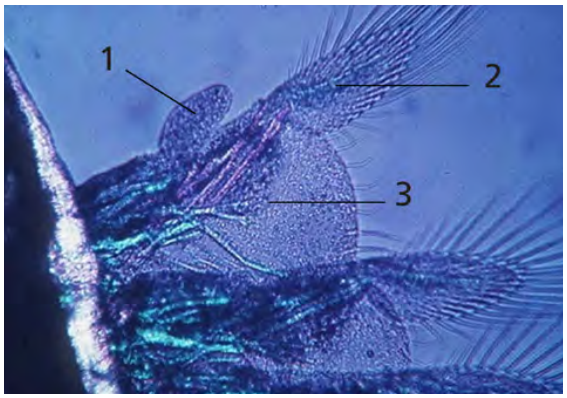


PLATE 11
Pre-copulation (riding position) of male and female *Artemia*
(1) Female uterus; (2) male penis

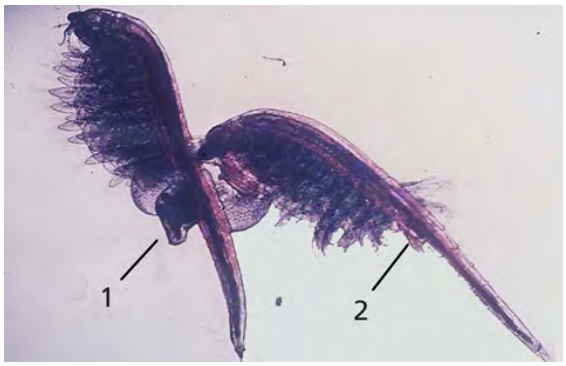


PLATE 12
Posterior thoracic region, abdomen and uterus of fertile female
(1) Ripe eggs in ovary and oviduct

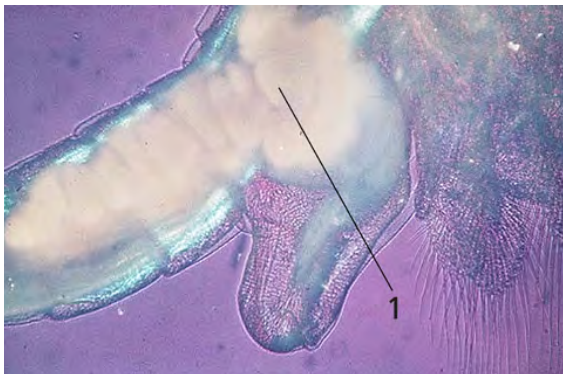


PLATE 13
Uterus of ovoviviparous *Artemia* filled with nauplii (first larvae are being released)

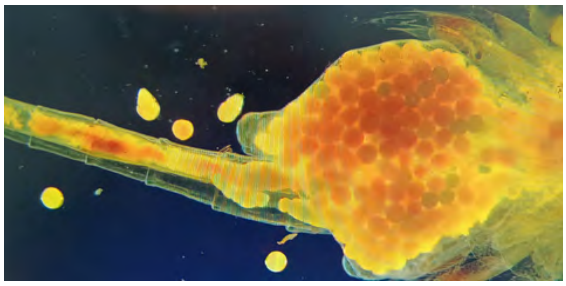


PLATE 14
Uterus of oviparous Artemia filled with cysts
(1) brown shell glands (darker colour)

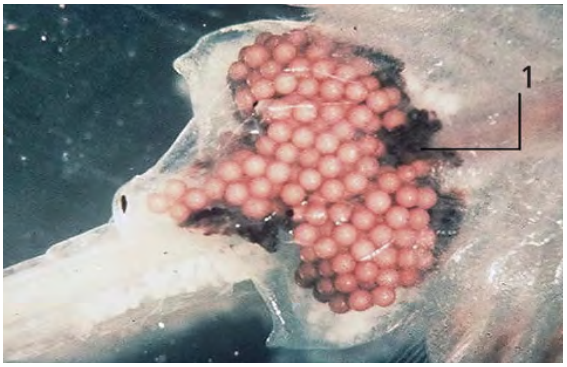
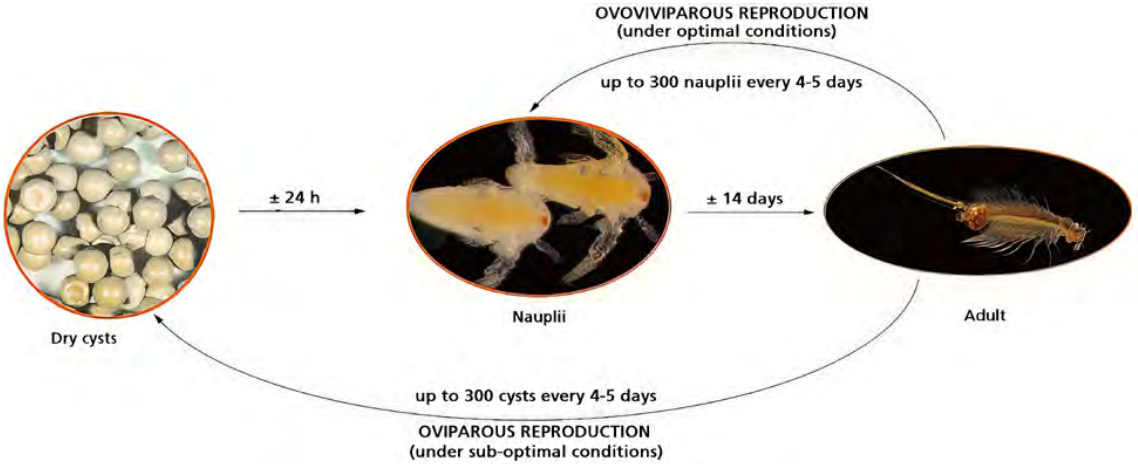


FIGURE 2
Typical life cycle of *Artemia franciscana*



Source: Authors' own elaboration.

adult in less than 10 days to a month and reproduce at a rate of up to 300 nauplii or cysts every 4–5 days (depending on strain, temperature and food availability) (Figure 2).

1.2. ECOLOGY AND NATURAL DISTRIBUTION

Artemia populations are found in several hundreds of natural salt lakes and human-induced salterns scattered throughout the tropical, subtropical and temperate climatic zones, along coastlines as well as inland. Lists of sites made so far (Persoone and Sorgeloos, 1980; Vanhaecke, Tackaert and Sorgeloos, 1987; Triantaphyllidis *et al*., 1994; Van Stappen, 2002) remain provisional as more extensive survey work should lead to the discovery of many more *Artemia* biotopes in different parts of the world. On the other hand, some *Artemia* populations, previously recorded, have disappeared in some locations where the salinity is too high or too low to sustain long-term survival of the population.

The distribution of *Artemia* is discontinuous: Not all highly saline biotopes are populated with *Artemia*. Although brine shrimp thrive very well in natural seawater, they cannot migrate from one saline biotope to another via the seas, as they depend on their physiological adaptations to high salinity to avoid predation and competition with other filter feeders. Their physiological adaptations to high salinity provide a very efficient ecological defence against predation, as brine shrimp possess:

- a very efficient osmoregulatory system;
- the capacity to synthesize very efficient respiratory pigments to cope with the low oxygen (O_2) levels at high salinities; and
- the ability to produce dormant cysts when environmental conditions endanger the survival of the population.

Different natural populations of *Artemia* at the subspecies level are generally referred to as "strains" in the *Artemia* literature, which is different from the standard FAO terminology that uses "strain" for a genetically differentiated farmed type, whereas in the wild it may be referred to as a "stock" or "population" (FAO, 2019). For the sake of congruence with the conventional *Artemia* literature, this manual will thus further use "strain" in the sense of a natural population occurring at a specific site. In addition to these natural populations, *Artemia franciscana* originating from Great Salt Lake and San Francisco Bay (both in the United States of America) have been introduced to a number of countries in the Global South for local cyst production to offset rising costs and limited availability from the original sites. For example, in Brazil,

China, Kenya, Thailand and Viet Nam, there have been inoculations with these original populations; afterwards from these countries there have been further inoculations into sites in Bangladesh, Cambodia, India, Myanmar, Sri Lanka and the United Arab Emirates. Introduction into new environments has initiated the development of locally adapted populations, which are also called "strains" in the *Artemia* literature, along with natural populations. A successful incipient "farmed type" (in the sense of FAO terminology) is the Vinh Chau *A. franciscana* strain in Viet Nam originating from San Francisco Bay cysts in 1986, which now has high thermal tolerance (>40 °C). Investigation of the underlying genetic basis of thermotolerance and other traits indicates that multiple genes and differential gene expression are involved (De Vos *et al*., 2021), although more research work on this Vinh Chau strain – and on other inoculated populations – is required.

Artemia strains have adapted to widely fluctuating conditions regarding temperature (6–40 °C), salinity and ionic composition of the biotope. Thalassohaline waters are concentrated seawaters with sodium chloride (NaCl) as the major salt. They make up most, if not all, of the coastal *Artemia* habitats where brines are formed by evaporation of seawater in salt pans. Other thalassohaline habitats are located inland, such as the Great Salt Lake in Utah, the United States of America. Athalassohaline *Artemia* biotopes are located inland and have an ionic composition that differs greatly from that of natural seawater: There are sulphate waters (e.g. Chaplin Lake, Saskatchewan, Canada; Lake Aibi, Xinjiang, China; Ebeyty Lake, Omsk region, Russian Federation); carbonate waters (e.g. Mono Lake, California, United States of America); and potassium-rich waters (e.g. several lakes in Nebraska, United States of America).

Artemia is a non-selective filter feeder of particulate organic matter (bioflocs), microalgae, small protozoa and bacteria/archaea. The *Artemia* biotopes typically show a very simple trophical structure and low species diversity; the absence of predators and food competitors allows brine shrimp to develop into monocultures. As high salinity is the common feature determining the presence of *Artemia*, the impact of other parameters (temperature, primary food production, etc.) may, at most, affect the abundance of the population and eventually cause a temporary absence of the species.

As *Artemia* is incapable of active dispersion, wind and waterfowl (especially flamingos) are the most important natural dispersion vectors; the floating cysts adhere to feet and feathers of birds and, when ingested, they remain intact for at least a couple of days in the digestive tract of birds (Proctor, Malone and Devlaming, 1967). Consequently, the absence of migrating birds is probably the reason why certain areas that are suitable for *Artemia* (e.g. salinas along the northeast coast of Brazil; see Box 5) are not naturally inhabited by brine shrimp.

Next to the natural dispersion of cysts, deliberate inoculation of *Artemia* in solar saltworks by artificial means has been a common practice in the past. Since the 1970s, humans have been responsible for several *Artemia* introductions in Africa, Asia, Australia and South America, either for salt production improvement or for aquaculture purposes. Additionally, temporal *Artemia* populations are found in tropical areas with a distinct wet and dry season (monsoon climate) through inoculation in seasonal salt operations (e.g. Bangladesh, Cambodia, India, Myanmar, the Philippines, Sri Lanka, Thailand and Viet Nam in south and southeast Asia) (Van Stappen *et al.*, 2019). In these pond systems, brine shrimp are mainly found in ponds at intermediate salinity levels (see Section 2.2). As *Artemia* have no defence mechanisms against predators, the lowest salinity at which animals are found is also the upper salinity tolerance level of possible predators (minimum 70 g/L and maximum 140 g/L depending on the locally occurring aquatic invertebrate and fish species). As a result of extreme physiological stress, especially when combined with food scarcity, *Artemia* dies off at salinities close to NaCl saturation, i.e. 250 g/L and higher. Although live brine shrimp can be found at higher salinity than 250 g/L, the need of increased osmoregulatory activity, requiring higher energy inputs, negatively influences growth and reproduction, eventually

leading to starvation and death. Cysts are produced in ponds having intermediate and high salinity (about 80 g/L to 250 g/L).

Introduction and managing brine shrimp populations in saltworks, where natural populations are not present, will improve profitability, even in situations where *Artemia* biomass and cyst yields are comparatively low. The presence of *Artemia* is not only essential for the control of algal blooms. The *Artemia* metabolites and/or decaying animals are also a suitable substrate for the development of halophilic bacteria and archaea, such as Halobacterium, in the crystallization ponds. High concentrations of these halophilic organisms – causing the water to turn wine red – enhance heat absorption, thereby accelerating evaporation, but at the same time reduce concentrations of dissolved organic matter. This in turn leads to lower viscosity levels, promoting the formation of larger salt crystals, thus improving salt quality (Davis, 2006; see Section 2.1.2).

1.3. TAXONOMY

The taxonomic classification of *Artemia* is as follows:

Phylum:	Arthropoda
Class:	Crustacea
Subclass:	Branchiopoda
Order:	Anostraca
Family:	Artemiidae
Genus:	*Artemia*

The genus *Artemia* is a complex of species, defined by the criterion of reproductive isolation. Early taxonomists assigned species names to populations with different morphologies, collected at different temperatures and salinities. Over time, the profusion of names was abandoned, and all brine shrimp were referred to as *Artemia* salina (Linnaeus, 1758). Some authors continue this erroneous practice today. Different species names have been assigned to reproductively isolated populations or clusters of populations, and the following zygogenetic (also called bisexual) species (having individuals of separate sexes) are recognized by consensus (with their overall distribution range) (Pilla and Beardmore, 1994; Beardmore, Pilla and Thomas, 1994; Browne and Bowen, 1991):

- *A. salina* (Linnaeus, 1758): Lymington, England (now extinct), Mediterranean area (in the past, the name *A. tunisiana* (Bowen and Sterling, 1978) has also been used for this species);
- *A. urmiana* (Gunther, 1990): the Islamic Republic of Iran (Lake Urmia), Crimean salt marshes;
- *A. sinica* (Cai, 1989): central and eastern China;
- *A. tibetiana* (Abatzopoulos *et al*., 1998): Lagkor Co Lake, Tibet, China;
- *A. persimilis* (Piccinelli and Prosdocimi, 1968): southern South America;
- *A. franciscana*: North, Central and South America (the population from Mono Lake, California, the United States of America, is generally recognized as a subspecies of *A. franciscana*: *A. franciscana monica* (Verrill, 1869).
- *A. sorgeloosi* (Asem *et al.*, 2023): Haiyan Lake, Tibet, China; and
- *Artemia amati* (Asem *et al.*, 2023): Kazakhstan.

Additionally, the numerous parthenogenetic *Artemia* populations occurring in Europe, Africa, Asia and Australia are often grouped under the species name *A. parthenogenetica* (Barigozzi, 1974; Bowen and Sterling, 1978).

Still, there is significant ongoing debate about the taxonomy of *Artemia.* The relatively high morphological plasticity in *Artemia* populations subjected to different environmental conditions has hindered the identification of the taxonomic entities encompassed within the genus *Artemia*. In addition, the mismatch between molecular and morphological evolution rates complicates the characterization of evolutionary lineages. Different research teams, using different methodological approaches, have been trying to disentangle the taxonomic problems derived from the complex biology of brine shrimps, and more details and controversies on the taxonomic status and phylogeny of *Artemia* species can be found in specialized literature (Naganawa and Mura, 2017; Asem *et al.*, 2020; Sainz-Escudero *et al*., 2021).

The coexistence of two species in the same saline habitat is possible: Co-occurrence of parthenogenetic and zygogenetic populations has been reported in Mediterranean salterns (Amat *et al.*, 1995) and in Lake Urmia, the Islamic Republic of Iran (Agh *et al*., 2007). In addition, commercial aquaculture ventures have seeded salterns with imported cysts on many occasions, where the introduced populations may co-occur with local ones; for example, *A. franciscana* has been introduced in places in Africa, Asia and Australia over the past 40 years. Because new populations are constantly being characterized, scientists are urged to use the denomination "*Artemia* sp." unless they have sufficient molecular, morphological and/or other evidence to identify the species name.

Recently the genome of *Artemia franciscana* has been published and this will facilitate a lot of new research on the differences between the various species and strains of *Artemia* (see Box 1).

BOX 1

The *Artemia* genome unraveled

by *Peter Bossier*

The genome of *Artemia franciscana* (Kellogg, 1906) has recently been sequenced (De Vos *et al*., 2021), which means that now a fairly good insight into the DNA sequence of the *Artemia* genome is publicly available. To assemble the genome, high-throughput sequencing technology (Illumina and PacBio DNA sequencing) was used. To reveal this genomic information, a highly inbred line of A. *franciscana* had to be reared in the laboratory over multiple generations, and this biological material was used for the sequencing analysis.

As it stands, the *Artemia* genome contains 26 057 so-called contigs, or known reassembled fragments of substantial length. The longest fragment in the assembly has a length of 855 000 base pairs; the N50 value (i.e. the value at which 50 percent of the obtained genome sequence is contained in fragments that are larger than the indicated size) was 112 000 base pairs, and this value is below the median N50 of other known crustacean genomes. This indicates that there is still room for improvement to further "reconstruct" the sequence of the genome.

Additionally, the structural content of the *Artemia* genome was compared to the genome of other crustacean species. The *A. franciscana* genome showed 2 percent exonic space, meaning that only 2 percent of the DNA sequence is actually involved in making proteins, similar to what has been observed in whiteleg shrimp (*Litopenaeus vannamei*) (3 percent), while introns (DNA segments not coding for proteins) made up 29 percent of the genome, exceeding the value observed in other arthropod genomes. As it stands, 21 828 genes were identified in *Artemia*. As a comparison, the human genome has about 30 000 genes. The annotation (allocating a function to each of the individual genes, for example, linking it to a certain protein or enzyme) was not very successful, in the sense that many genes remain without a known function, which also illustrates that there is still room for improvement in the exercise of annotation.

As a case study of "gene discovery", the paper by De Vos *et al.* (2021) also describes how *Artemia* nauplii reacted when exposed to salinity stress. Under salinity stress, an

BOX 1 (CONTINUED)

instantaneous increase in salinity from 30 g/L to 200 g/L was associated with the differential expression of 674 genes, signifying that in one way or another these genes (and the proteins or enzymes they code for) are related to the processes of how *Artemia* copes with salinity stress. Again, only 42 percent of these genes have a known function, indicating that much remains to be learned about how exactly *Artemia* reacts when exposed to strong salinity fluctuations.

It is anticipated that the availability of the information on the *Artemia* genome will strongly contribute to future efforts to further unravel how *Artemia* can cope with extreme environmental conditions. It is also expected that this knowledge will contribute to a more in-depth understanding of the many life-cycle processes in this unique organism. This may help, for example, in breeding specific farmed types of *Artemia*, based on traits of importance for commercial application (e.g. small cyst size, favourable fatty acid metabolism). These farmed types of *Artemia* might thus complement the existing natural resources. So far, however, there have been few scientific studies on heritability and selection of advantageous traits in *Artemia*, all limited to controlled laboratory conditions. But in order to safeguard the sustainable provision of *Artemia*, in line with the United Nations Strategic Development Goals, development of new *Artemia* applications through strain selection and selective breeding is considered as one of the critical issues to be addressed, as emphasized by the newly established International *Artemia* Aquaculture Consortium at the occasion of the Global Conference on Aquaculture, in Shanghai, China, in September 2021.

The worldwide distribution of brine shrimp in a variety of isolated habitats, each one characterized by its own ecological conditions, has furthermore resulted in the existence of numerous geographical populations, so-called "strains", or genetically different populations within the same species; in particular, parthenogenetic *Artemia* with its di-, tri-, tetra-, and pentaploid populations display a wide genotypic variation. Among these strains, a high degree of genetic variation and a unique diversity of quantitative characteristics have been observed. Some of these characteristics (e.g. the nutritional value of cysts and freshly hatched nauplii) are phenotypical and change from year to year or season to season. Others, however (e.g. cyst diameter, growth rate, resistance to high temperature) are strain-specific and remain relatively constant. They have become genotypical as a result of long-term adaptations of the strain to the local conditions (see Section 1.4). According to standard FAO terminology used to distinguish between "wild" and "farmed" aquatic species, some of these populations can be considered "wild" (such as the feral *A. franciscana* population from Great Salt Lake, United States of America), whereas others are the result of human and/or natural selection processes, such as the *A. franciscana* population from Vinh Chau, Viet Nam, and are to be considered as "farmed" species.

1.4. STRAIN-SPECIFIC CHARACTERISTICS

While the nutritional value of *Artemia* as live food can be enhanced by the so-called enrichment procedure (see Section 1.4.6), other qualities favourable for aquaculture use can be obtained by proper choice of a suitable strain. Nowadays, *Artemia* cysts are commercially available from various production sources in China, Russian Federation, United States of America, Viet Nam and Central Asian countries. Knowledge of the characteristics (both genotypical and phenotypical) of a particular cyst product can greatly increase the effectiveness of its usage in a fish or shrimp hatchery.

1.4.1. Size and energy content of cysts and larvae

The nutritional effectiveness of a food organism for larvae is primarily determined by its ingestibility and, as a consequence, by its size and form (see Section 3.2.1). Many

strains can be differentiated based on the biometrical characteristics of their cysts and nauplii. Despite small variations between batches of the same strain, possibly caused by environmental and/or processing factors, generally the cyst diameter of different production batches of the same strain remains rather constant. Other biometrical characteristics, such as cyst volume, cyst dry weight, instar I naupliar length, individual naupliar weight and naupliar volume and energy content, show a high correlation with the cyst diameter (see Table 1 and Table 2). Consequently, biometrical parameters, in particular the cyst diameter, are good tools to characterize *Artemia* strains and help to define the origin of unknown or even mixed cyst samples.

Some general correlations can also be made between species and size: Parthenogenetic *Artemia* generally produce large cysts; among the bisexual species, *A. tibetiana* has the largest cyst size, and *A. franciscana* and *A. persimilis* have small or intermediate-size cysts with a thin chorion.

TABLE 1
Biometrical data (average and standard deviation) of *Artemia* cysts and instar I nauplii from various origins

Common name (in Latin alphabet)	Species	Cyst diameter (µm)	Decapsulated cyst diameter (µm)	Chorion thickness (µm)	Instar I length (µm)
Great Salt Lake, United States of America	*A. franciscana*	245.1 ± 13.8	219.6 ± 10.6	12.8	486.7 ± 33.2
San Francisco Bay, United States of America		237.5 ± 24.7	225.9 ± 21.8	5.8	435.0 ± 16.8
Vinh Chau, Viet Nam		224.9 ± 11.9	207.3 ± 11.9	8.8	399.7 ± 15.4
Bolshoe Medvezhe, Russian Federation	*A. parthenogenetica*	257.5 ± 12.9	242.3 ± 13.5	7.6	469.4 ± 18.3
Bolshoe Yarovoe, Russian Federation		262.7 ± 18.2	244.8 ± 18.4	8.9	473.8 ± 24.1
Ebeyty, Russian Federation		259.5 ± 10.9	242.8 ± 12.6	8.3	467.8 ± 38.9
Kuchukskoe, Russian Federation		261.4 ± 15.3	248.6 ± 13.4	6.4	446.9 ± 34.5
Kulundinskoe, Russian Federation		251.3 ± 9.8	240.0 ± 10.7	5.7	490.8 ± 35.9
Lake Aibi, China		292.6 ± 16.2	270.7 ± 12.0	10.8	490.4 ± 19.6
Tanggu salt ponds, Bohai Bay, China		273.7 ± 14.6	256.6 ± 12.0	8.5	486.2 ± 28.2
Lagkor Co, China	*A. tibetiana*	322.2 ± 16.1	308.7 ± 19.0	6.8	529.2 ± 35.6
Qixiang Co, China		293.9 ± 11.8	278.7 ± 13.1	7.6	509.0 ± 34.7

Source: Authors' own elaboration (ARARC, China and LAARC).

TABLE 2
Individual dry weight and energy content of *Artemia* instar I nauplii, hatched in standard conditions (salinity 35 g/L, 25 °C) from cysts of various origins

Locality	Dry weight (µg)	Energy content (10^{-3} joule)
San Francisco Bay, United States of America	1.63	366
Great Salt Lake, United States of America	2.42	541
Tanggu salt ponds, Bohai Bay, China	3.09	681

Source: Authors' own elaboration (ARARC, China and LAARC).

1.4.2. Hatching quality of cysts

Comparative studies of the hatching success of cysts of different origin show a considerable variation in hatching parameters, as defined in Section 3.2.2. However, none of these parameters is strain-specific, as they are influenced by a wide array of factors, such as harvesting, processing, storage and hatching techniques as well as production conditions affecting the parental generation (Lavens and Sorgeloos, 1987). For optimal use of *Artemia* in aquaculture, the hatching characteristics of any batch of cysts used should be known. More information in this respect is given in Section 3.2.2.

1.4.3. Growth rate of nauplii

Standard culture tests with brine shrimp from different geographical origin show important differences in growth rate even within the same species, but not among batches of the same strain. Although in the field the population growth of *Artemia* is determined by many factors, the choice of a strain with a high potential growth rate will have a positive impact on maximal production output.

1.4.4. Temperature and salinity tolerance

Both temperature and salinity significantly affect survival and growth, the effect of temperature being more pronounced. A broad range of temperatures and salinities meets the requirements for >90 percent survival. Strains from thalassohaline biotopes share a common temperature area of preference in the range of 20–25 °C, where mortalities are <10 percent. Interaction between temperature and salinity is limited; substantial differences in tolerance between strains have been recorded at low salinities (around 5 g/L) and high temperatures (30–34 °C). At elevated temperatures, the survival of the Great Salt Lake *A. franciscana* strain is significantly higher than for other "wild" strains, whereas the "farmed" Vinh Chau *A. franciscana* strain (see Section 1.2) can tolerate even higher temperatures (up to 40 °C) than the Great Salt Lake strain.

1.4.5. Life history traits and reproductive capacity

Life history and reproductive characteristics of *Artemia* strains are important factors when introduction of brine shrimp in a new habitat is considered, especially when competition with a local strain is to be expected. These competitive abilities are related to factors such as the length of the pre and post-reproductive period, total lifespan, number of offspring per brood, broods per female and time in-between broods.

Because *A. franciscana* has a very large number of offspring per brood, a large number of broods per female and a fast development time to sexual maturity, this species is favoured over other bisexual species and parthenogenetic *Artemia* (Browne and Halanych, 1989; Browne *et al.*, 1984, 1993). Age at first reproduction is a key factor determining the population growth rate and the rate of colonisation of new environments with limited nutrient resources. Consequently, if environmental preferences and nutritional factors do not interfere, *A. franciscana* generally outcompetes parthenogenetic strains, the latter in their turn predominating over the other bisexual species.

As a consequence, wild genetic *Artemia* spp. resources need monitoring and protection. This genetic diversity represents the source of novel traits, enables adaptation to envitonmental change such as from climate change or human impacts, and forms the basis for selective breeding for futher aquaculture. Inoculation experiments in natural habitats always represent a potential risk for local natural biodiversity; therefore, a risk assessment should always be undertaken prior to introduction – including screening of candidate strains and of possible local populations – as well as a study of prevailing environmental conditions. Thus, the socioeconomic advantages that may follow from the introduction of more productive allochthonous strains have to be carefully balanced against the possible loss of natural biodiversity.

1.4.6. Nutritional value

In the late 1970s, when many fish and shrimp hatcheries started to go commercial, switching from one source of *Artemia* to another provoked unexpected problems. Very significant differences in production yields were even obtained with distinct *Artemia* batches of the same geographical origin. The pattern of total lipids and fatty acid composition, as well as the metabolization of fatty acids by *Artemia*, seemed to differ widely from strain to strain, and even from batch to batch, as a consequence of the fluctuations in biochemical composition of the primary producers (mainly unicellular algae) available to the adult population (Léger *et al.*, 1987; see Table 3). In general,

cyst products from inland resources turned out to be more constant in composition, however, often at suboptimal low levels. Appropriate techniques have thus been developed to improve the lipid profile of deficient *Artemia* strains, taking advantage of the indiscriminate filter-feeding behaviour of *Artemia* (see Section 3.1.7).

TABLE 3
Crude protein, crude lipid and fatty acid profile of decapsulated *Artemia* cysts and newly hatched *Artemia* nauplii of strains of commercial relevance

	Populations							
	GSL	VC	BY	EBL	AIBI	TSP	LC	QC
	Decapsulated *Artemia* cysts							
Crude protein (% DW)	52.7	46.1	53.0	53.3	50.0	48.0	51.9	56.4
Crude lipid (% DW)	11.7	18.7	12.1	18.6	8.1	10.7	22.7	21.3
	Fatty acids (mg/g DW)							
C16:0	16.4	18.0	12.2	12.5	20.6	16.1	26.7	18.58
C16:1ω7	3.5	15.8	3.3	7.0	27.9	15.0	70.9	16.45
C18:0	7.2	4.4	7.2	6.3	6.1	5.2	9.6	5.69
C18:1ω9	26.4	19.2	19.1	23.7	33.2	24.8	50.5	44.63
C18:1ω7	8.4	13.3	7.0	13.7	14.1	10.1	25.6	20.65
C18:2ω6	9.7	4.1	5.5	10.3	6.0	9.8	7.0	17.10
C18:3ω6	1.3	1.3	0.5	0.5	0.3	1.4	8.3	17.16
C18:3ω3	42.6	3.6	26.8	35.9	10.0	10.4	4.3	9.84
C20:5ω3	1.5	17.0	1.2	3.2	14.5	14.5	53.2	30.53
C22:6ω3	–	–	–	–	–	0.7	0.8	1.25
	Newly hatched *Artemia* nauplii							
Crude protein (% DW)	54.4	N.D.	N.D.	N.D.	N.D.	54.5	56.2	55.6
Crude lipid (% DW)	9.3	N.D	N.D.	N.D.	N.D.	14.3	25.6	24.1
	Fatty acids (mg/g DW)							
C16:0	20.1	18.3	N.D.	N.D.	14.8	15.1	23.7	16.6
C16:1ω7	4.6	16.9	"	"	13.9	15.5	65.1	20.7
C18:0	8.2	5.1	"	"	6.2	5.0	9.1	6.4
C18:1ω9	30.6	20.7	"	"	28.7	21.3	51.0	50.9
C18:1ω7	9.9	15.6	"	"	15.2	11.9	25.9	12.0
C18:2ω6	9.7	5.1	"	"	8.1	8.4	6.1	13.6
C18:3ω6	1.2	1.3	"	"	0.7	8.2	7.0	16.2
C18:3ω3	40.2	4.3	"	"	27.5	2.0	3.6	7.3
C20:5ω3	2.0	19.6	"	"	14.7	16.1	51.6	30.9
C22:6ω3	1.0	-	"	"	-	-	0.6	0.7

Dash (–) denotes below detection level.

Note: AIBI = Lake Aibi, China; BY = Bolshoe Yarovoe, Russian Federation; DW = dry weight; EBL = Ebeyty, Russian Federation; GSL = Great Salt Lake, United States of America; LC = Lagkor Co, China; N.D. = no data; QC = Qixiang Co, China; TSP = Tanggu salt ponds, Bohai Bay, China; VC = Vinh Chau, Viet Nam.

Source: Authors' own elaboration (ARARC, China and LAARC).

A number of other compounds also appear to be variable: nutritional components, such as the total amount of free amino acids, pigments (canthaxanthin), vitamin C, minerals and trace elements, as well as contamination with chemicals, such as pesticides and heavy metals. In most cases, these variations are not strain-specific but just correspond to different production conditions. Despite this, their effects on larviculture success are usually not considered as significant.

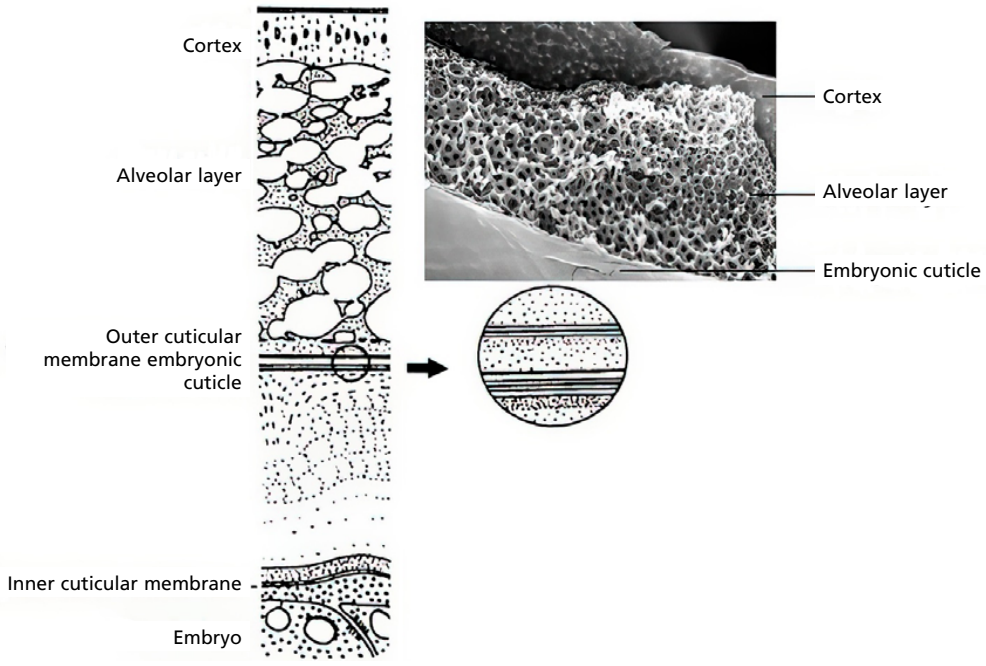
1.5. CYST BIOLOGY

1.5.1. Cyst morphology

The cyst shell consists of three layers (Figure 3):

- 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 Section 3.1.4).
- Outer cuticular membrane: This multilayer membrane acts as a molecular "sieve" or permeability barrier, allowing only molecules with the size of the carbon dioxide (CO_2) molecule or smaller to pass.
- 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).

FIGURE 3
Schematic diagram (left) and scanning microscopic images (right) of the ultrastructure of an *Artemia* cyst



Left: modified from Morris and Afzelius, 1967. **Right:** from Wang and Sun, 2007.

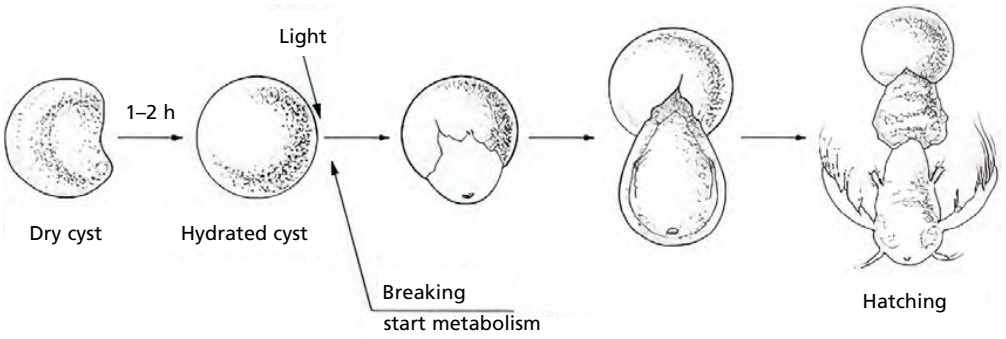
Sources: Morris, J.E. & Afzelius, B.A. 1967. The structure of the shell and outer membranes in encysted Artemia salina embryos during cryptobiosis and development. *Journal of Ultrastructure Research*, 20(3-4): 244-259. https://doi.org/10.1016/S0022-5320(67)90285-7
Wang, S.F. & Sun, S.C. 2007. Comparative observation on the cyst shells of seven Artemia strains from China. *Microscopy Research and Techniques*, 70: 663-670. https://doi.org/10.1002/jemt.20451

It is generally assumed that the embryo is an undifferentiated gastrula, which is ametabolic at water levels below approximately 10 percent and which can be stored for long periods without losing its viability (see Section 3.1.2). The viability is affected when cysts are stored at water levels higher than 10 percent (start of metabolic activity) and when they are exposed to oxygen; that is, the presence of oxygen results in the formation of free radicals that destroy specific enzymatic systems in the ametabolic *Artemia* cysts.

1.5.2. Physiology of the hatching process

The development of an *Artemia* cyst from incubation in the hatching medium until nauplius release is shown in Figure 4.

FIGURE 4
Development of an *Artemia* cyst from incubation in seawater until nauplius release



Source: Authors' own elaboration (ARARC, China).

When incubated in seawater, the biconcave cyst swells up and becomes spherical within 1 to 2 h. After 10 to 20 h hydration, the cyst shell (including the outer cuticular membrane) bursts (= breaking stage) and the embryo surrounded by the hatching membrane become visible. For most *Artemia* strains, a light trigger, at least during the initial hours of hatching incubation following full hydration, is essential to switch on the hatching metabolism (Vanhaecke, Cooreman and Sorgeloos, 1981; Van der Linden, Blust and Decleir, 1985; El-Magsodi *et al*., 2016).

The embryo then leaves the shell completely and hangs underneath the empty shell (the hatching membrane may still be attached to the shell; this stage is called the "umbrella" stage). 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. A hatching enzyme (active at pH >8.0), secreted in the head region of the nauplius, weakens the hatching membrane. Soon, the hatching membrane breaks open (= hatching stage), which enables the free-swimming nauplius, head first, to liberate itself into the hatching medium.

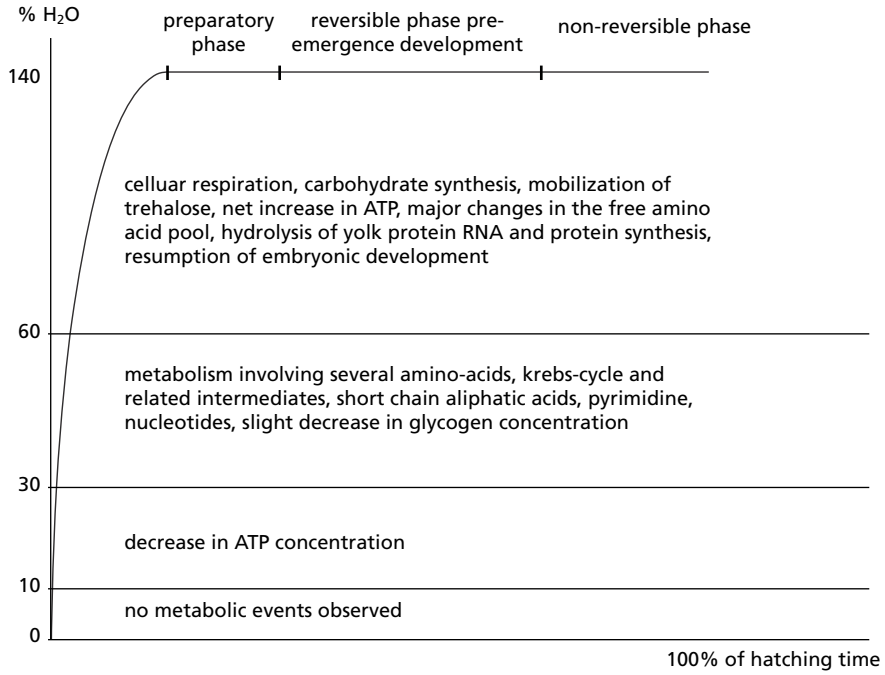
Dry cysts are very hygroscopic and take up water at a fast rate (that is, within the first hours of incubation in seawater, the volume of the hydrated embryo increases to a maximum of 140 percent water content [Figure 5]). However, the active metabolism of the embryo is resumed once a water content of 60 percent has been reached and provided that environmental conditions are favourable (e.g. oxygen, temperature; see Section 3.1.5).

The aerobic metabolism in the cyst embryo assures the conversion of the carbohydrate reserve trehalose into glycogen (as an energy source) and glycerol. 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 that will result in the shell bursting, and less energy reserves will thus be left in the nauplius (Figure 6).

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

FIGURE 5

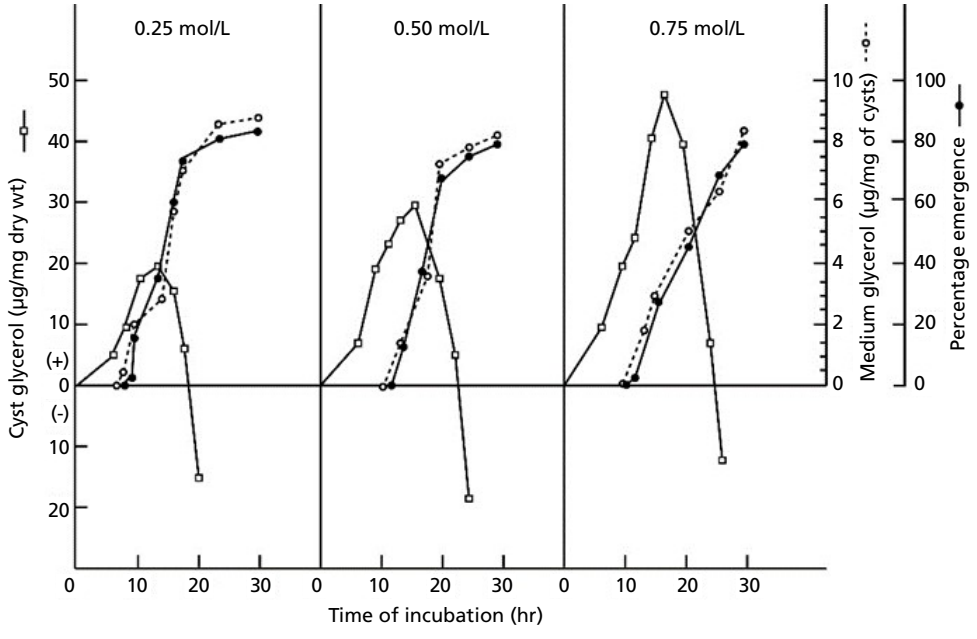
Cellular metabolism in *Artemia franciscana* cysts as a function of hydration level



Source: Lavens, P. & Sorgeloos, P. 1987. The cryptobiotic state of *Artemia* cysts, its diapause deactivation and hatching, a review. In: P. Sorgeloos, D.A. Bengtson, W. Decleir & E. Jaspers, eds. *Artemia Research and Its Applications*, pp. 27–63, Vol. 3. Wetteren, Belgium, Universa Press.

FIGURE 6

Relationship between the concentration of glycerol in the cysts, the glycerol level in the medium and the percentage of cysts in the breaking stage (emergence) versus the time of incubation of *Artemia franciscana* cysts at three different concentrations of NaCl (0.25 molar NaCl = 14.6 g/L salinity)



Source: Clegg, J.S. 1964. The control of emergency and metabolism by external osmotic pressure and the role of free glycerol in developing cysts of *Artemia salina*. *Journal of Experimental Biology*, 41: 879–892. https://doi.org/10.1242/jeb.41.4.879

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 that will result in the shell bursting, and less energy reserves will thus be left in the nauplius (Figure 6).

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.

1.5.3. Effect of environmental conditions on cyst metabolism

Dry cysts (water content from 2 percent to 5 percent; see Annex 9 for determination of water content) are very resistant to extreme temperatures, with hatching viability not being affected in the temperature range from –273 °C up to about 60 °C, and up to 90 °C for only short exposures being tolerated.

Hydrated cysts have far more specific tolerances with mortalities occurring below approximately –18 °C and above +40 °C, and active cyst metabolism situated between ±4 °C and ±33 °C: Within this range, the hatching percentage remains constant, but the nauplii hatch earlier as the temperature is higher. A reversible interruption of the metabolism, with viability not being affected, occurs between –18 °C and +4 °C and between ±33 °C and ±40 °C. These temperature ranges and their upper and lower limits, however, may vary slightly from strain to strain.

As for other environmental conditions, optimal hatching outputs are reached in the pH range of 8.0-8.5. Therefore, the addition of sodium bicarbonate ($NaHCO_3$) up to 2 g/L or regular addition of sodium hydroxide (NaOH), as practiced in many hatcheries, 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. As for oxygen, an increased hatching has been reported with increasing oxygen level in the range of 0.6-2.0 mg/L, and maximal hatching is 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 (see Section 3.1.5 on optimal hatching conditions).

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 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 is in practice generally situated in the range of 15–35 g/L (although it can be as low as 5 g/L).

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.

1.5.4. Diapause

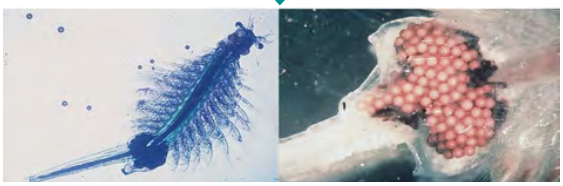
Because *Artemia* is an inhabitant of biotopes characterized by unstable environmental conditions, its survival during periods of extreme conditions (desiccation, extreme temperatures and high salinities, among others) is ensured by the production of diapausing embryos. *Artemia* females can indeed easily 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 and salinity changes, for instance). 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 cannot be distinguished from a non-living embryo. Upon the interruption of diapause, cysts enter the stage of quiescence, meaning that development can be resumed at the moment they are brought into favourable hatching conditions, eventually resulting in hatching: In this phase, the developmental arrest is uniquely dependent on external factors (see Figure 7). As a result, synchronous hatching metabolism occurs, resulting in a fast start and consequent development of the population shortly after the re-establishment of favourable environmental conditions. This allows for effective colonization in temporal biotopes.

FIGURE 7
Schematic diagram explaining the specific terminology used in relation with diapause of *Artemia* embryos

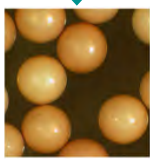
Gastrulae

Diapause induction



Encysted embryos

Deposition in habitation



Diapausing cysts

Diapause deactivation
(e.g. desiccation)

Quiescent cysts

Hatching under favourable
environmental circumstances

Instar I nauplii

Source: Authors' own elaboration (ARARC, China).

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, and hence the difficulty of predicting the effect on hatching outcome. When working with new or relatively unknown strains, the relative success or failure of certain methods has to be discovered empirically.

In many cases, the removal of cyst water content is an efficient way to terminate the state of diapause. This can be achieved by drying the cysts at temperatures not exceeding 35–40 °C or by suspending the cysts in a saturated NaCl brine solution (300 g/L). 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 do not yield a sufficiently high hatching quality, indicating that a more specific diapause deactivation method is necessary.

The following procedures have proven to be successful when applied with specific sources of *Artemia* cysts:

- Freezing: This "imitates" the natural hibernation period of cysts originating from continental biotopes with low winter temperatures (for example, Great Salt Lake, in the United States of America, and lakes in Siberia, the Russian Federation, and Kazakhstan; see Table 4).
- 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 on the optimal dose/period to be applied and the maximal effect that can be obtained (see Table 5). 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 (Van Stappen, Lavens and Sorgeloos, 1998; Robbins *et al.*, 2010).

In general, other diapause termination techniques (cyclic dehydration/hydration, decapsulation, γ radiation, other chemicals, etc.) 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) rather than from an increased hatching itself.

TABLE 4
Effect of cold storage at different temperatures on the hatchability of *Artemia* cysts from Kazakhstan (non-specified lake)

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

Source: Authors' own elaboration (LAARC).

TABLE 5
Dose-time effect of H_2O_2 incubation treatment on the hatching percentage of *Artemia* cysts from Kazakhstan (non-specified lake); hatching percentage of control (without H_2O_2 incubation) = 18.6 percent

Incubation time (min)	Concentration of H_2O_2 solution (%)				
	1.0	5.0	10.0	20.0	27.0
1	31.4	50.8	62.4	53.8	42.2
5	36.6	64.6	69.2	58.2	47.9
10	46.4	36.7	39.7	68.5	45.0
15	48.8	74.7	76.1	48.6	28.2
20	47.1	76.2	74.2	66.5	17.6
30	49.1	47.7	45.3	25.6	9.5

Source: Van Stappen, G., Lavens, P. & Sorgeloos, P. 1998. Effects of hydrogen peroxide treatment in *Artemia* cysts of different geographical origin. Arch. *Hydrobiol. Spec. Issues Advanc. Limnol.*, 52: 281–296.

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2. *Artemia* production in salt lakes and salt ponds

2.1. DESCRIPTION OF DIFFERENT *ARTEMIA* HABITATS

2.1.1. Natural salt lakes

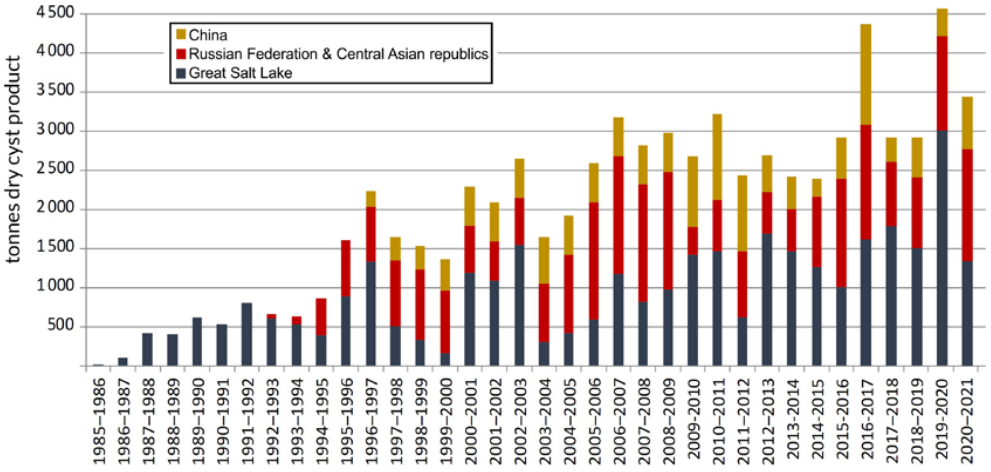
2.1.1.1. Introduction (by Gilbert Van Stappen)

As described in Section 1.2, *Artemia* occurs naturally in numerous saline lakes, varying widely in size, topography, biotic and abiotic conditions (Triantaphyllidis, Abatzopoulos and Sorgeloos, 1998; Van Stappen, 2002). Typically, these lakes are located in so-called endorheic drainage basins (hence their qualification as "terminal lakes"), i.e. basins that receive water from tributary rivers but that have no outflow to other external waterbodies, such as other rivers or the sea or ocean. As a result, the water level and salinity in these lakes equilibrate mainly through the incoming water volumes from rivers, but also from precipitation and direct runoff from surrounding areas, versus evaporation, with underground seepage as a less visible additional factor. In these lakes, brine shrimp population densities are usually low and mainly fluctuate as a function of the local conditions, with food availability, temperature and salinity being the most important ones. The size of these lakes, even the smallest ones, makes efficient management in terms of maximizing *Artemia* exploitation very difficult, if not impossible. Consequently, harvesting cysts and/or biomass from natural salt lakes is basically extensive harvesting of (part of) the standing crop present in the lake.

In the 1960s, two companies in the United States of America started to market *Artemia* cysts collected from the salt ponds in the San Francisco Bay, California, and from the Great Salt Lake (GSL), Utah. The GSL continued to be the main resource of cysts until the 1990s. However, with aquaculture demand expanding and the harvests from GSL appearing to fluctuate over the years, the threat of a possible cyst shortage on the market triggered cyst harvesting companies and other stakeholders towards – among others – the exploitation of alternative salt lakes, aiming to diversify and secure future *Artemia* cyst supply. New resources, mainly in continental Asia (e.g. China, Kazakhstan, Siberia, Russian Federation, Turkmenistan and Uzbekistan), were explored from the late 1990s and onwards for their production potential and characteristics of the local *Artemia* populations. In many of these lakes, *Artemia* exploitation on a regular basis started (Lavens and Sorgeloos, 2000; Dhont and Sorgeloos, 2002).

Currently, the geographical origin of cysts from natural salt lakes commercially available on the world market is rather diverse: The cysts may not only originate from the Great Salt Lake but also from salt lakes in Central Asia and inland China, with a relative share that has been fluctuating over the years (Figure 8). Diversification of *Artemia* cyst resources is crucial, as many inland salt lakes, small or large, are highly vulnerable to the effects of climate change, such as desertification, reduced salinity through excessive precipitation or glacier melting, or major changes in temperature regime. As these lakes are often located in (semi–) arid areas with limited freshwater resources, the fragile balance of salt lake ecosystems may also suffer from human activities in the lakes' drainage basin, especially reduced water inflow (in extreme cases resulting in complete desiccation of the lake), as water from incoming rivers is diverted for agriculture and industrial and urban purposes. Additionally, these terminal salt lakes may see accumulation of pollutants originating from the waterbodies located in their drainage basin. These factors may have an effect on the local *Artemia* population

FIGURE 8
Fluctuations in *Artemia* cyst harvests (in tonnes of commercial dry product) from lakes in China, from the Russian Federation and Central Asian Republics, and from the Great Salt Lake, United States of America



Source: INVE, Belgium.

and on the cyst quantities available for harvesting (Gajardo and Beardmore, 2012). As the brine shrimp population is the only or dominant aquatic metazoan in these salt lakes, it is also an important element in the ecosystem; *Artemia* biomass has a crucial role as a source of food for migrating waterbirds. Hence, large-scale exploitation should be coupled with population studies in the field, aiming to define maximal sustainable yields and to organize sustainable harvesting programmes.

Therefore, the following sections in this manual, describing, respectively, *Artemia* production in the Great Salt Lake, in Chinese lakes and in Russian lakes are intended to mirror the diversity of products that find their way to the market and the diversity of environments from where these products originate. Additionally, this chapter offers some selected reading on specific salt lakes – Lake Urmia in the Islamic Republic of Iran; Lake Aral (also known as the Aral Sea) in Kazakhstan/Uzbekistan; and Lake Kara-Bogaz-Gol in Turkmenistan) – illustrating extreme cases of long-term temporality relating to *Artemia* presence (and harvests) and explaining potential interference of climate change and human factors.

2.1.1.2. Great Salt Lake: a case study on sustainable management of the Artemia *population* (by *Phil Brown*, *Brad Marden* and *Thomas Bosteels*)

Great Salt Lake topography and ecology

The Great Salt Lake (GSL) of Utah, United States of America, is a 3 600 km^2 hypersaline terminal lake occupying the lowest portion of a 56 000 km^2 watershed at the eastern edge of North America's expansive Great Basin. The GSL is a mosaic of environmental variability and wildlife habitat, ranging from freshwater wetlands to brackish embayments to hypersaline open waters. Three rivers empty into GSL from the surrounding mountains, feeding expansive wetlands on the eastern margins of the lake before flowing into the open bays. The wetlands and playas support a diverse array of migratory and resident waterfowl and shorebirds. The seasonally brackish bays can support zooplankton assemblages and some fish near the river mouths.

The main body of GSL has been divided by a solid fill causeway constructed across the middle of the lake in 1959; this causeway restricts water flow, alters salinities, and thereby modifies the ecology of each bay (Plate 15). The small culverts, present in the causeway from the beginning, were replaced with a 60 m wide opening in a deeper

location with an adjustable berm in 2017. Despite this, water exchange across the causeway is limited and the bays remain hydrologically distinct. Gunnison Bay, north of the causeway, receives freshwater only through direct precipitation and groundwater inflow, and evaporation keeps salinity at or near saturation most years. Gilbert Bay in the south is 65 km long and receives the inflow of all three rivers, and salinity has ranged from 100 g/L to 150 g/L over the past 10 years. It supports the overwhelming majority of the lake's resident *Artemia franciscana* population during most years.

PLATE 15
Map of the Great Salt Lake depicting river inflow, causeway and causeway breach locations

Source: Satellite image courtesy of the Earth Science and Remote Sensing Unit, viewed in April 2021 NASA Johnson Space Centre, United States of America (https://eol.jsc.nasa.gov).

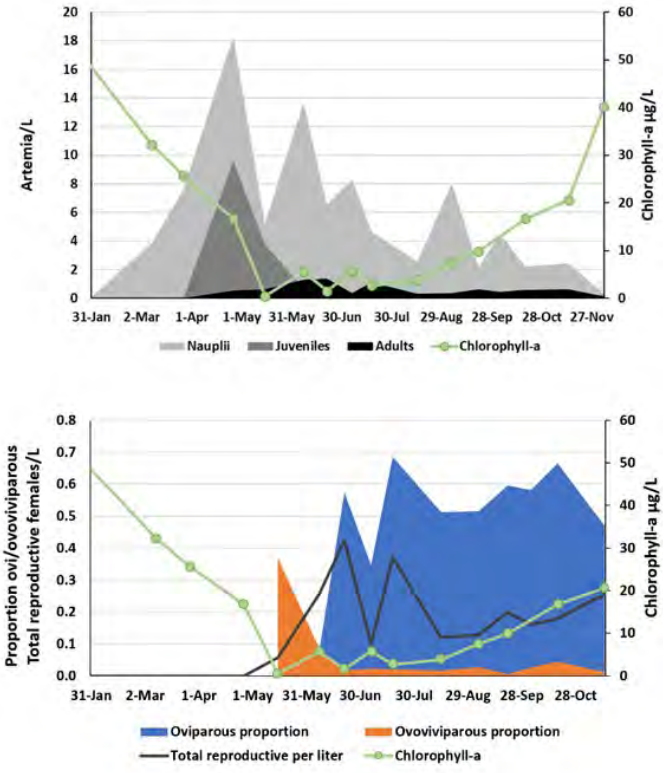
The GSL *Artemia* population provides important ecosystem functions and services at local and hemispheric scales. Gilbert Bay is a productive aquatic system, and the *Artemia* rapidly convert the abundant phytoplankton biomass into forms usable by both wildlife and humans. At the local scale, *Artemia* control eutrophication to maintain water clarity. Phytoplankton chlorophyll–α concentrations in Gilbert Bay average 40–50 µg/L in the *Artemia*-free months and can substantially exceed it, resulting in eutrophic conditions that would be of concern in less saline systems vulnerable to harmful cyanobacterial blooms, anoxia and fish die–offs. However, in hypersaline Gilbert Bay, eutrophication is of little concern because the *Artemia* are able to quickly consume the phytoplankton, reduce chlorophyll–α to less than 1 µg/L, and increase water clarity to the benefit of human recreational use and industrial salt extraction. At the hemispheric scale, *Artemia* are the link between the abundant phytoplankton biomass and energetic needs of migratory bird species. Gilbert Bay is an annual staging location for up to 4.5 million eared grebes (*Podiceps nigricollis*), which feed primarily on *Artemia* to gain weight for their energy reserves in preparation for continued migration south to their overwintering grounds, and several duck species, which utilize both *Artemia* adults and cysts during harsh winter months. Gilbert Bay is also the largest single supplier of *Artemia* cysts for aquaculture operations across the globe.

Great Salt Lake Artemia *population dynamics*

The annual population cycle of GSL *A. franciscana* is shaped by the surrounding climate and seasonal environmental conditions within the lake. It is located in a continental climate with cold winters lethal to free-swimming *Artemia*, and the population overwinters as diapausing cysts. The life cycle begins anew in March when the cysts typically begin hatching in response to warming temperatures and increased freshwater inflow from annual snowmelt. Maturation and growth are slow for the first weeks but proceed rapidly as temperatures continue to warm. Nauplii mature into juveniles and adults in April (Figure 9). The first reproduction by live birth (ovoviviparity) of nauplii occurs in late April and May through the largest brood sizes of the year. The resulting pulse of second generation nauplii and the abundant algal food

FIGURE 9

Densities per litre of broad *Artemia* age classes and chlorophyll–α values in 2018 (top): Shift in reproduction from live birth (ovoviviparity) to cysts (oviparity) in 2018, with densities per litre of total reproductive females (bottom)



Source: GSLBSC, United States of America.

supply create an exponential increase in population size and simultaneous peaks of first and second generation *Artemia* in May or early June. An abundant phytoplankton food supply greets the first and second generation *Artemia* each year because the lake does not freeze over and therefore phytoplankton in winter is limited by nutrients and self-shading rather than by ice cover. *Artemia* are efficient grazers, and their rapid population growth soon depletes this resource. As the phytoplankton is grazed down, water clarity can increase from an overwinter low of less than 0.5 m to a full 6 m. Food and oxygen simultaneously become limiting for the *Artemia* and population numbers drop severely because of starvation and oxygen stress.

The period of early summer starvation is a stress event which appears to influence the *Artemia* population throughout the late summer and autumn. *Artemia* demographics proceeding from this point are distinctly different. The phytoplankton is released from heavy grazing pressure when the *Artemia* decline and begins to rebound. The *Artemia* respond to this in turn with increases in live birth, but the reproductive mode generally has shifted toward cyst production (oviparity) and away from the live birth that dominated prior to phytoplankton depletion (Figure 9). The phytoplankton and *Artemia* continue to fluctuate opposite of each other, with modest phytoplankton gains grazed by smaller increases in the *Artemia* population. Each cycle of increase and decline is less extreme than in early summer. The stability in primary producer and grazer interactions continues into September and October. On average, the overall *Artemia* population density remains relatively static in these months, but abundances of cyst-bearing females and their clutch sizes increase. Cyst densities within Gilbert Bay rise throughout August and September, and usually peak in October. Adult densities decline in November with falling water temperatures and may persist in lower numbers well into December, during which temperatures below 7 °C usher in the end of the free-swimming population. A typical year in the GSL results in three or four often overlapping generations of *Artemia*.

The above demographic patterns are descriptive of a typical year seen in the past 25 years of monitoring data, and can differ when environmental conditions, notably salinity, deviate from an optimal range. *Artemia* are proficient osmoregulators and can physiologically tolerate a wide range of salinity from brackish to hypersaline to near-saturation, but in natural environments they are controlled by predation and competition across much of the lower salinity spectrum. Predation by the aquatic insect Trichocorixa verticalis (Plate 16) greatly diminished the GSL *Artemia* population during a period of unusually high runoff, which reduced Gilbert Bay salinities to < 60 g/L from 1985 to 1987 (Wurtsbaugh and Berry, 1990), and viable populations of the related *Trichocorixa reticulata* have been observed in *Artemia* habitats of 100 g/L

PLATE 16
Dorsal view of *Trichocorixa verticalis*

© SEC

(Herbst, 2006). Furthermore, GSL salinities declining from 120 g/L to 76 g/L during the late 1990s correlated with poor *Artemia* reproduction and shifts in phytoplankton assemblage that may have been unfavourable (Stephens and Birdsey, 2002; Belovsky and Perschon, 2019). Salinities below 120 g/L, therefore, carry a risk of both top-down predatory and bottom-up phytoplankton controls on the *Artemia* population.

Regarding the upper salinity tolerance, laboratory and field data show a decline in GSL *Artemia* abundance and survival above 160 g/L (Marden, Brown and Bosteels, 2020); this is generally supported by studies on other *Artemia* populations involving survival and reproduction (Dana and Lenz, 1986; Triantaphyllidis *et al.*, 1995; Abatzopoulos *et al.*, 2003). The available evidence from the literature and field observations indicates that the optimal salinity range for the GSL *Artemia* population is 120 g/L to 160 g/L, a range which Gilbert Bay has stayed within 20 g/L for 22 of the last 26 years despite substantial interannual variations in precipitation and water supply. The extensive *Artemia* population monitoring on GSL has demonstrated the population's ability to quickly rebound once salinities have returned to the optimal range.

In addition, because of the limited mixing between upper and deeper, more saturated and denser water layers, the latter act as a nutrient processor and sink as they accumulate organic matter from the productive *Artemia* habitat above. The isolation of these limiting nutrients within these deep layers highlights the need to maintain nutrient input to the upper water layers from the rivers. While nutrient dynamics within GSL are thus primarily driven by internal cycling of the nutrient pool, annual nutrient gains and losses from the productive upper water layers of GSL can become substantial over the course of multiple years. Riverine nitrogen amounts to nearly 10 percent of Gilbert Bay's nitrogen load annually (Naftz, 2017), demonstrating the necessity of continued nutrient supply to the lake in the face of annual losses.

The durability and dormancy of GSL *Artemia* cysts are instrumental in their ability to persist in the lake. A crucial environmental condition that free-swimming *Artemia* in any location cannot survive is seasonality of habitat. In salterns and shallower saline lakes, seasonality may be the loss of habitat or suitable salinities due to evaporation, but in the permanent GSL it is the lethal winter temperatures. However, the suite of protective adaptations (see Section 1.5) cannot entirely block environmental stresses, and *Artemia* cysts will lose viability over time in the natural environment. In the harsh natural environment of the GSL system, cyst viability remains high over a single winter (Marden, Brown and Bosteels, 2020), but multi-year exposure to the heat, ultraviolet radiation and precipitation-caused hydration events on the shoreline will substantially lower viability, which has consequences for its storage conditions (see Section 3.1.2).

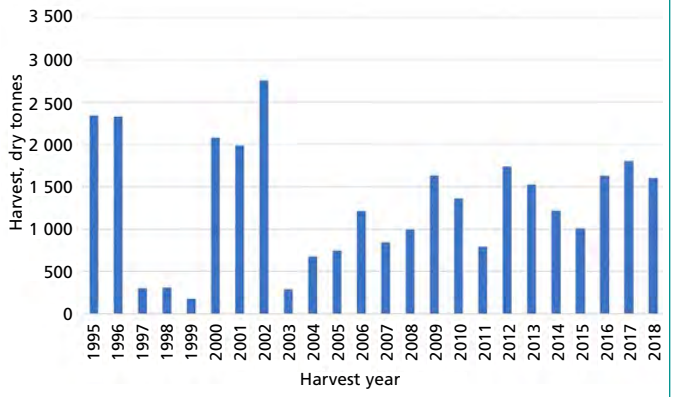
GSL Artemia *harvest management*

Commercial interest in GSL *Artemia* began decades ago, in the 1950s, but it was initially limited to the use of *Artemia* adult biomass for the tropical fish hobbyist market (Sturm, Sanders and Allen, 1980). The harvest shifted to *Artemia* cysts, as the ability of the dormant embryos to be utilized in commercial aquaculture was

recognized and developed. Harvest pressure for those first decades remained low, and harvest management by the overseeing government agency (Utah Division of Wildlife Resources–UDWR) reflected this. Management was limited to a simple permit system and the charging of small royalties. However, the tandem expansion of commercial demand and harvesting acumen on GSL in the late 1980s and early 1990s (Kuehn, 2002) necessitated a change in management strategy. The UDWR increased permit fees and shortened the harvest season during this time. Still, continually increasing harvest pressure in the mid-1990s concerned many in the harvest industry, who requested that the UDWR consider a more active management of the resource.

As conservation concerns mounted, a cooperative adaptive management began to take shape. The UDWR formed a multidisciplinary technical advisory group to examine existing ecological information and direct future research. The UDWR capped the number of permits at 79 in 1996 and further shortened the harvest season to four months (Stephens and Birdsey, 2002). A harvest management approach began that prioritized a minimum stock of *Artemia* cysts that must remain in the lake to repopulate it in the spring. Estimates of this minimum were rudimentary and subject to assumptions and caution during the first several years due to limited field data, but continued data collection soon enabled a UDWR management model which correlates the post-harvest cyst density with the following year's peak cyst production. The model is loosely based on the so-called Ricker stock recruitment curve (Ricker, 1954) and predicts optimal autumn cyst production when 21 cysts/L are left in the lake the prior spring (Belovsky and Perschon, 2019). The management model is adaptive and incorporates data from each year, but changes in the model over time have been too minor to modify the 21 cysts/L management threshold for ending the harvest. The management model has operated effectively for two decades, but is specific to GSL. The 21 cysts/L threshold is not applicable to other *Artemia* populations and species in dissimilar lake and pond systems, each of which will require individual research programmes to establish appropriate management strategies.

FIGURE 10
Annual fluctuations in *Artemia* cyst harvest (in final dry weight) from the Great Salt Lake



Source: GSLBSC, United States of America.

Implementation of this adaptive science-based cooperative management strategy between the brine shrimp industry and resource managers has thus led to consistency in the harvest size (Figure 10), after a period (1980s and 1990s) when harvesting tonnage was variable. Through cooperative management, the needs of local ecology, local economics and global aquaculture have been met for more than two decades, which is a rare convergence of positive outcomes in natural resource management situations through an approach that incorporates the lake system as a whole and the interests of the various stakeholders.

Managing the challenges to the modern day GSL ecosystem

The local and global ecosystem services conferred by GSL are unfortunately repaid by ecosystem challenges of local and global origin. Nearly 2.5 million people live in the adjacent metropolitan areas and the GSL watershed still includes extensive agriculture, all of which influence the quantity and quality of water reaching the lake. GSL is vulnerable to the same ecological damage suffered by numerous other terminal lake systems across the globe if consumptive water uses continue to go unchecked. Already, cumulative human water use has reduced GSL volumes substantially (Null

and Wurtsbaugh, 2020), and although global climate change is predicted to increase precipitation in Utah, increased rainfall in lieu of snowpack will require changes in water infrastructure in order to maintain riverine inflows into the lake. If left unchecked, declining river inflows could have impacts on the GSL *Artemia* population through increased salinities and a reduction in habitat volume. Fortunately, recent legislative and stakeholder efforts have already secured increased water flows to GSL, and additional infrastructure budgets are being implemented to further mitigate these issues.

Dust generation from exposed GSL lakebeds may expand the impacts of water loss beyond the aquatic ecosystem and towards human health and economics. Dust from regional playas is deposited in urban areas and mountain snowpack (Goodman *et al*., 2019), which can harm human respiratory health and hasten snowpack melting within the GSL watershed (Skiles *et al.*, 2018), as has been demonstrated for other desiccating inland salt lakes (see Box 2 and Box 3). As a result of federal and state laws mandating pollution discharge reductions, most heavy metals have been decreasing in GSL sediments since 1950, and recent systematic ongoing research is tracking contaminants such as heavy metals in the GSL ecosystem, but has so far indicated relatively stable and low levels in the biota. Nevertheless, continued focus on water inflows will be required to avoid excessive dust generation.

The adverse ecological impacts of reduced river inflow to the open waters of GSL, and how these can be mitigated, are being carefully considered by various Utah state agencies and committees. Another important tool for implementing management recommendations from these collaborative efforts is the control berm at the 2017 opening in the railroad causeway separating the Gilbert and Gunnison Bays. Salinity interchange between bays is governed in part by the elevation of the berm, and can be adjusted in the future according to mass balance modeling (Loving, Waddell and Miller, 2000) to compensate for changing lake elevations, with the 120–160 g/L salinity optimum providing an ecologically defensible management target for any such modifications of the berm. Years of research on Gilbert Bay have demonstrated a high assimilative capacity of nutrients, such as nitrogen and phosphorus, of this unique hypersaline food chain, quickly leading to nutrient limitation in summer. Even though the vast majority of nutrients are cycled throughout the different trophic levels in GSL and therefore remain in the ecosystem, preservation of nutrient inflows is important for the long-term protection of the *Artemia* population in the lake. These concerns are being addressed through research and stakeholder involvement coordinated by the Utah Division of Water Quality. The combination of nutrient and salinity management, both important drivers of the ecosystem, has buffered the *Artemia* population against changes in lake levels resulting from the present drought cycle.

Conclusions

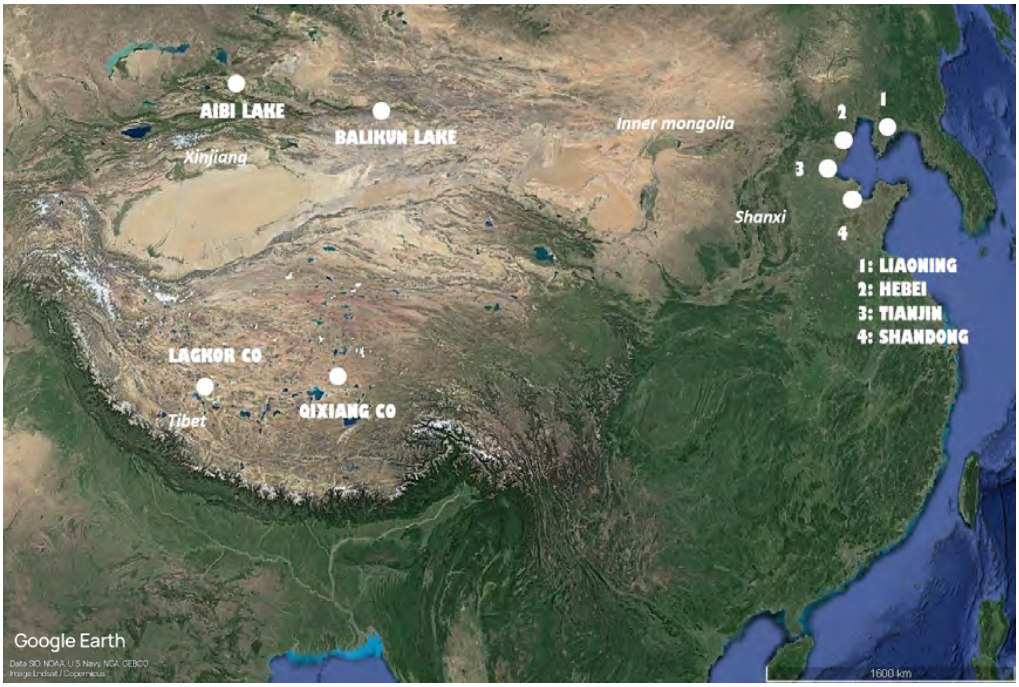
The GSL *Artemia* population is a critical component of local ecosystem functioning and global aquaculture production. The population has been examined in detail through long-term monitoring programmes, as briefly described in this section. While the GSL shares the same risks and threats as other saline lakes, the network of public and private stakeholders with a long-term interest in the continued health of the GSL ecosystem has provided cooperative management strategies that successfully mitigate these risks.

2.1.1.3. Chinese Artemia *resources* (by *Liying Sui*)

In China, *Artemia* are found in more than 100 natural habitats (including salt lakes and coastal solar saltworks). Three indigenous *Artemia* species (i.e. *Artemia sinica* and *A. tibetiana* and *A. sorgeloosi*) and several parthenogenetic populations have been identified in Chinese salt lakes (Triantaphyllidis *et al*., 1994; Xin *et al*., 1994; Van Stappen, 2002; Zheng and Sun, 2013; Asem *et al.*, 2023). *A. sinica* is found in the

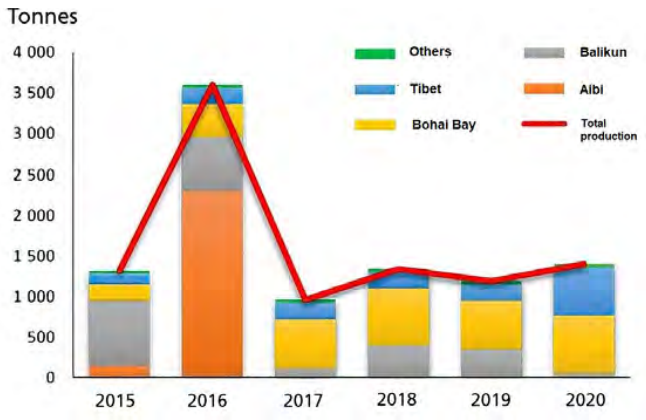
salt lakes of Inner Mongolia, in Shanxi Province, and in the north of Hebei Province; *A. tibetiana* and *A. sorgeloosi* are distributed in most of the Tibetan salt lakes, whereas the other areas are dominated by parthenogenetic *Artemia*. Commercial harvest of *Artemia* cysts is mainly conducted in inland salt lakes (with Lake Aibi, Lake Balikun, Qixiang Co, also called Kyebxang Co, and others as the most important lakes) and in coastal salt ponds in Bohai Bay region (Figure 11 and Figure 12).

FIGURE 11
Main *Artemia* cyst harvesting sites in China



Source: Google Earth viewed in April 2023 and ARARC, China.

FIGURE 12
Raw *Artemia* cyst harvests from the main Chinese harvesting sites

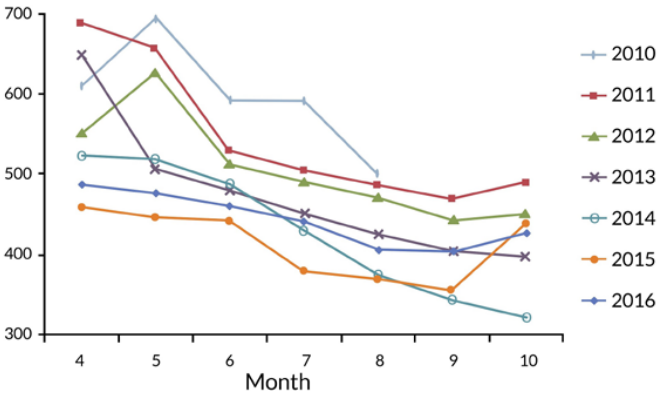


Source: AAC, China.

Lake Aibi is the largest *Artemia* cyst producing site in China. The commercial harvest in Lake Aibi started in 1999, with annual raw cyst yields peaking at 2 200 tonnes in 2012 and 2016, while the lowest harvest (142 tonnes) was obtained in 2015 (Figure 12). Harvesting cysts in Lake Aibi has been prohibited by the government since 2017. Moreover, being a very shallow lake (minimum 0.7-0.8 m, maximum 3 m), the salinity (minimum 45 g/L, maximum 240-250 g/L) of the water varies remarkably from season to season and from year to year, with corresponding changes in the lake surface area (Figure 13) and in the development of the *Artemia* population. The hatching quality of Aibi *Artemia* cysts also differs significantly when harvested from salinities < 85 g/L or > 180 g/L; better cyst hatching quality is usually obtained when cysts are harvested from a salinity range of 120-150 g/L (Plate 17).

Artemia resources also exist in the salt lakes on the Qinghai-Tibet Plateau, where low oxygen level, low precipitation and high UV radiation are the major climatological features. Most of the salt lakes in Tibet, China, are carbonate or sulphate waters, and are located in Nagqu and Ngari regions with an average altitude of 4 000-4 500 m (Zheng, 1997) (Plate 18). In 2020, the total raw cyst yield in Tibetan salt lakes (mainly from Qixiang Co and Lagkor Co) reached over 800 tonnes. The *Artemia* population originating from Lagkor Co was identified as *A. tibetiana* in the 1990s (Abatzopoulos, Zhang and Sorgeloos, 1998) and from Haiyan Lake

FIGURE 13
Variation in the water surface area of Lake Aibi in the period 2010–2016



Source: Guo Yan, XARI, China.

PLATE 17
Harvest of *Artemia* cysts from Lake Aibi in China

PLATE 18
Harvest of *Artemia* cysts from Qixiang Co

as *A. sorgeloosi* in 2023 (Asem *et al.*, 2023). However, by means of molecular markers and mitochondrial genome sequencing, it has been found that *Artemia* populations in different Tibetan salt lakes have differentiated to some extent (Wang *et al.*, 2008). The *Artemia* cyst resources in Qixiang Co and Lagkor Co have been commercially

harvested and processed since the 1990s. In general, the *Artemia* cysts from Tibetan salt lakes are larger, are dark-red in colour and contain higher levels of highly unsaturated fatty acids (HUFAs) – i.e. arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (see Section 1.4.6) – than other *Artemia* species and strains. Moreover, generally, a better separation can be obtained between the empty cyst shells and nauplii upon hatching. Because of these characteristics, Tibetan *Artemia* strains better meet the nutritional and production needs of marine fish (e.g. grouper) and shrimp (e.g. *Litopenaeus vannamei*) larviculture (Xing *et al*., 2022; Liu *et al*., 2022).

China has the world's largest market demand of *Artemia* cysts: Annually, Chinese aquaculture consumes around 1 700 tonnes of dry product (which is about 50 percent of the global cyst consumption). *Artemia* cysts are mainly used in the larviculture of penaeid shrimp, freshwater prawn (*Macrobrachium* sp.), Chinese mitten crab (*Eriocheir sinensis*) and marine fish (e.g. grouper, sole). Since recently, *Artemia* cysts are also demanded for the hatcheries of snail species (e.g. the freshwater golden apple snail, *Pomacea canaliculata*) and largemouth bass (e.g. *Micropterus salmoides*). Whereas annual domestic production is generally in the range of 1 000–1 500 tonnes of raw cysts (Figure 12), China is also the world's largest importer of *Artemia* cysts: Annually, 2 000–3 000 tonnes raw *Artemia* cysts are imported from Kazakhstan, Russian Federation, and Uzbekistan. Moreover, China annually exports 400–600 tonnes of dry cyst product (information provided by the *Artemia* Association of China, Tianjin, China).

2.1.1.4. **Artemia *in lakes in the Russian Federation*** (by *Liudmila Ilinichna Litvinenko*, *Alexander Ivanovich Litvinenko*, *Elena Grigoryevna Boyko*)

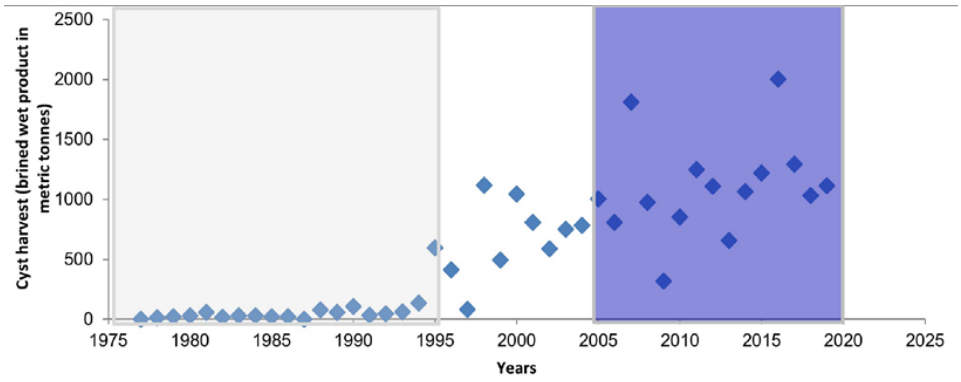
History of Artemia *exploitation in the Russian Federation*

Hypersaline lakes in the Russian Federation with a known *Artemia* population are located in the forest-steppe, steppe and semi-desert areas along the southern border of the country, mainly in the area from the Urals to the Altai Mountains but also beyond this zone. Though there are more than 100 lakes with an *Artemia* population being reported, with a total area estimated at 1 600 km², only a minority of lakes are regularly exploited (Van Stappen *et al.*, 2009; Litvinenko *et al.*, 2015; Litvinenko, Litvinenko and Boiko, 2016). This is due to the highly fluctuating conditions in the ecosystem of these often shallow salt lakes, which only in some years are productive enough to allow for commercial harvesting. The main commercial lakes are located in the regions of Altai, Kurgan, Omsk, Novosibirsk and Tyumen in western Siberia, the Russian Federation. *Artemia* cysts have been harvested in the Russian Federation since the 1970s. In general, in the history of exploitation of Russian lakes, three different periods can be distinguished (Figure 14), as explained below. It is important to note that, in contrast to other *Artemia* harvesting areas, where cyst harvests are generally expressed either as a raw product (i.e. as harvested from the lake) or as a dry product (i.e. brined, rinsed with freshwater and subsequently dried), harvests in the Russian Federation are expressed as "brined wet cysts", i.e. a raw harvested product, rinsed with brine and stored in brine for so-called conditioning (diapause termination). Throughout this text, harvesting amounts are thus reported as "brined cysts".

1977–1994

Exploitation was mainly done to meet the domestic needs of fish farms in what was then the Soviet Union. Some cyst product was also used for aquarium fish, and a significant part also in poultry farming. The main resource was Lake Bolshoe Yarovoe, with an area of 67 km² (exploration of *Artemia* resources in the Altai region started in the 1980s); occasionally, harvesting also took place in other lakes, such as Maloe Yarovoe (Altai) and Siverga (Tyumen). During this period, annual harvests ranged between 1 and 270 tonnes of brined cysts, with an average of 65 tonnes, and a slow increase towards the end of this period.

FIGURE 14
Evolution of *Artemia* cyst harvests in the Russian Federation (tonnes of brined wet cysts)



Sources: Litvinenko, L.I., Litvinenko, A.I., Boiko, E.G. & Kutsanov, K. 2015. *Artemia* cyst production in Russia. *Chinese Journal of Oceanology and Limnology*, 33(6): 1436–1450. https://doi.org/10.1007/s00343-015-4381-6
Litvinenko, L.I., Litvinenko, A.I. & Boiko, E.G. 2016. *Brine shrimp Artemia in Western Siberian lakes*. Novosibirsk: Nauka. 295 pp. (in Russian)

Litvinenko, L.I., Bizikov, V.A., Kovacheva, N.P., Saenko, E.M., Vesnina, L.V., Kutsanov, K.V., Semik, A.M. & Parshin-Chudin, A.V. 2019. *Methodological recommendations for assessing the stock and forecasting the recommended volume of production (catch) of Artemia*. Moscow, Russian Federation, VNIRO Publishing House. 50 pp. (in Russian) www.vniro.ru/files/publish/bizikov_artemii.pdf
Internet Resources of Federal Agency for Fishery. In Russian. https://fish.gov.ru/otraslevaya-deyatelnost/ekonomika-otrasli/statistika-i-analitika

1995–2004

This period was characterized by the threat of a global shortage of cysts, linked with the temporary decline in cyst production in the Great Salt Lake, in the United States of America, and hence there was an increased commercial interest in cyst harvesting worldwide, including in the Russian Federation. The decline in Great Salt Lake triggered the advancement of commercial exploitation of the country's *Artemia* resources, ranging from large, highly productive lakes such as Bolshoe Yarovoe and Kulundinskoe to many smaller lakes scattered throughout western Siberia, the Russian Federation. Several companies involved in cyst harvesting and processing were established during these years. Regular harvesting took place in 26 lakes with a total area of 1 130 km^2, spread over the five regions of western Siberia (Altai, Kurgan, Omsk, Novosibirsk and Tyumen). Together with the increase in the number of commercially exploited lakes, the volume of harvested cysts also increased to 84–1 119 tonnes brined product annually (average 643 tonnes). During this period of substantial expansion, cysts from the Russian Federation also made their appearance on the world markets. Most of the product was exported to China.

2005–present

Harvesting has been conducted in about 70 lakes with a total area of 1 460 km^2, spread over the five regions listed above as well as the Chelyabinsk region, of which 40–50 lakes are harvested regularly. The volume of cyst harvests has further increased, reaching an average of 1 207 tonnes of brined cysts. In the past five years, about 1 500 tonnes of brined cysts have been annually harvested, with a record harvest of 2 000 tonnes brined product in 2016. Up to 20 organizations participate in the harvesting process and are active in many lakes, primarily Lakes Bolshoe Yarovoe, Maloe Yarovoe, Kuchukskoe, Ebeyty, Ulzhay, Medvezhye and Kulundinskoe, which are the most important lakes for *Artemia* harvesting. In general, no more than 10 percent of the harvested cysts are used in the country itself, where they are mainly used in larviculture of sturgeon (different species of the genus Acipenser), Coregonus whitefish, and catfish (different Clarias species). Most of the cysts are exported in brined form, mainly to China, whereas the rest are exported as dry product to Southeast Asian countries (Indonesia, Thailand and Viet Nam), Europe and Latin America. Some of the cysts are also used for aquarium fish and in the cosmetic and pharmaceutical industry.

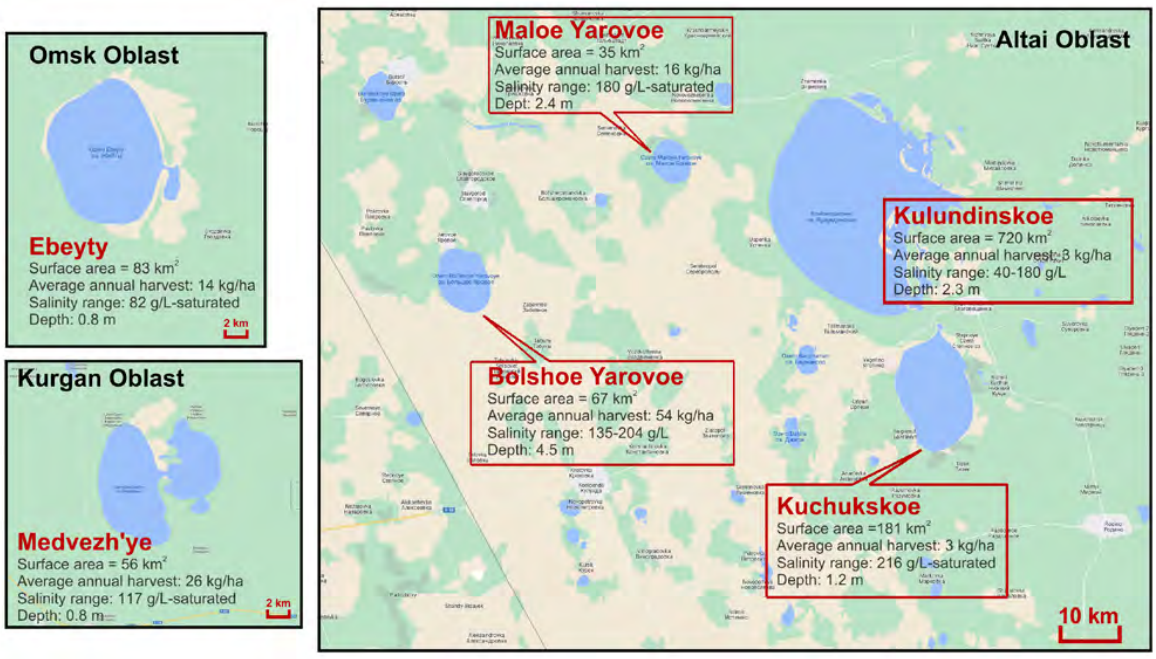
Characteristics of Artemia *populations in the Russian Federation and present status of* Artemia *exploitation*

Out of about 100 lakes with a known *Artemia* population, only about 20–30 of them are of permanent commercial importance. In the other lakes, harvesting is not regularly done or not done at all because of their unstable productivity or their location within a natural reserve or another protected area.

At present, key commercial lakes (Bolshoe Yarovoe, Medvezhye, Maloe Yarovoe, Ebeyty, Kulundinskoe and Kuchukskoe) produce 83 percent of all harvested cysts in the country. The largest lake is Lake Kulundinskoe (720 km^2), while the most productive is the deep-water Lake Bolshoe Yarovoe with an average annual harvest of 54 kg/ha of brined cysts, followed by Lake Medvezhye (26 kg/ha) and Lakes Maloe Yarovoe and Ebeyty (16 and 14 kg/ha, respectively). Lakes Kulundinskoe and Kuchukskoe (Figure 15) have the lowest productivity, harvesting about 3 kg/ha.

FIGURE 15

Lakes in the Russian Federation – Altai, Omsk and Kurgan regions ("oblast") – with the highest commercial importance for *Artemia* exploitation; production is reported as brined cysts



Sources: Litvinenko, L.I., Litvinenko, A.I., Boiko, E.G. & Kutsanov, K. 2015. Artemia cyst production in Russia. *Chinese Journal of Oceanology and Limnology*, 33(6): 1436–1450. https://doi.org/10.1007/s00343-015-4381-6

Litvinenko, L.I., Litvinenko, A.I. & Boiko, E.G. 2016. *Brine shrimp Artemia in Western Siberian lakes*. Novosibirsk: Nauka. 295 pp. (in Russian)

Litvinenko, L.I., Bizikov, V.A., Kovacheva, N.P., Saenko, E.M., Vesnina, L.V., Kutsanov, K.V., Semik, A.M. & Parshin-Chudin, A.V. 2019. *Methodological recommendations for assessing the stock and forecasting the recommended volume of production (catch) of Artemia*. Moscow, Russian Federation, VNIRO Publishing House. 50 pp. (in Russian) www.vniro.ru/files/publish/bizikov_artemii.pdf

Google Earth viewed in April 2021.

The most unstable biotopes with a significant interannual salinity variation are found in Lakes Kulundinskoe and Ebeyty, where salinity can fluctuate four to fivefold over different years; in the other lakes, the variability is lower, approximately 1.5 to threefold.

Because these lakes are stretched over a mainly longitudinally extended geographical area, they have different seasonal dynamics (e.g. temperature regime), which determines the life cycle and period of development of their respective *Artemia* populations: The period in which active *Artemia* stages may be found is generally about 180 days in the lakes of western Siberia, the Russian Federation, i.e. from the second half of April to the first ten days of October. Additionally, other conditions vary extensively, such as average depth (from 0.3 to 4.5 m); average annual salinity (70–250 g/L); surface area (0.3

to 1 300 km²); and salt composition (chloride for the majority of lakes; sulphate in e.g. Lakes Ebeyty and Kuchukskoe; carbonate in Lakes Tanatar and Petukhovskoe). As a result of the climatic regime and of temperature variations between years, the number of brine shrimp generations in these lakes may vary from one to maximally four. Consequently, female fecundity over the total lifespan also may show considerable variations.

Parthenogenetic populations predominate in the salt lakes. However, a few bisexual populations also have been found, some of which belong to the species *Artemia* sinica and *A. amati*, whereas for others the bisexual species status has not been fully ascertained yet (Van Stappen *et al*., 2009; Shadrin and Anufriieva, 2012; Litvinenko *et al*., 2015, 2018; Asem *et al.*, 2023).

Harvesting/processing procedures and exploitation policy

Artemia harvesting has been regulated in the Russian Federation since 2002. Monitoring of *Artemia* cysts stocks is carried out every year, as described below, resulting in a forecast of harvestable quantities for the year to come.

In shallow lakes, the collection of cysts is mainly done manually at the shoreline; only in Lake Bolshoe Yarovoe is harvesting also done from the water surface using boats and pumps (Plate 19). Primary washing of cysts with the brine of the lake is carried out on the shore immediately after harvesting, then the cysts are put into bags and sent to cold storage (a temperature from 0 to -20 °C) for subsequent processing. The duration of cold storage may depend on the timing of further processing procedures, but generally the cysts are kept for about 6 months in a cold warehouse to ensure diapause termination (= conditioning). During this period, the hatching quality is regularly monitored, upon which a decision is made on subsequent processing, which includes washing in freshwater, disinfection (treatment with hypochlorite) and drying (Plate 19). However, in addition to the export of dry cysts, most cysts are shipped as brine-washed cysts to consumer countries, with China as the most important country.

In the Russian Federation, *Artemia* harvesting and trading are controlled and regulated by the Federal Agency for Fishery (Rosrybolovstvo), which officially recognized, in 2009, the nation's *Artemia* resources as a valuable type of biological resources. Rosrybolovstvo oversees the monitoring of fisheries (including *Artemia*) stocks, and also issues harvesting and export licences. The scientific institution subordinate to Rosrybolovstvo is the Russian Federal Research Institute of Fisheries and Oceanography (VNIRO), which monitors the stocks of *Artemia* cysts, formulates recommendations for stock assessment and determines the recommended harvesting volumes (Litvinenko *et al.*, 2019). The essence of this assessment methodology is to determine the total stock of cysts in the entire lake ecosystem (including the bottom, the water column, coastal accumulations, and cysts within the female brood sacs – with an extrapolation for the younger stages observed during the monitoring period). The formulas for calculating the total stock and harvestable quantities take into account the number of generations to be expected in that year and the conditions such as salinity and water depth. The recommended harvestable volume is issued for the next year and is defined as the difference between the total estimated stock and the stock of cysts necessary for the reproduction of *Artemia* for the following year.

The harvesting period is regulated by the Ministry of Agriculture. In western Siberia, the Russian Federation, cyst harvesting is banned from December until May/June. Harvesting *Artemia* shrimp biomass is prohibited in the period August-October. Although harvesting without permission is prohibited, poaching is a significant problem, as the salt lakes in western Siberia are distributed over an often sparsely populated area extending about 1 400 km. However, since cysts are included in the list of strategically important biological resources as decreed by the Government of the Russian Federation, legislation has been more enforced recently and poaching has decreased.

PLATE 19

Harvesting *Artemia* cysts

From the shoreline (a, b) or by boat (c); brine separation with lake water and sieving (d, e); cold storage of brined cysts (f); washing and cleaning of cysts in freshwater (g); followed by dewatering in centrifuge (h), drying (i) and packaging (j)

BOX 2

How ecological changes in inland salt lakes may create opportunities for *Artemia* exploitation

by *Philippe Léger* and *Eddy Naessens*

During the 1980s and the first half of the 1990s, nearly all *Artemia* cyst needs for the emerging marine shrimp and fish hatchery market were provided for by the Great Salt Lake (GSL), Utah, United States of America (Lavens and Sorgeloos, 2000). In the winter of 1994/95, the harvest of *Artemia* at the GSL suffered a steady decline to less than half of the 1991/92 season's harvest, a development that threatened the fulfilment of the growing market's needs. The situation prompted INVE Aquaculture, Belgium, to explore new sources of *Artemia*. As a first step, the company set up a dedicated *Artemia* Task Force. Its mission was to identify natural *Artemia* production sites, characterize the product, assess its commercial potential, research the resources' ecology and assess the parameters for sustainable harvesting, and establish local infrastructure and teams for harvesting and processing the local product.

In January 1995, the INVE *Artemia* Task Force identified an immense brine reservoir (18 000 km²) known as the Kara-Bogaz Gol in Turkmenistan as a new potential *Artemia* resource. The gulf (translated from the Turkmen language as "Dark Throat") is connected with the Caspian Sea through a natural channel in the isthmus separating the two waterbodies (see Plate A). With the water level in the sea being higher than in the gulf, this opening was believed to be the reason why the level of the Caspian Sea was dropping since the 1930s. As a remedy, in 1980, the opening between the Caspian Sea and the gulf was closed off by a dam. The consequence of this intervention was that the gulf, located in an extremely arid climate, dried up completely (see plates on page 37), while salt storms caused

BOX 2 (CONTINUED)

Satellite views (2020) with the location of Kara-Bogaz-Gol as a gulf of the Caspian Sea (a) with the isthmus separating it (b) and the connecting channel (c)

Source: Google Earth viewed in February 2020.

Satellite view of the dry Kara-Bogaz-Gol in 1987

Source: Google Earth viewed in June 1987.

environmental and health problems in the wider region. In 1992, as the Caspian Sea level was rising again, the dam was opened. Inflowing water into the gulf caused the massive salt crust to gradually dissolve. In January 1995, when the site came into the picture for *Artemia* exploration, water levels on both sides of the dam were close to equal and salinity in the gulf was about 180 g/L. This had prompted an *Artemia* population to develop and for a few years the INVE *Artemia* Task Force could harvest and process several hundreds of tonnes of raw product annually. The window of opportunity was, however, narrow, as the Caspian

BOX 2 (CONTINUED)

seawater inflow slowed down, the bottom salt crust dissolved, and salinity again increased to levels where the *Artemia* population ceased to reproduce; consequently, harvests were stopped around the turn of the century.

During its presence in Turkmenistan, the same task force also explored other sites bordering the Caspian Sea, eventually also prospecting the Aral Sea some 500 km northeast of Kara-Bogaz Gol. At one time, around the middle of the twentieth century, the largest inland brackish-water lake and fishing ground of the then Soviet Union, the Aral Sea did undergo a catastrophic change resulting from the diversion of its tributary rivers for agriculture irrigation purposes. Over the decades, the Aral Sea dried up gradually and its salinity increased (Mirabdullayev *et al.*, 2004; Aladin *et al.*, 2018). In 1997, exploration of *Artemia* started, and its presence was documented for the first time in 1998 (Arashkevich *et al.*, 2009). INVE conducted an ecological study (as it had also done at Kara-Bogaz Gol), in cooperation with the Academy of Sciences of Uzbekistan, the Karakalpak Institute of Bioecology and Ghent University, and later followed up by a NATO–financed programme on the sustainable exploitation of the Aral Sea *Artemia* resource (Marden *et al.*, 2012). Currently, more than 100 tonnes of raw product are still harvested every year.

The shift from "GSL–only" *Artemia* to *Artemia* from Kara-Bogaz Gol, the Aral Sea and other resources also resulted in a shift from the use of *Artemia franciscana* to a variety of parthenogenetic strains with sometimes very different quality characteristics. Consequently, this occasionally required different processing and application procedures to be worked out. Undeniably, the exploration and use of *Artemia* resources other than GSL has significantly increased, diversified and stabilized the global cyst supplies ever since the early 2010s.

BOX 3

Past, present and future of *Artemia* in Lake Urmia, the Islamic Republic of Iran

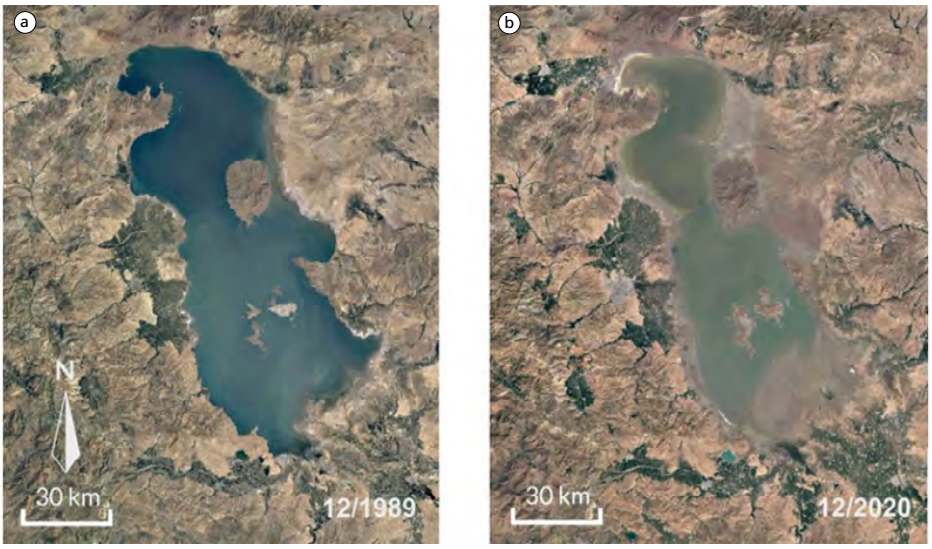
by *Naser Agh*

Located in the northwest of the Islamic Republic of Iran, Lake Urmia has a surface area of 5 500 km^2 and is home to *Artemia urmiana*, which coexists with a local parthenogenetic population of *Artemia* (Agh *et al.*, 2007). The area is a unique wetland for thousands of migrating and local birds, is listed as a Ramsar Site (Convention on Wetlands of International Importance Especially as Waterfowl Habitat), and is known as a UNESCO Biosphere Reserve. According to a population assessment study conducted in the mid-1990s, natural production of *Artemia* cysts used to exceed a few thousands of tonnes of raw product annually, and it was therefore considered as an important natural resource of brine shrimp, especially for aquaculture in the country and the region (Van Stappen, Fayazi and Sorgeloos, 2001).

However, towards the end of the 1990s, the effects of climate change and human intervention started to appear, causing severe damage to the lake and its ecosystem. Freshwater inflow decreased year by year, whereas evaporation increased gradually, which resulted in the lake shrinking to an estimated 10 percent of its previous water volume (see plates on page 39). A renewed resource assessment, conducted in the early 2000s, estimated the annual production to have decreased with a factor of 30 as compared to the 1990s. Lake water became supersaturated with brine and the *Artemia* population completely disappeared for about 10 years. Eventually, the total dissolved solids of the lake water increased to values above 500 g/L, as the lake's major salt component changed from NaCl to magnesium chloride ($MgCl_2$). Billions of tonnes of salt precipitated on the lakebed, changing the geomorphology of the lake and converting it into a shallow playa.

BOX 3 (CONTINUED)

Satellite view of Lake Urmia in 1989 (a) and 2020 (b)



Source: Google Earth viewed in December 1989 (left) and December 2020 (right).

With increased inflow of freshwater as of 2019, mainly attributed to increased precipitation, the *Artemia* cysts buried in the salt started to hatch once again, and since then a relatively dense population of *Artemia* has been witnessed again in the lake. However, the lifespan of these *Artemia* has decreased to 3–4 months due to the rapid evaporation in the summer season and the still relatively high salt concentration in the water. During 2019–2020, the annual production of *Artemia* cysts in Lake Urmia was estimated to be about 200 tonnes of raw product, but no commercial harvesting was permitted. The production of *Artemia* is harvested from the lake with an ecosystem that has become sensitive and fragile, and it strongly depends on annual inflow of sufficient amounts of freshwater to temporarily reduce the water salinity, allowing the animals to complete their life cycle.

Global warming, unscientific and unmanaged expansion of agricultural activities, and uncontrolled extraction of groundwater are leading the Islamic Republic of Iran to experience major water crises that affect the environment and many of its wetlands, including Lake Urmia. The future of the lake's ecosystem and its *Artemia* populations is highly uncertain and strongly depends on the future policy of the government and the methods it will adopt for solving this problem. In 2006, at an international conference organized at Urmia University, an expert panel of scientists jointly proposed a strategy for adaptive management and rehabilitation of the lake by trying to restore a lake of decreased size (about 2 800 km^2) – similar to what has been realized in an area of former Lake Aral, Kazakhstan – creating a south and a north wing. If the plan is implemented, the salinity in the south wing will be managed at about 120 g/L, whereas the north wing will remain saturated with brine. This programme would facilitate an estimated annual production of about 1 000 tonnes of raw *Artemia* cysts from the south wing, and the north wing could be used as a huge source for extraction of NaCl and various other minerals.

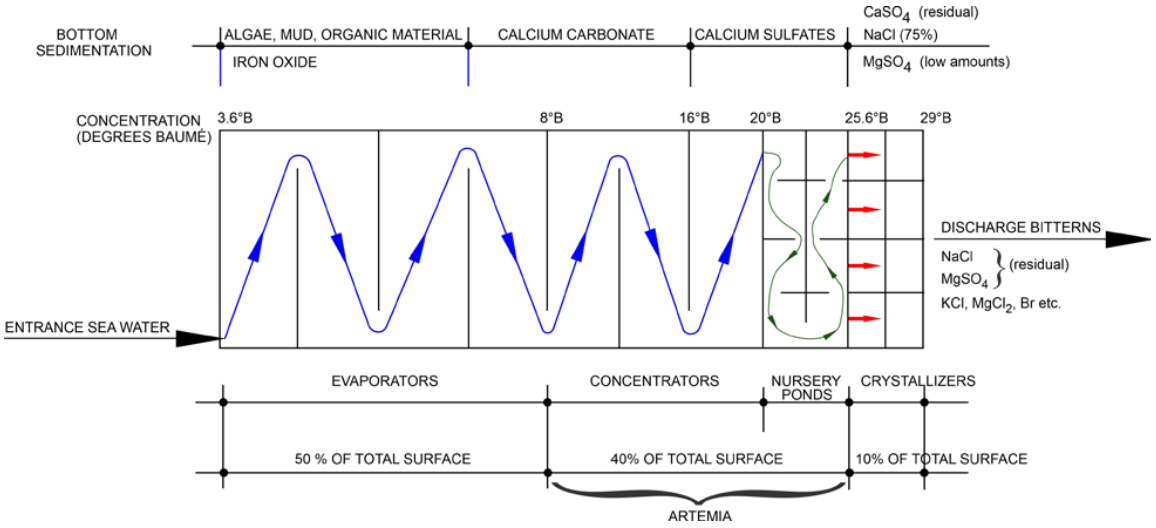
2.1.2. Permanent solar salt operations (by *Hector Teruel*)

2.1.2.1. Principles of solar salt production

Solar saltworks are enterprises where seawater or any water of higher salinity, such as saline groundwater, is introduced by pumping or by gravitation (due to tidal differences in water levels) into a series of interconnected ponds, where the water salinity increases through solar evaporation. These ponds are called evaporators,

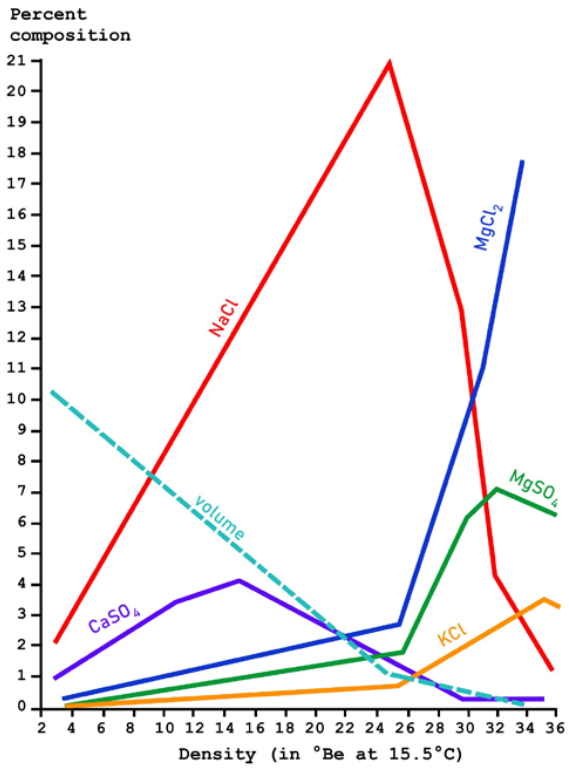
concentrators, precrystallizers (or nursery ponds) and crystallizers, depending on the degree of salinity that is maintained in these compartments of the system (Figure 16).

FIGURE 16
General layout of solar saltworks



Source: Bradley, H.L. & Venkatesh Mannar, M.G. 1984. *Guidelines for the establishment of solar facilities from seawater, underground brines and salted lakes*. UNIDO/IS.330/REV.1. https://downloads.unido.org/ot/47/93/4793176/10001-15000_12271.pdf

FIGURE 17
Fractional crystallization of salts



Source: Bradley, H.L. & Venkatesh Mannar, M.G. 1984. *Guidelines for the establishment of solar facilities from seawater, underground brines and salted lakes*. UNIDO/IS.330/REV.1. https://downloads.unido.org/ot/47/93/4793176/10001-15000_12271.pdf

Consequently, the dissolved salts begin to precipitate (crystallize) according to their specific solubility in a process known as fractional precipitation (Figure 17). As the salinity increases, salts with low solubility such as carbonates and sulphates precipitate first. Once the seawater has evaporated to about one tenth of its original volume and has reached the "salting point", so-called "mother brine" is pumped into the crystallizers where NaCl precipitates. This salt represents 79 percent of total salts per litre of seawater, with $MgCl_2$, magnesium sulphate ($MgSO_4$), potassium chloride (KCl), calcium chloride ($CaCl_2$), calcium carbonates ($CaCO_3$), and bromides as the most important other salts (Bradley and Venkatesh Mannar, 1984).

2.1.2.2. Operation of permanent solar saltworks

Permanent solar saltworks are found in geographical areas where climatological conditions allow for operation throughout the year. The main favourable factors are low annual rainfall, strong winds, impermeable soils and appropriate salinity at the entrance of the first evaporator pond (Bradley and Venkatesh Mannar, 1984). Permanent saltworks are typically highly mechanized

and can be classified according to their size, from small (10 000–50 000 tonnes of salt production/year), medium (50 000–100 000 tonnes/year), and large productive capacity, for example the saltworks in Australia, Brazil and France, which produce more than 1 million tonnes of high-quality salt annually. In this type of saltworks, pond sizes can range from a few hectares to several hundred hectares, each with depths of 0.5 m up to 1.5 m.

The geometrical characteristics of these operations are variable but generally follow a number of rules allowing the best structuring of the evaporating surfaces, and thus the highest efficiency. The evaporation ponds, having lowest salinity, are generally the largest in the circuit because the water with the lowest salinity has a higher rate of evaporation because of its lower surface tension compared to higher salinity water. The pond dimensions and their spatial organization (Plate 20) are designed in such a way that the water is forced to flow along the longest possible route per unit of surface area, in which the outflow of each pond in the circuit keeps a certain salinity and a steady overflow. This continuous flow is what distinguishes a permanent saltwork from a saltwork operating seasonally. After passing through the evaporation area and the subsequent concentration area, the high salinity water or brine has reached concentrations approaching saturation of sodium chloride. Along the path of the water flow, salts such as carbonates, iron oxides and gypsum (calcium sulphate dihydrate, $CaSO_4 \cdot 2H_2O$) have fractionally precipitated, and the brine now contains 270–285 g/L of total salts (or, converted into so-called degrees Baumé, which express density: 22–23 °Bé; see Annex 1). At this point, the brine is pumped into deeper nursery ponds, where the gypsum is left for several days to supersaturate and to precipitate as much as possible. When the brine reaches its maximum saturation point (25.6 °Bé or 314 g/L) and the precipitation of sodium chloride begins, it is drained by gravity into crystallizer ponds, where harvestable "salt floors" will form. This crystallization zone is generally 10 percent or more of the total saltwork area, depending on the initial salinity of the seawater entering the first evaporator pond. The crystallization ponds are very regular in shape with a solid pond floor to facilitate harvesting operations and the manoeuvring of heavy harvesting and transport machinery. When the salt layer on the bottom of the crystallizer has reached a certain thickness (which depends on the climate) and the brine has a density of up to 29 °Bé (375 g/L total salts), the latter is drained towards the sea or towards bittern ponds, when direct drainage of the residual brines into the marine environment is not possible or not allowed. The salt crust is collected mechanically, and salt is transported to the factory for washing and processing. After the washing process, the final purity of the sodium chloride can reach up to 99.5 percent, with minimal quantities of calcium ions (Ca^{2+}), magnesium ions (Mg^{2+}) and sulphate ions (SO_4^{2-}) remaining in the product.

PLATE 20

Saltworks of Salin-de-Giraud, France (a), and saltworks in Bonaire, Netherlands Antilles (b): lower-salinity ponds coloured green; crystallizers coloured reddish

Source: Google Earth viewed in March 2022.

2.1.2.3. Biological aspects of solar saltworks and role of Artemia

Saltworks are not just to be considered as a kind of factory for salt production, but also as an ecosystem that must be handled with great care to achieve a permanent healthy state (Davis, 2000, 2009). Appropriate management ensures that each area, consisting of ponds with similar salinity, develops a specific and interactive flora and fauna to avoid hydrobiological imbalances that could negatively affect salt production in terms of salt quantity and/or quality.

In an optimally operating saltwork, benthic mats consisting of conglomerations of unicellular algae contribute to maintaining oxygenation of the water column, control the pH to prevent acidity of the biological environment, and help in sealing the pond bottom to avoid brine leaks through the subsoil of the ponds. In the water column, typically phytoplankton develops in monocultures of single or of few species, depending on the salinity and other pond conditions, and these prevail over other species because of the competition for nutrients, contributing to colour differences of different ponds in the system. High algal cell density promotes brine coloration, thus helping to increase the evaporation rate by means of the higher caloric absorption and reduced reflection of solar rays.

Artemia is another vital factor in balancing this system: It is the most abundant or only zooplankton species often naturally occurring in permanent saltworks, typically within the salinity range of 12–21 °Bé (about 120–240 g/L). In saltworks, *Artemia* should not only be considered as a valuable by–product. The presence of brine shrimp also influences salt quality as well as quantity. Adequate control and management of brine shrimp populations is thus important to maintain the ecological balance of the salt production system and thus improves profitability of the salt operation, even in situations where *Artemia* biomass and cyst yields are relatively low (Davis, 2000, 2009).

First, because of its non-selective filtering behaviour, *Artemia* ingests microalgae that could develop into an unbalanced factor if their populations are not regulated through *Artemia* grazing. In saltworks, algal blooms are common, not the least because of the increase of nutrient concentration with evaporation. The presence of algae in low salinity ponds is beneficial, as the coloration promotes evaporation. At elevated salinity, however, if present in large numbers, algae and more specifically their dissolved organic excretion and decomposition products will prevent early precipitation of gypsum because of the increased viscosity of the water. In this case, gypsum, which precipitates too late in the crystallizers together with the sodium chloride, will contaminate the salt, thus reducing its quality. Furthermore, accumulations of dying algae, which turn black when oxidized, may also contaminate the salt and may be the reason for the production of small salt crystals. In extreme situations, the water viscosity might even become so high that salt precipitation is completely inhibited and salt flakes accumulate at the water surface.

Moreover, faecal pellets of *Artemia* sink to the pond floor, where they promote the growth of the bottom mats. *Artemia* metabolites and disintegrating biomass, following mortality because of food shortage and osmotic shock when *Artemia* enters with the water flow into precrystallizers and crystallizers (22–25 °Bé or about 260-310 g/L total salts), are a substrate for the proliferation of halophilic archaea and bacteria populations, such as species belonging to the genus Halobacterium. These microbiota colour the waters of these ponds intensely pink-orange due to the high concentrations of carotenoids, bacterioruberin for example, in the vacuoles of their cytoplasm. This coloration allows for an enhanced caloric absorption that increases the temperature of the water generally by 7–8 °C, but sometimes even up to 15 °C. This stimulates evaporation despite the high surface tension of the liquid. Without this intense colour, evaporation would slow down, reducing the production in the crystallization area. The proliferation of these halophilic microbiota also reduces the concentrations of dissolved organic matter in the brine. This in turn leads to lower viscosity levels, promoting the formation of larger salt crystals, and thus improving salt quality (Davis, 2000, 2009).

The *Artemia* population density generally depends on the local conditions of the moment, such as food availability, temperature and salinity (see Box 4). The availability of pumping facilities and intake canals could allow for manipulation of nutrient intake and salinity, and sometimes fertilization can increase yields. Still, given the size of these operations, this type of manipulation may have an effect that is just limited in time or space, and typically the numbers of animals per unit of volume or surface area in this type of salt operations are low. Moreover, the rather stable conditions prevailing in the ponds of these saltworks often result in stable brine shrimp populations in which the ovoviviparous reproduction mode dominates, leading to decreased cyst yields as described, for example, for the saltworks in northeastern Brazil (see Box 5). *Artemia* can also be farmed using unconventional water resources such as salty underground waters, as is particed in the Islamic Republic of Iran (see Box 6).

BOX 4

Biological saltwork management at Sama Salt Company, Al Aryam, Abu Dhabi, United Arab Emirates

by *Hector Teruel*

Because of the oligotrophic condition of the Persian Gulf water, especially along the local "sabkha" (coastal mudflats) environments, the brines from the crystallizers remained completely transparent, preventing optimal evaporation and crystallization as an effect of the intense surface tension and the reflection of a high percentage of the incident radiation on the white salt bottom.

By fertilizing with urea and triple superphosphate, with a N:P (nitrogen to phosphorus) ratio of 5:1, microalgae developed in the first evaporators. When their concentration was sufficient, water was transferred by overflow to the evaporators, where water salinity was 110 g/L or more (10 °Bé). In these evaporators, a repeated inoculation of *Artemia franciscana* nauplii from the Vinh Chau strain (Viet Nam) was also done, which after a month developed into a dense population consisting of adults, juveniles and also second generation nauplii through ovoviviparous reproduction. This population dispersed towards the other evaporators until reaching the salinity of 23–24 °Bé (280–290 g/L), where they cannot survive because of osmotic pressure and lack of food. In these ponds, the decomposition of *Artemia* contributed to the development of an intense pink coloration, a product of the proliferation of the archaea *Halobacterium*.

Finally, the crystallizers, previously transparent, stained in a very significant way, enhancing evaporation owing to a temperature increase of up to 7–8 °C as compared to the 34 °C measured when brine is transparent. Monitoring of the evaporation rate following this biological intervention showed a 100 percent increase, which translated into an increased growth of the thickness of the salt floors with, on average, 1.6 mm/day.

Sama Salt Company salt ponds, Abu Dhabi, United Arab Emirates: before (a) and after (b) biological management

BOX 5

Artemia history and current practices in Brazil

by *Marcos Rogerio Camara*

Permanent saltworks are a relevant part of coastal wetland systems in the semi–arid zone of northeastern Brazil. The largest Brazilian salt fields (size range of 775–3 000 ha) are located in the state of Rio Grande do Norte (see plate), where ideal conditions for this activity, such as long dry seasons (stretching from June to January), strong winds, high solar incidence and high evaporation rates, are found. The total area associated with the marine salt industry in Rio Grande do Norte amounts to 41 718 ha, of which 30 642 ha are exploited for salt production activities. The resulting scenario is that marine salt production in Brazil (6 million tonnes in 2017) comes mostly from Rio Grande do Norte (>95 percent).

Geographical location of Rio Grande do Norte salt fields in Brazil

Source: Google Earth viewed in November 2021.

Artemia franciscana populations are found in northeastern Brazil as a result of inoculations made in the salt ponds of Macau (Rio Grande do Norte) in 1977 with cysts from a San Francisco Bay (California, the United States of America) stock. In the following years, introduced *Artemia* were dispersed to neighbouring saltworks by local workers (for aquacultural purposes and to aid in salt production), by wind and by waterbirds (Camara, 2001).

A significant portion of brine shrimp cysts available in Brazil is currently harvested in saltworks in Rio Grande do Norte as a by-product of the marine salt industry. On a yearly basis, a substantial part (approximately 20 percent) of the 20 tonnes of cysts used by the Brazilian aquaculture industry is harvested in local salt fields. The collection of *Artemia* cysts is mostly performed by local entrepreneurs and their families. No formal cyst harvest

BOX 5 (CONTINUED)

management occurs. A rudimentary restocking is occasionally done by inoculating nauplii or by transplanting biomass harvested in higher salinity ponds into lower salinity ponds.

Low productivity and fluctuations in cyst yields are intrinsic characteristics of this artisanal (and basically unregulated) form of extractive aquaculture. Brine shrimp cysts float and accumulate in spots in local evaporation ponds. Once a spot of cysts is found, fishers intensively work the area with nets and buckets to scoop out the cysts from the water, collecting them also at the shoreline (see plate a). Harvested cysts are taken to local processing plants (see plate b) for later use in local shrimp (*Litopenaeus vannamei*) hatcheries (Wainberg *et al*., 2011). Brine shrimp biomass is also harvested, and then frozen, freeze–dried or made into flake diets for larval/postlarval shrimp.

Brine shrimp (*Artemia franciscana*) cyst and biomass production in the saltworks in northeastern Brazil

(a) Collecting the cysts from the shoreline; (b) washing the harvested cysts with brine

© M. Rogerio Camara, FURGN

Cyst harvesting in Brazilian salterns (for use in local aquaculture) has exerted a selective pressure on A. franciscana for over four decades, and it is likely that harvesting has affected brine shrimp populations in multiple ways (population size, phenotypes, fitness components and genetic variation, to name a few). In general, there has been a reproductive shift to ovoviviparity (direct production of free–living nauplii) in local brine shrimp populations (Camara, 2020). The sustainable harvesting of *A. franciscana* cysts in Brazilian salterns is critical for the long-term occurrence of the brine shrimp populations, but also for social and economic reasons. Careful management of this essential resource requires a science-based approach with eyes open for the possible effects of unregulated exploitation before it seriously impacts the viability of this emerging form of extractive aquaculture.

BOX 6

Artemia culture using unconventional water resources

by *Naser Agh*

In Southeast Asia, *Artemia* pond production using evaporated seawater dates back to the 1980s. This kind of production expanded quickly to other countries in Asia and to Africa and Latin America as well. In the Islamic Republic of Iran, *Artemia* pond production started about 25 years ago, towards the end of the twentieth century, in coastal saltworks along the Persian Gulf. Later, *Artemia* culture was introduced into other water resources, in regions far away from the open sea. A number of water sources can be used for culturing *Artemia*: salty underground water, desalination effluents, effluents from water treatment and agriculture mixed with saline ground or surface water. Some of these sources may be less rich in nutrients than seawater, and sometimes they also have a different ionic composition compared to seawater. Therefore, using such water sources for *Artemia* culture requires adapted management in terms of feeding and pond fertilization. Before starting any large-scale production, laboratory culture tests should be done using the water from the different

BOX 6 (CONTINUED)

sources, or after mixing with locally available salty water, to investigate if the medium is suitable for *Artemia* culture in terms of ionic composition and the absence of any toxic compounds.

An example of using such a system for *Artemia* culture is a 25-ha pond that was made by constructing a dam at an agricultural drainage system in Kerman Province in the central area of the Islamic Republic of Iran. Brackish groundwater is pumped up to maintain the salinity between 80 and 150 g/L during several months of the year for *Artemia* biomass and cyst production. In addition, a 2-ha pond has been constructed for green water production, which is supplemented with fermented wheat and rice bran to feed the *Artemia*. Beetroot molasses is added as a carbon source at an adequate rate to stimulate biofloc formation as additional food for the organism. Annually, 2 tonnes of raw product of *Artemia franciscana* cysts and about 20 tonnes of biomass are produced at this site for consumption in local shrimp hatcheries.

25-ha pond constructed at an agricultural drainage system in Kerman Province, Islamic Republic of Iran

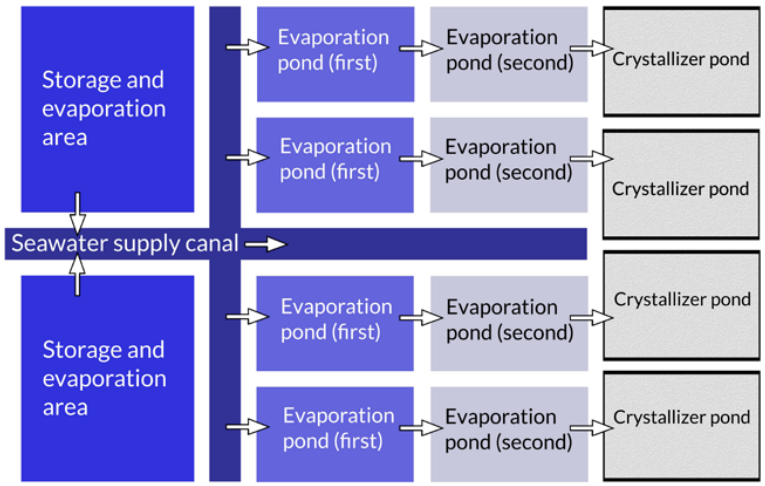
Special attention should be given to the effluents of desalination plants: Annually, billions of cubic metres of these effluents having a salinity ranging between 50 and 70 g/L make their way into the sea, locally polluting the coastal ecosystem while also possibly having a biological impact on the life cycle, growth and reproduction of corals, seagrasses, invertebrates and fish. Many of these active desalination plants are located in the Persian Gulf area, where their effluents cause increased salinity and pose a threat to marine life. Transferring these effluents to artificial wetlands for *Artemia* culture could be a way to recycle them, contributing to assured local production of *Artemia* cysts required by the growing aquaculture industry in the region, creating employment, and increasing the living standard of local rural communities. In dry countries, as the Islamic Republic of Iran, such a wetland creation programme can replace the dried and drying inland wetlands used by local and migrating waterfowl for food, reproduction and overwintering.

2.1.3. Seasonal solar salt operations (by *Nguyen Van Hoa* and *Gilbert Van Stappen*)

This section discusses small artisanal saltworks (known also as salt fields, salt farms or salt streets) in the tropical–subtropical belt that are only operational during the dry season. In these artisanal saltworks, ponds are only a few hundred square metres in size and typically have depths of 0.1 m to 0.6 m. Figure 18 shows the layout of a typical artisanal salt farm (Vinh Tien Salt Cooperative – Viet Nam). Most salt farms only operate for a few months of the year, when the balance evaporation/precipitation is positive. Salt production is abandoned during the rainy season, when evaporation ponds are often turned into fish or shrimp ponds. Although salt production in these so-called salt streets is based on the same chemical and biological principles as in large permanent salt farms, production methods differ slightly. A complete overview on all aspects of seasonal *Artemia* pond production can be found in Section 2.2. However, a broad outline of the production is summarized below.

At the beginning of the production season, all ponds are filled with seawater. Water is supplied by tidal inflow, but small portable pumps, windmills and/or manually operated water–scoopers are also used, allowing for better manipulation of water and salinity levels.

FIGURE 18
Layout of a typical artisanal salt farm (Vinh Tien, Viet Nam)
(1) Storage and evaporation area = 0.4–1 ha and salinity = 10–50 g/L; (2) evaporation pond (first series) = 0.2–0.5 ha and salinity = 40–120 g/L; (3) evaporation pond (second series) = 0.2–0.5 ha and salinity 80–160 g/L; and (4) crystallizers = 0.2–0.5 ha and salinity of 120 g/L until all water evaporates



Source: CTU, Viet Nam.

Water evaporates and, usually just before the next spring tide, all the water, now having a higher salinity than seawater, is concentrated in one pond. All other ponds are refilled with seawater, which once again is evaporated and concentrated in a second pond. This process is repeated until a series of ponds is obtained in which salinity increases progressively, but not necessarily gradually. For the remainder of the season, water is kept in each pond until the salinity reaches a predetermined level and is then allowed to flow into the next pond holding water of a higher salinity. The salinity in the different ponds is thus not kept constant as in permanently operated saltworks. Occasionally, to further increase evaporation, ponds are not refilled immediately but left dry for one or two days. During that time, the pond bottom heats up, which further enhances evaporation. Once the salinity reaches 260 g/L, water is pumped to the crystallizers, where the sodium chloride precipitates. *Artemia* thrive in ponds where salinity is high enough to exclude predators (generally above 80–90 g/L).

As seasonal systems are often small, they are fairly easy to manipulate. Hence, higher food levels and thus higher brine shrimp densities can be maintained. Also, environmental factors fluctuate, such as temperature (shallow ponds), oxygen level (high algal density, use of organic manure) and salinity (discontinuous pumping), creating an unstable environment. This, together with the fact that population cycles are yearly interrupted, seems to favour oviparous reproduction.

Integrated systems in which *Artemia* culture (at high salinity) is combined with the culture of shrimp or fish (stocked in the ponds with lower salinity) also exist. Intensive *Artemia* culture in ponds can also be set up separately from salt production. Ponds may be filled with the effluent of fish/shrimp hatcheries and/or grow–out ponds. As salinity in these systems is often too low to exclude predators (45 g/L to 60 g/L), intake water is screened, using filter bags or crossflow sieves. Agricultural waste products, including rice bran and soybean meal, can be used as supplemental feeds. For more details on the operation of seasonal *Artemia* pond production, see Section 2.2.

2.2. POND PRODUCTION (by *Nguyen Van Hoa* and *Gilbert Van Stappen*)

Although *Artemia* pond production is practiced in different countries and regions, the information in this manual is largely based on the methodology as applied in the Soc Trang and Bac Lieu areas of the Mekong Delta, Viet Nam: It is in this area that working protocols have been developed using long-term experience of salt farmers and researchers, which have proven successful in the long run (Hoa and Sorgeloos, 2015; Van Stappen *et al.*, 2019). As such, many aspects of the methodology are directly applicable to other areas. However, at specific places in the text, explicit reference is made to the particularities of the Vietnamese context, such as its climatological conditions, which may be different elsewhere. It should also be emphasized that these production protocols have been worked out for the Vietnamese strain (Vinh Chau strain) of the species *Artemia franciscana*, which originates from an inoculum with the San Francisco Bay strain *A. franciscana* in the 1980s (Quynh and Ngoc Lam, 1987; Clegg *et al.*, 2000; Hoa, 2002; Kappas *et al.*, 2004). Consequently, several factors described – such as the *Artemia* population's temperature tolerance, maturation time, fecundity and estimations of production yields – are not applicable as such when other *Artemia* strains/species are used for pond production, especially not when working in environmental conditions widely different from those in the Mekong Delta.

2.2.1. Site selection

Integrating *Artemia* production in an existing operational solar saltwork or shrimp/fish farm will be more cost-effective than constructing an *Artemia* production pond system from zero. Ponds can be constructed close to evaporation ponds with the required salinity, or ponds already existing in the salt operation system can be modified.

Although the next section does not give a detailed account of all aspects related to pond construction and site selection, it does summarize those aspects that should be specifically applied for *Artemia* pond culture. For more detailed information, the reader should refer to specialized handbooks for pond construction, such as de la Cruz, 1983; Kumar, 1992; FAO, 2017; and Sneyers and Ingawa, 2005.

2.2.1.1. Climatology

The presence of sufficient amounts of highly saline water is imperative, especially to keep *Artemia* predators out of the system, although filtration techniques to prevent predators from entering culture ponds can be applied for short-term cultures (a filtration less than 70 µm). Therefore, *Artemia* culture is mostly found in areas where evaporation rates are higher than precipitation rates during extended periods of the year (e.g. a dry season of more than four months in the tropical-subtropical belt). Evaporation rates depend on temperature, wind velocity and relative humidity. Especially when integrating *Artemia* ponds in fish/shrimp farms, practioners should have knowledge of the area's evaporation rates. On the other hand, the presence of local solar salt farms is a clear indication that *Artemia* pond culture is possible during at least part of the year.

As temperature also influences population dynamics directly, this climatological factor should receive special attention. Too low temperatures will result in slow growth and reproduction, whereas high temperatures can be lethal. Note that optimal culture temperatures are strain-dependent (see Section 1.4.4).

2.2.1.2. Topography

The land on which ponds are constructed should be as flat as possible to allow easy construction of ponds with regular shapes. A gradual slope can eventually facilitate gravity flow in the pond complex. The choice between dug out (entirely excavated) and level ponds (pond bottom approximately at the same depth as the surrounding land and water retained by dykes or levees) will depend on the type of ponds already in use. Locating the *Artemia* ponds lower than all other ponds is good practice, as in *Artemia*

production the water flow into the ponds is much more significant and frequent than the outflow; usually, ponds are only drained at the end of the culture season. Making use of gravity or tidal currents to fill the ponds, even if only partially, will reduce pumping costs.

2.2.1.3. Soil conditions

Because long evaporation times are needed to produce high salinity water, leakage and/or infiltration rates should be minimal. Heavy clay soils with minimal contents of sand are therefore the ideal substrate (Hoa, 2014). As leakage is one of the most common problems in fish/shrimp farms and even in large saltworks, construction of a small pilot unit at the selected site, prior to embarking on the construction of large pond complexes, might avoid costly mistakes.

An additional problem might be the presence of acid sulphate soils, often found in mangrove or swamp areas. Sometimes yellowish or rust-coloured particles can be observed in the surface layers of acid sulphate soils. When exposed to air, such soils form sulphuric acid H_2SO_4, resulting in a pH drop in the water. At low pH, it is very difficult to stimulate an algal bloom. As algae constitute an important food source for the *Artemia* population, yields are low in such ponds. Treatment of acid sulphate soils is possible but costly (see further).

The presence of lots of organic material on the pond bottom might also cause problems. Especially when used for dyke construction, such type of soil tends to shrink, thus lowering the dyke height considerably. Moreover, problems with oxygen depletion at the pond bottom, where organic material is decomposing, can arise. Using such soils over several years will lower the organic content, but nevertheless during the first years of operation this kind of problem may persist (Hoa, 2014).

2.2.1.4. Water supply and drainage canals

The main canal, as used for the intake of seawater for the salt production process and to stock fish/shrimp during the rainy season, also brings seawater into the main reservoir; a system of canals further distributes water to and between the evaporation ponds and finally into the crystallization area.

2.2.2. *Artemia* production models

2.2.2.1. Overview of Artemia *production models*

Since the 1980s, different *Artemia* production models have been developed in the Soc Trang and Bac Lieu salt fields of the Mekong Delta, Viet Nam, in order to cope with fluctuations in market demand and with variable investment capacity of the farmers (Son, 2010; Hoa and Sorgeloos, 2015; Vinh *et al.*, 2019). The models listed in Table 6 are not mutually exclusive, but are defined depending on the criterion considered (Hoa *et al.*, 2011). They are further explained in the sections below. Many of the principles and management practices mentioned in Section 2.2.2 will be further explained from Section 2.2.3 onwards.

TABLE 6
Overview of *Artemia* production models

General approach	Degree of investment	Pond management			End product
Monoculture	Extensive	Stagnant	One cycle	Stocking with nauplii	Cysts
Integrated culture	Intensive	Flow-through	Multiple cycles	Stocking with older stages	Biomass

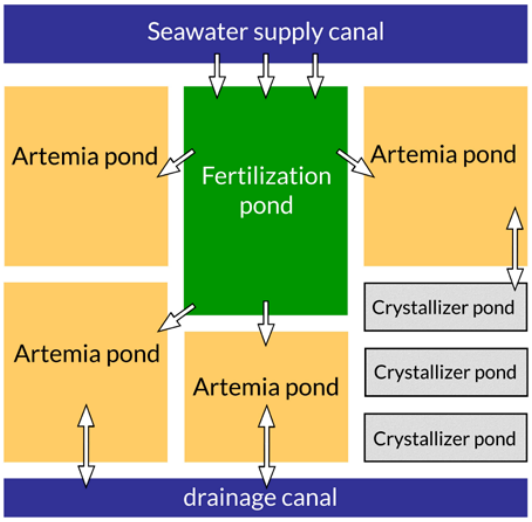
Source: Hoa, V.N. & Van, N.T.H 2019. *Principle of Artemia culture in solar saltworks*. N.V. Hoa & P.K.H. Lam, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2946-5. 219 pp.

2.2.2.2. Monoculture versus integrated models

Monoculture model

The monoculture model is specially designed for *Artemia* culture solely, including the pond system where *Artemia* production takes place, the fertilization pond (usually accounting for 15–20 percent of the total surface area of the system), and the system of supply and drainage canals. In this model, the entire salt field area is transformed into an *Artemia* culture area. For small-scale farmers, it is generally beneficial to be connected with the area of other farmers in the same location, so that the water supply system can be shared. Additionally, the salt crystallization area can be converted into *Artemia* ponds, or can still be used for salt production, using effluent water from the *Artemia* ponds (Figure 19).

FIGURE 19
General design of an *Artemia* farm with allocation of ponds to specific functions



Source: Hoa, V.N. & Van, N.T.H 2019. *Principle of* Artemia *culture in solar saltworks*. N.V. Hoa & P.K.H. Lam, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2946-5. 219 pp.

Integrated culture model

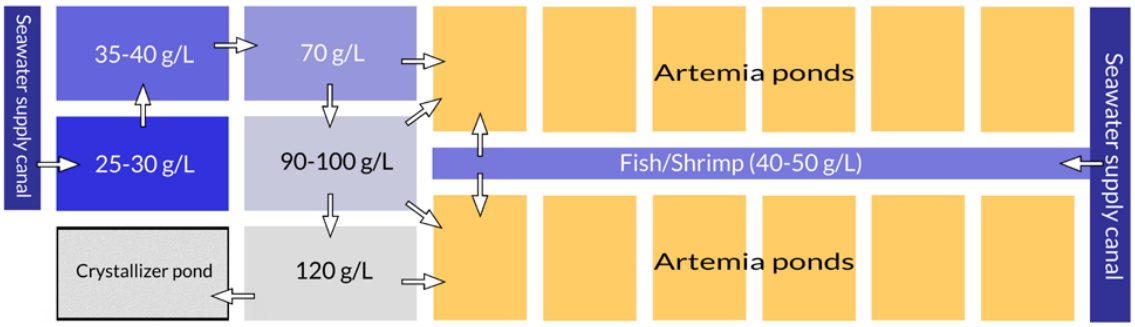
The integrated culture model is defined as a (simultaneous) combination of *Artemia* farming with other activities, e.g. *Artemia*–salt, *Artemia*–shrimp/fish–salt, *Artemia*–cattle/poultry-salt, as follows:

- *Artemia*-salt: Saline water from the salt production area is used to regulate the salinity in the *Artemia* ponds and, vice versa, effluents from the *Artemia* ponds can be used to produce salt. The effluent from the *Artemia* ponds has been filtered by *Artemia* (and thus concentrations of microalgae, organic matter, etc., have been reduced). Consequently, salt produced from these effluents, because of reduced water viscosity, can yield better salt quality.
- *Artemia*-shrimp/fish-salt: The interaction between *Artemia* and the salt component is as described above, while shrimp/fish are cultured in lower salinity in the same farming system (Figure 20). For example, tilapia can be cultured in the fertilization pond, shrimp/fish can be fed pellet diets, and fertilizers can stimulate primary production. Consequently, the water from these shrimp/fish culture ponds will receive many nutrients, therefore stimulating algae growth, and this water can be used as a food source for *Artemia*. On the other hand, fresh *Artemia* biomass (of different size as needed) can be used for nursing/culturing shrimp/fish in different stages (Bengtson, Léger and Sorgeloos, 1991). The combination of *Artemia* with shrimp/fish offers the possibility of using *Artemia* as a "biofilter" because of its

ability to "clean" the aquaculture environment (i.e. extractive aquaculture; see Kongkeo and Wilkinson, 2005).

- *Artemia*-cattle/poultry-salt: Similar integration of *Artemia*-salt takes place as described above, while cattle/poultry can be farmed in cages or constructions directly above the *Artemia* ponds or the fertilization ponds. Leftover feed and waste released from the cages will drop into the ponds, stimulate algal blooms, and is also a direct food source. At the same time, biomass collected from *Artemia* ponds is considered to be a source of protein and can be mixed with cattle/poultry feed. In this integrated system, however, it is important to consider that any prophylactic or curative medical compounds administered to the cattle or poultry may end up in the faeces and thus will find their way into the aquatic food chain.

FIGURE 20
Example of an *Artemia*-shrimp/fish-salt integrated culture model



Source: Hoa, V.N. & Van, N.T.H 2019. *Principle of* Artemia *culture in solar saltworks*. N.V. Hoa & P.K.H. Lam, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2946-5. 219 pp.

2.2.2.3. Extensive versus intensive culture models

Extensive culture model

As in other extensive aquaculture models, in this model the culture system requires less investment; it only takes advantage of highly saline water available in the salt field at the end of the dry season, water that cannot be used for any other aquaculture purpose. In these conditions, a high amount of organic matter and/or microalgae is available, and neither fertilization nor adaptation of the salt field is applied for *Artemia* culture. Farmers just inoculate (stock) *Artemia*, using newly hatched nauplii or transferring part of the grown-out *Artemia* biomass from other ponds. The *Artemia* population will then develop and reproduce. In this culture model, because of lower management efforts, the pond is more susceptible to contamination with *Artemia* predators and/or food competitors, and therefore the brine shrimp density is often low. Low density along with absence of supplementary feeding leads to relatively low and unstable productivity, fluctuating, for example, in the range of 10–20 kg wet cysts per ha/season or a few hundred kilograms of wet weight biomass.

Intensive culture model

In comparison to the extensive culture model, the intensive culture model is clearly different in terms of:

- The infrastructure is larger scale, with a better water supply and drainage system, is operational throughout the full season, and is less affected by environmental fluctuations such as temperature and salinity.
- Systems are operated by qualified/experienced technical staff, and a sufficient labour force and machines (e.g. pumps, aerators) are in place.
- Production is more stable; ponds can operate for a long term, ensuring sufficient food for the *Artemia* population (microalgae and/or supplementary feed).

- Fertilization is managed according to a protocol ensuring a proper ratio and content of N:P to stimulate growth of suitable microalgae for *Artemia* (e.g. diatoms/Bacillariophyta).
- Biofloc technology, while ensuring an optimal C:N (carbon:nitrogen) ratio, may be applied to increase the amount of feed in the pond while stabilizing the environment.

Obviously, production systems can differ in their degree of intensification and thus be semi-extensive or semi-intensive. Given the scale and the requirements for active management, the operational cost of intensive systems is significantly higher (typically 1.5–2 times) than in the extensive model. On the other hand, the intensive culture model is typically at least two- to threefold higher in productivity, with yields in the range of 50–70 kg wet cysts/ha over a season of four months in semi-intensive conditions and yields up to 200 kg wet cysts/ha for intensive systems.

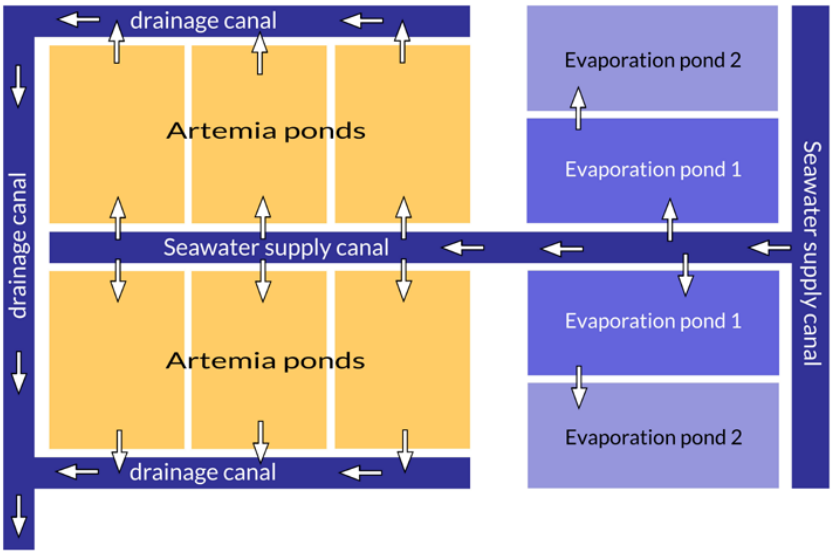
2.2.2.4. Stagnant versus flow–through culture model

Stagnant culture model

Generally, in this model, management in each pond is carried out independently, and stocking or restocking of a pond does not affect the other ponds. When any problem occurs in one culture pond, such as low survival rate, invasive predators and appearance of Lab-Lab (phytobenthos), the operation of the other ponds is not affected even though they share the same water supply and drainage system (Figure 21). This system is suitable for salt field areas that have recently been converted into *Artemia* culture ponds and when there is no intention to turn them into specialized intensive *Artemia* farms. Since each pond is involved in production, the farming area is expanded maximally; however, practical experience shows that only 50–60 percent of the *Artemia* culture area is economically effective. The obvious limitation for the stagnant culture system is the spatial separation of each pond, so that each pond can be managed separately. This culture model also requires more direct labour for pond management.

FIGURE 21

Example of a stagnant *Artemia* monoculture model, associated with an evaporation area to increase salinity and *Artemia* culture ponds



Source: Hoa, V.N. & Van, N.T.H 2019. *Principle of* Artemia *culture in solar saltworks*. N.V. Hoa & P.K.H. Lam, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2946-5. 219 pp.

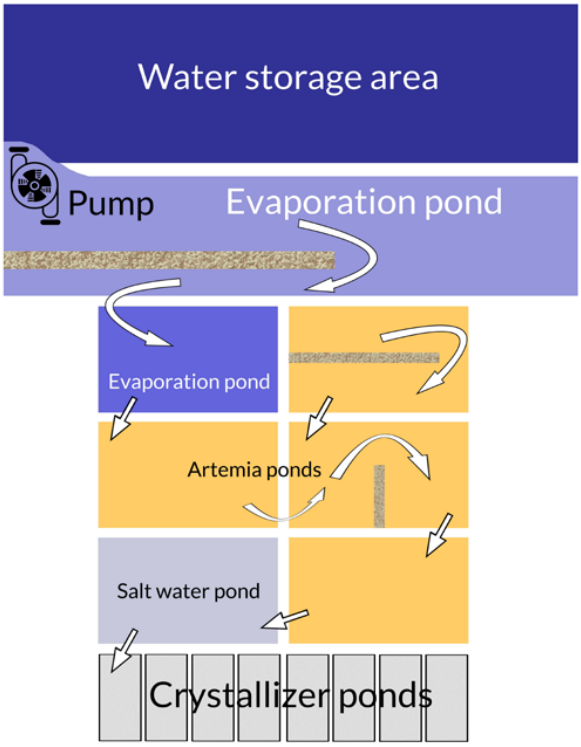
Flow-through culture model

The flow-through model differs from the stagnant model by the following characteristics:

- Its large scale, suitable for integrated production, includes many connected ponds that are operated together.
- Higher investment costs are needed, e.g. for construction, stronger dyke system, better drainage and high-capacity pumping stations.
- Its general operation requires substantial knowledge and experience.

The design and water flow in this model are completely different from the stagnant culture model (Figure 22): The pumping stations provide water for the whole system; and the water level and salinity are different between the ponds in the system, with the first pond having the lowest salinity and the last pond being close to saturation and ready for crystallization.

FIGURE 22
Example of a flow-through *Artemia* culture model



Source: Hoa, V.N. & Van, N.T.H 2019. *Principle of* Artemia *culture in solar saltworks*. N.V. Hoa & P.K.H. Lam, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2946-5. 219 pp.

In this model, the ratio of the *Artemia* culture area to the total farm area is low, as *Artemia* culture can only take place in areas with a salinity in the range of 80–100 g/L. The advantage is the higher yield of cysts/biomass (per unit area) compared to the stagnant system thanks to the frequent green water supply, leading to a high density of reproducing females. As the system requires higher construction costs, it is not suitable for systems that may be converted back to salt fields at some point in time (as this would require additional construction costs).

2.2.2.5. One-cycle versus multiple-cycle culture model

One-cycle culture model

In this context, "cycle" implies that the time and activities needed for pond renovation, preparation of highly saline water, *Artemia* inoculation ("stocking"), pond

management, harvesting and termination of the production (the "crop") run from the start until the end of the dry season. This culture model requires highly experienced staff to manage the ponds such that the *Artemia* population is maintained in a stable highly productive state, with a steady balance between the different age components (nauplii, juveniles, subadults and adults) until the end of the crop, i.e. the end of the dry season (4–5 months). In a stable population, adults may die after having participated in reproduction, but the next generation will come up and replace the previous one. However, if the environment becomes unfavourable, e.g. when low or unstable availability of food creates problems because the feeding efficiency of the larval *Artemia* stages is lower than in the adults, then the recruitment of the next generation is jeopardized, eventually leading to a diminishing population and lower yield if these food conditions persist.

Multiple-cycle culture model
In favourable conditions, *Artemia* reproductive activity tends to increase gradually within about 2–3 weeks from the time of stocking, producing high cyst or biomass yields for a certain period, and then gradually declines. When the reduction in harvested quantities affects the economic efficiency of the operation, farmers may stop the culture, carry out pond renovation, stock the ponds with *Artemia* again, and manage a new reproducing population (= second cycle/crop) as long as the dry season lasts. A second cycle may also be started when nearly all females have switched to ovoviviparous reproduction, and when the primary interest is in cysts. Depending on the duration of the dry season and weather conditions in general, and assuming that the farmer has full control over the *Artemia* populations of the successive cycles, the yields of cysts or biomass over the whole season will be significantly higher than in the one cycle model (Baert *et al*., 1997).

2.2.2.6. Inoculation models

Culture model stocking newly hatched Artemia *nauplii (hatching model)*
Ideally, the culture is started by hatching cysts in standardized hatching conditions, resulting in high hatching of good quality instar I nauplii. However, in reality, the hatching conditions in the field sites may be below standard, resulting in lower hatching efficiency. Whereas a gram of good quality product of Vinh Chau *Artemia* cysts can produce 300 000–320 000 nauplii under standard conditions, in practice this hatching efficiency value is often 250 000–280 000 nauplii. Based on the volume of the culture pond(s) and the intended stocking density (usually 100 nauplii/L but sometimes up to 150 nauplii/L), the farmer should estimate or calculate the number of cysts that need to be hatched.

Culture model stocking ongrowing Artemia *stages (transfer model)*
When the conditions are favourable in the pond after stocking, after 10–15 days (Vinh Chau) *Artemia* will reach the adult stage and start reproducing, with the first broods usually being nauplii (ovoviviparous reproduction), which leads to a burst in population density. Taking advantage of this reproductive trait, the farmer can use a screening net with an appropriate mesh size (1 mm) to keep the adults within the pond, only allowing the nauplii to enter a new pond when there is a difference in water level between the releasing pond and the receiving pond.

The transfer is terminated as soon as the larval density in the new pond is sufficient (approximately 100 nauplii/L). This operation should not affect the healthy condition of the original population, except for a number of collided animals within the net due to the gravity drainage from one pond to another. Transfer of part of the population can also be carried out with any developmental stage of the population if the density in the first pond is too high. The advantage of this model is not only that it saves on

cysts for stocking the ponds, on time, labour and electricity for hatching, but also that stocking ponds with older stages shortens the development time of the population and the reproduction phase is reached faster.

2.2.2.7. Cyst versus biomass production model

Cyst production model
In the initial period of *Artemia* production (since the 1980s) in salt fields in the Mekong Delta, the production of cysts to supply aquaculture hatcheries was the target (Rothuis, 1987). In theory, in order to stimulate brine shrimp females to produce cysts, three conditions must be fulfilled: (1) highly saline water (>80 g/L); (2) fluctuating oxygen content in the water; and (3) sufficient availability of iron (derived from the chlorophyll in microalgae). Practice (both in ponds and in the laboratory) shows that reproductive activity of the females generally starts with ovoviviparous reproduction, then switches to oviparity (although ovoviviparous reproduction may also continue), while the life cycle is ended again with ovoviviparous reproduction (Lavens and Sorgeloos, 1984, 1987a; Hoa and Hong Van, 2019). There is a clear correlation between, on the one hand, an environment full of food with healthy females in the population and, on the other hand, very high fecundity (over 100 cysts or nauplii/brood). Depending on the degree of intensification, the farmer can obtain 30–50, 70–120 or 150–200 kg wet cysts per ha/crop of 3–4 months from extensive, semi-intensive and intensive farming systems.

Biomass production model
In this model, farmers mainly harvest biomass according to different stages (sizes) to supply nurseries, hatcheries or broodstock farms. Protocols have been developed to manage the population once it has reached the reproductive stage, targeting at periodical biomass harvesting (Baert *et al.*, 2002): The most effective procedure is to harvest every 3 days 30 kg of fresh biomass/ha (Anh, 2009; Anh *et al.*, 2010). Moreover, in this model, farmers can collect cysts as a side product, at approximately 30 kg wet cysts per ha/crop. Thus, depending on the sales value and profit expected from cyst versus biomass production, farmers can fully choose between either culture model.

2.2.3. Pond adaptation and preparation

2.2.3.1. General requirements
After choosing an appropriate culture model (see Section 2.2.2) the next step is to design the system for *Artemia* production, which may include different components: (1) the reservoir; (2) the green water or fertilization pond (in which algal bloom is stimulated); (3) the *Artemia* culturing area; and (4) the salt crystallization area. These components are allocated next to the accompanying water inlet and drainage system. The relative surface area and proportions of these components are designed to make the most efficient use of the area for production (normally the water surface area allocated to *Artemia* production itself reaches a maximum of 75–80 percent of the whole area). Depending on the model applied, i.e. intensive or semi-intensive culture, the *Artemia* production system can be designed with or without a fertilization pond (Table 7). In absence of a fertilization pond, farmers can fertilize in the reservoir and/or the water supply canal, and then pump the green water into the *Artemia* ponds. The area of the fertilization pond in case of intensive farming systems depends on the soil profile and fertility as well as on the nutrient availability in the natural seawater. Generally, this pond occupies approximately 20–25 percent of the entire water surface.

TABLE 7
Example of the surface area of *Artemia* culture ponds and fertilization pond according to production model (Shrimp-Salt Cooperative in Vinh Phuoc, Vinh Chau, Soc Trang, Viet Nam)

Type of pond	Semi-intensive		Intensive	
Type of pond	Area (ha)	Percentage	Area (ha)	Percentage
Culture pond	3.2	100	3.70	78
Fertilization pond	-	0	1.05	22
Total	**3.2**	**100**	**4.75**	**100**

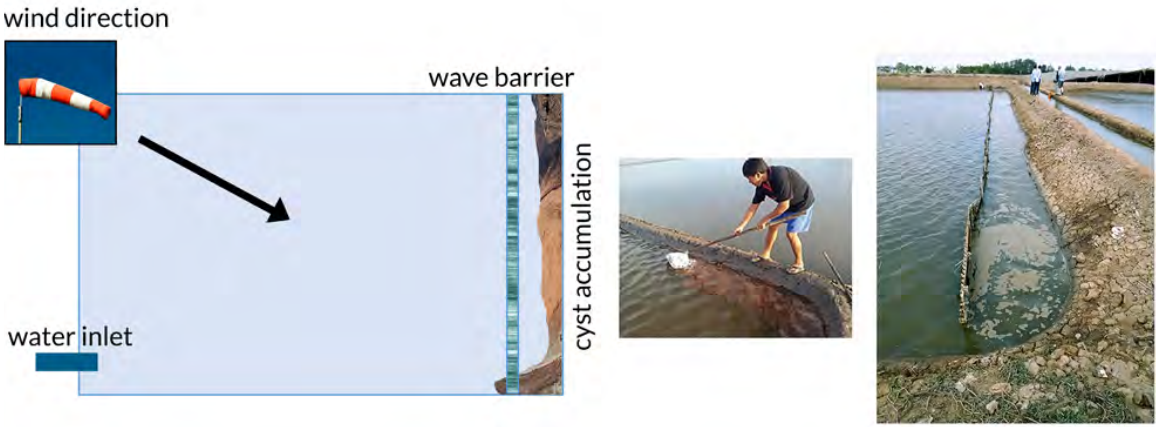
Source: Hoa, V.N. & Van, N.T.H 2019. *Principle of Artemia culture in solar saltworks*. N.V. Hoa & P.K.H. Lam, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2946-5. 219 pp.

2.2.3.2. Size, layout and orientation of Artemia culture ponds

Individual pond areas in the range of 0.5–1.0 ha are the most suitable for *Artemia* culture in an extensive or semi-intensive model for reasons of convenience of pond management and limitation of labour costs, especially for the stagnant culture model. For intensive ponds, the appropriate size of an individual pond is about 0.3 ha because of the higher requirement for specific management techniques (e.g. related to water supply and drainage protocols, feeding procedures, aeration type being installed). In the intensive system, a green water pond is often used to supply food to the *Artemia* culture ponds (see, for example, Figure 20). Ponds are usually constructed in the shape of a rectangle, with the length being 3–4 times the width.

Several pond production activities are affected by the direction of the prevailing winds on the pond surface, for example, the dispersion of the nauplii at the time of stocking, dispersion of fertilizers and feed, and most importantly the accumulation of the floating cysts into a corner for more convenient harvesting. Therefore, when constructing a pond, the long side of the rectangle should more or less coincide with the prevailing wind direction (Figure 23). However, because strong winds often create foam close to the shore on the surface of the saline pond water, cysts are easily trapped into this foam. If the foam with the trapped cysts is blown out of the ponds by the wind, cysts are lost. Foam production can be reduced by the construction of obstacles (so-called "cyst barriers") using coconut leaves or bamboo sticks in the corner of the pond or along its shortest side. These cyst barriers are also effective in lowering wave height behind the barrier, thus facilitating cyst accumulation and harvesting (Figure 23).

FIGURE 23
Example of an *Artemia*-shrimp/fish-salt integrated culture model



Source: Hoa, V.N. & Van, N.T.H 2019. *Principle of Artemia culture in solar saltworks*. N.V. Hoa & P.K.H. Lam, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2946-5. 219 pp.

2.2.3.3. Pond depth

In traditional salt fields, the water level in the pond is often very shallow, i.e. 10–20 cm, so the temperature fluctuates at a very high rate and beyond the *Artemia* tolerance level (>40 °C on the hottest days in the Mekong Delta). Moreover, the shallow water level in

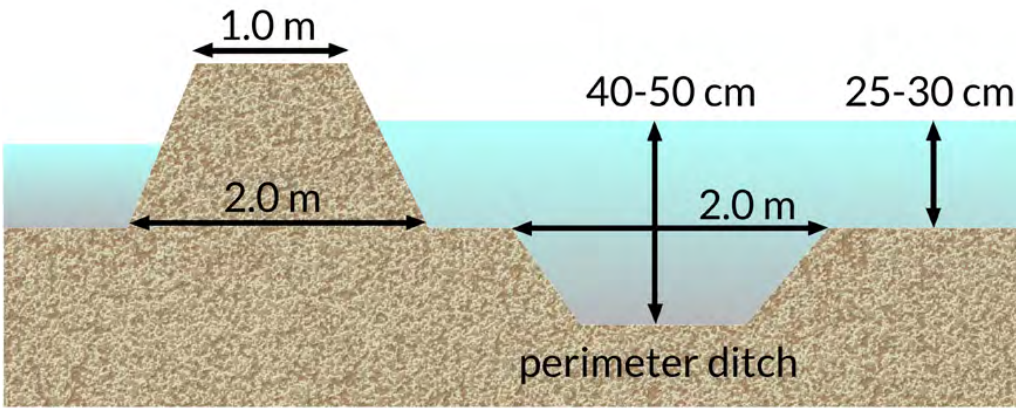
the pond and high transparency of the water column stimulate the growth of benthic macroalgae (phytobenthos, known as "Lab-Lab"). Development of phytobenthos is undesirable, as it is too large for *Artemia* to ingest and prevents normal development of microalgae (macroalgae remove nutrients more efficiently from pond water than microalgae). Floating phytobenthos reduces evaporation rates and hampers cyst collection (see Section 2.2.5.5).

To reduce temperature stress and proliferation of bottom algae, and thus to optimize *Artemia* production, the ponds should be deep enough (Figure 24). It is recommended to excavate the existing salt production pond and to raise the pond's dyke, allowing to maintain a water level of at least 50 cm from the pond bottom. Moreover, especially when ponds are shallower (e.g. 30 cm), peripheral ditches should be dug out (2–3 m wide, at least 20–30 cm but typically 50 cm or even more in depth) not only to help to increase the pond volume but that also to provide refuge for the *Artemia* population when higher temperature occurs at the superficial water layer at the end of the dry season. In any case, the pond and/or its peripheral ditches should provide for >50 cm of water depth. The earth excavated from the ditches can be used to heighten the dykes.

On the other hand, it should also be noted that ponds should not be dug out too deep (e.g. >1 m depth), as evaporation rates depend upon the ratio pond surface (in m^2) over pond volume (in m^3); in deeper ponds, a decreased ratio leads to a slower increase in salinity, as the temperature is usually lower in the bottom layers than at the surface (i.e. stratification). Also, more water is needed to fill deeper ponds. In regions where no permanent stocks of highly saline water are available, all this slows down the evaporation process, prolonging the time needed to obtain water of salinity sufficiently high for *Artemia* inoculation (i.e. in Viet Nam, more than one extra month is needed to completely fill ponds with a deep peripheral ditch) and thus delaying the crop. Alternatively, the area in which *Artemia* is cultured can be reduced while the area allocated for evaporation is increased.

However, if availability of brine waters is not critical, deeper ponds are recommended as they allow for much higher *Artemia* production yields. Moreover, in deep ponds (such as practiced in a number of *Artemia* farms in Thailand, a 1.5 m water depth is used), it is easier to maintain high salinity during the dry season.

FIGURE 24
Cross–section of an *Artemia* culture pond, a peripheral ditch and a dyke with recommended dimensions



Source: CTU, Viet Nam.

Sometimes salt producers, interested in *Artemia* production, may explicitly wish not to convert their salt field into an optimally functioning *Artemia* farming system and thus not to bring substantial changes in the layout of their salt field, described above, to their full extent. This approach may be inspired by several objectives (Vinh *et al.*, 2019):

- To keep the freedom to switch the production strategy between *Artemia* and salt at any time, as a function of market demand, estimated profit margin according to the production model, etc.
- To save on costs and time needed for optimal pond construction/adaptation – operating deeper *Artemia* ponds requires more saline water, as described above, and thus a longer preparation time than, for example, the period of about 30 days in the Mekong Delta, which is normally sufficient to obtain saline water of 80–100 g/L when working with shallow water (as is the case in the salt production process). This factor is even aggravated in the context of climate change, with extreme weather appearing more often: A longer preparation period shortens the period of *Artemia* production and delays it into the dry season. Suitable temperatures for *Artemia* culture, however, generally occur in the early season. Later in the dry season, extremely hot temperatures are more frequently recorded, causing higher mortality and reduced reproduction in *Artemia*.

2.2.3.4. Pond floor and dykes

Preventing leakage in ponds is essential. The pond should be located in a place with clayish soil structure, and its floor and dykes need to be compacted carefully during construction (Plate 21). While heightening the dykes, cracks often appear in the soil structure, which are sources of leakage. To minimize this, first it is needed to wetten the old surface of the dyke, to add new soil on top of it and to compact it again. Next, calcium oxide (CaO) and clayish soil can be used not only to fill holes and cracks but also to kill crab and fish living in those crevices. Moreover, to limit excessive erosion, the dyke should be constructed with a slope corresponding with a surface:base width ratio of 1:2 (Figure 24). Rather than manual compaction, using machinery allows for tighter and stronger construction of the pond elements. At the start of the dry season, and before filling the pond with water, it is good practice to scrape away the bottom mud layer and to leave the pond bottom to dry for 2–3 days to stimulate the activity of aerobic bacteria in the pond bottom. These bacteria decompose the organic matter, release nutrients from the substrate, and remove toxic compounds such as hydrogen sulphide (H_2S) and ammonia (NH_3).

PLATE 21
Manual compacting of dykes

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2.2.3.5. Liming

Normally, ponds used to culture *Artemia* do not need liming. The highly saline water often has a hardness of more than 50 mg calcium carbonate ($CaCO_3$) per litre (because of the presence of carbonates). Liming ponds with such hardness will not further improve yields. Liming, however, can be considered when culture water has a pH of less than 7.5 and when stimulating an algal bloom is difficult.

The chemicals used for liming are the oxides, hydroxides, and silicates of calcium and magnesium. The liming substances most often used in aquaculture are agricultural calcium carbonate ($CaCO_3$), CaO or quicklime, and calcium hydroxide ($Ca(OH)_2$) or hydrated lime. The use of lime will increase the pH of the pond environment quickly, up to about 10, and may kill pathogens and organisms surviving on the bottom. After about three weeks, the pH decreases to 7.5 and normal mineralization occurs. Recommended doses vary between 500 and 1 000 kg of $CaCO_3$ per hectare. The lime

requirement is highest for clay bottoms, acid bottoms, and when the pond water has a low concentration of Ca^{2+} and Mg^{2+}.

Whereas drying can be beneficial for most soils, this is not true for acid sulphate soils (see above), which are often found in mangrove areas. When exposed to air, the pyrite of these soils oxidizes to form sulphuric acid (Hoa, 2014). Liming of these soils is possible but requires high quantities of lime. A simpler method to reduce acidity is flushing ponds repeatedly after oxidation (exposing the soil to air). This procedure, however, can take a long time. Therefore, such type of bottom is usually kept submerged and extra layers of oxidized acid-free soil are added on top of the original substrate. In general, culturing brine shrimp in regions with acid sulphate soils should be avoided.

2.2.3.6. Predator control

Predators of *Artemia* are wading birds, crustaceans such as crab, and fish species such as tilapia. Moreover, aquatic insects (Corixidae) and a variety of zooplankton organisms, such as cyclopoid copepods, rotifers and the protozoan ciliate Fabrea salina (Plate 22), can act as food competitors or predate on *Artemia* nauplii.

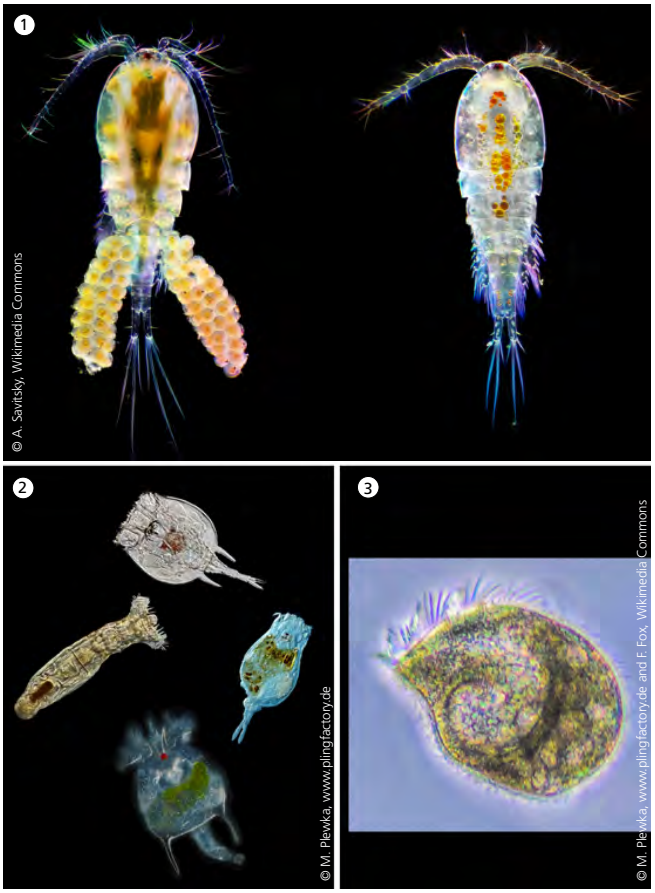
Predation by birds can be avoided by the use of scarecrows installed in the pond. As ponds often cannot be drained completely, some predator control may be needed before filling the ponds. Predating fish and crustaceans, left in puddles, may be killed using rotenone (0.05 mg/L to 2.0 mg/L); tea–seed cake (15 mg/L); a combination of urea and hypochlorite (5 mg/L urea and 24 h later 5 mg/L calcium hypochlorite $Ca(OCl)_2$,); or derris root (1 kg/150 m^3). Dipterex® (2 mg/L) kills smaller predators such as copepods but is also very toxic for shrimp. The degradation of rotenone and chlorine to non–toxic forms is fairly rapid (24–48 h). If on the other hand tea-seed cake or Dipterex® are used, ponds should be flushed prior to stocking *Artemia*.

During pond operation, aquatic predators can be avoided by filtering the intake water, where the monk is installed (Plate 23), through a screen, preventing fish or crustacean eggs and larvae to enter the system. In case ponds are managed independently (stagnant ponds each with separate inlet and outlet system), a screen should be installed as a filter in front of each water intake. In a flow-through system where water in different ponds is managed with a common water supply and drainage canals using pumping stations, usually a fine fish net (seine) is installed at the first water pumping station (Plate 23). To restrict the presence of small food competitors and predators, filter boxes can be used made of stainless-steel screen (mesh size 120 µm) to filter the inlet water. However, this kind of filter

PLATE 22

Predators and food competitors of *Artemia*

(1) Copepods; (2) Rotifers (clockwise starting from top: *Platyias quadricornis*[1], *Euchlanis deflexa*[1], *Brachionus quadridentatus*[2], *Rotaria citrina*[1]; (3) *Fabrea salina*

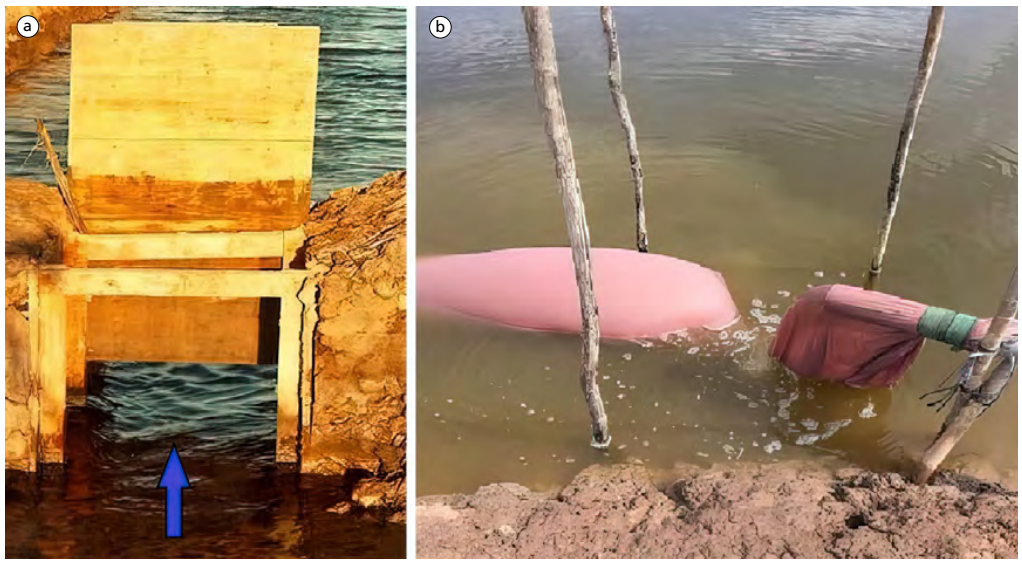


Source: (1) A. Savitsky, Wikimedia Commons.
(2) [1] M. Plewka, www.plingfactory.de and [2] F. Fox, Wikimedia Commons. (3) B.T. Larson, University of California, San Francisco, United States of America.

box usually has a high cost, and is less effective when used for a large inlet surface area or for a source of water containing lots of suspended materials such as large size algae, zooplankton and organic particles. Therefore, the most effective way to prevent and/or eliminate these organisms is to raise the salinity level of the pond up to 80-100 g/L or more within a few days before *Artemia* inoculation, or to use derris root or rotenone; 24 h after its application, the pond can be inoculated.

PLATE 23

Monk located at water inlet (arrow indicates water direction) (a); fine fish net/cloth bag to trap incoming fish (b)



2.2.3.7. Production of high salinity water

General procedures for evaporation of seawater

After pond adaptation/renovation has been completed, the whole production system is filled with seawater for evaporation by the action of wind and solar energy, with the aim of obtaining saline water of at least 80 g/L. Saline water preparation has high priority, as this period is considered as "lag–time". The duration of this evaporation process depends on the weather conditions, the area of water exposed to sunlight, the initial salinity and the volume of saline water needed for the production process.

Depending on the farmer's experience and local geographical characteristics, farmers in the Mekong Delta, for example, have protocols to operate through "thin water layer" (i.e. shallow water) or "thick water layer" (i.e. deep water). Moreover, frequent raking is commonly applied to create water turbulence and hence to speed up the rate of evaporation. In the climatological conditions of the Vinh Chau–Soc Trang salt fields, saline water preparation takes at least a month, and several factors may interfere negatively with the course of the evaporation process:

- Late termination of the rainy season.
- The seawater intake, often being located near estuaries, generally receives a mixture of freshwater and seawater. Consequently, the salinity at the very first inlet of the production system is very low, typically in the range of 12–15 g/L, at the beginning of the production season.
- In certain areas (in the Mekong Delta), the beginning of the dry season often coincides with a cold wind regime (i.e. northeast monsoon), which lowers the water temperature and hence slows down the evaporation process.

Therefore, in order to speed up the evaporation process and hence advance the *Artemia* production process, a number of adaptations of the production protocols have been worked out, as described below.

Use of saturated brine to obtain high–salinity water

Using brine water to shorten the time needed for evaporation is a method that has been widely applied in salt production in many different countries in the world, a procedure carried out as follows: By the end of the *Artemia* crop, water from the culture ponds or evaporation ponds that still has high salinity can be collected and stored for the next season in a reservoir, i.e. a pond with a depth of at least 1 m, that does not leak or have any contaminants. To retain the brine water throughout the rainy season, an overflow outlet should be installed to drain the superficial rainwater layer, and thus to minimize the risk that rainwater dilutes the saline water. Experience gained in the Soc Trang and Bac Lieu areas shows that, when using a pond with a depth of 1–1.2 m to store brine water with an initial salinity of 100–120 g/L, water salinity drops to 45–55 g/L after 5–6 months of storage in the rainy season. Depending on the salinity and total volume of the reservoir, the *Artemia* culture area of the next season can be tailored in size. Mixing this water with natural seawater or water from the evaporation ponds can ensure that the target salinity of 80–100 g/L for *Artemia* inoculation is quickly reached.

When using brine water to produce saltwater of 80–100 g/L, it should be noted that if the brine water has too high salinity, a number of salts have precipitated, which changes the chemical composition of the saltwater (see Section 2.1.2). Because the tolerance of different *Artemia* strains to these chemical imbalances is not always well documented, it is recommended that brine water used for this process should not have a salinity level higher than 120 g/L.

Use of crude salt to obtain high salinity water

The *Artemia* farmer can also increase salinity by adding crude salt to the evaporation pond or directly into the *Artemia* culture ponds, but this should only be done using poor quality salt products (also known as "black salt" Plate 24), which are cheap and have a low market demand. Practical experience in the conditions of the Mekong Delta shows that, for an *Artemia* culture area of 1 800 m² and when using about 30 tonnes of salt added to seawater with an initial salinity of 30 g/L, the time to prepare 80 g/L saline water in a pond with 10 cm depth can be shortened from the usual 3–4 weeks to 1–2 weeks. As mentioned above, the economic efficiency of this practice should be considered, taking into account the extra investment needed. In conditions when salt is cheap and unmarketable, valorizing this product to advance the *Artemia* stocking time and thus to extend the *Artemia* production season can be a profitable policy.

PLATE 24

Crude ("black") salt (right) can be used to accelerate the salinity increase in *Artemia* ponds

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2.2.4. Food management

2.2.4.1. Types of food supplied and monitoring nutritional status of the Artemia *population*

Artemia is a continuous and non-selective filter feeder, and different factors can affect its filtration rate, digestion rate and assimilation rate, including the quality and quantity

of the feed used, the developmental stage and physiological status of the animals and the abiotic culture conditions. An appropriate *Artemia* feed should meet the following criteria:

- It is easily (commercially) available, or can be easily produced.
- It has a low purchasing or production cost.
- It has an appropriate size (<50 µm particle size for *Artemia* adults).
- It is highly digestible and has a suitable nutrient composition (leading to a favourable feed conversion rate).
- It is storable without quality loss (for feeds other than live microalgae).
- It has low solubility or leaching of nutritional components (for feeds other than live microalgae).

Whereas standard feeding protocols are available for culturing *Artemia* in the laboratory with microalgae or other food types, food management for pond culture requires daily observation and, if needed, adjustment.

In *Artemia* pond culture, microalgae are mainly used as food, growing directly in the *Artemia* pond or supplied through the green water from the fertilization pond (Le *et al.*, 2019; Van Stappen *et al.*, 2019). However, as microalgae availability generally fluctuates, as a function of the nutrient sources provided and abiotic water parameters such as temperature and salinity, often leading to unstable algae development in the fertilization pond, additional food is generally provided as the key factor in ensuring the success of productivity. Other food sources used for *Artemia* culture are usually organic fertilizers, such as manure from pigs or other terrestrial animals. Additionally, to maintain a stable feeding level in the *Artemia* pond (especially when the green water supply does not meet *Artemia* requirements), the waste product from the feed additive monosodium glutamate ("ami-ami") or the by products from agro industrial processing (processing of rice, corn, soy, milk, etc.) are added as supplementary feeds into the *Artemia* ponds (Le *et al.*, 2019; Van Stappen *et al.*, 2019).

Supplementary feeds are commonly the subject of ongoing experimental work in ponds (Hoa *et al.*, 2013) to investigate how yields (and profits) can be increased by applying, for example, fermentation of cheap agricultural products such as rice bran, or by use of or supplementation with shrimp feed and fishmeal, directly administered into the pond (Anh *et al.*, 2009; Hoa and Van 2019). Moreover, a formulated *Artemia* dry feed has been produced by Can Tho University, Viet Nam (see Section 2.2.4.4), which can be used as supplementary feed in pond culture when there is a lack of green water. Also, biofloc technology can be applied in *Artemia* pond culture (Hoa and Hong Van, 2019) by adding a cheap carbon source into the fertilization pond to reach a C:N ratio of 10 or more, or directly into the *Artemia* culturing pond, to stimulate the development of heterotrophic bacteria: This technique helps in stabilizing the environment by converting nitrogen waste products into bacterial biomass that can be used as food by *Artemia*, thus saving on costs for other feed sources. When well fed both in terms of feed quantity and quality, *Artemia* will thus reach the adult stage within 10–15 days, increasing its body mass about 500-fold compared to newly hatched nauplii.

While there are thus a number of ways to satisfy the nutritional needs of *Artemia* in the pond, the key is to monitor and assess whether the food source used is suitable for *Artemia* growth. This assessment is based on the observation of individuals or the population as a whole:

- Are the faecal pellets well filled? This can be checked by keeping some animals in a recipient filled with pond water, collecting pellets from the bottom and checking pellets under a microscope. Are the pellets short or do the animals have tail–like long pellets (Plate 26)? If "tailing pellets" are observed together with only partly filled pellets, animals are underfed. If tailing pellets are observed, but pellets and guts are well filled, the food is not digested properly, which can be due to overfeeding or the presence of unsuitable algae.

- Are the individuals in the *Artemia* population uniform in size? What colour is commonly observed in *Artemia* (brightly coloured, opaque, pale, etc.)?
- Is *Artemia* actively swimming? Are they swimming in groups or alone, close to the water surface or to the bottom? (When lacking feed, *Artemia* tend to stay close to the pond bottom, grazing on the bottom layer of algae.)
- Does the population show a normal time to develop and become sexually mature; when are riding couples observed? (For Vinh Chau *Artemia*, reproduction should start 10–15 days after inoculation.)
- How is the fecundity of the females (as shown when dissecting ovaries)? Do they show high fecundity (brood size) or not? Are the ovaries brightly or darkly coloured? What is the ratio of females carrying an empty ovary?
- What is the balance between different age classes of the population, i.e. nauplii, juveniles, subadults, adults? (see Section 2.2.5.4.)
- Are any dead *Artemia* accumulating in the downwind corner?

Correct observation largely depends on the interest and experience of the farmer; full and correct observation allows for adjustments in pond management, when needed, thus leading to a longer culture period with higher and stable productivity.

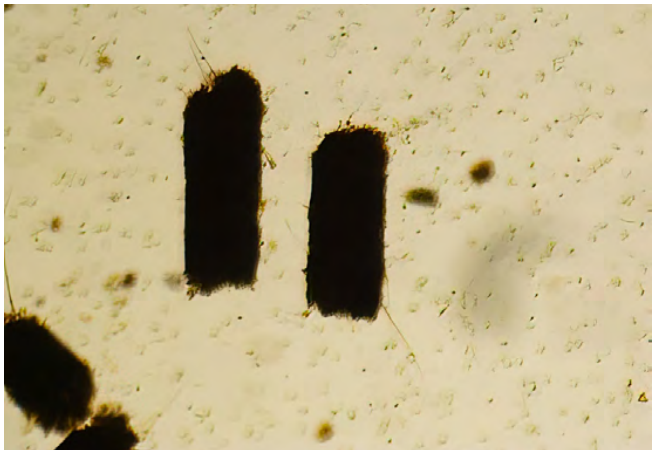
PLATE 25
***Artemia* with filled gut (a) and an underfed *Artemia* (b)**

2.2.4.2. Green water production

General principles of green water production

Artemia cultured in ponds need to be provided with microalgae as a main food by a combined use of organic and inorganic fertilizers while keeping a N:P ratio ≥3:1 (Geider and La Roche, 2002; Khoi, Guong and Merckx, 2006a). These microalgae come from two main sources: (1) from the fertilization pond, which is considered as "indirect fertilization" and which is often also the main source of green water supply; and (2) grown within the culture ponds through "direct fertilization" into the *Artemia* ponds. Each strategy of fertilizing has its own advantages and disadvantages. In practice, the amounts of fertilizers and manure applied to the fertilization pond are much higher than the amounts applied to

PLATE 26
Short, well-filled faecal pellets



the *Artemia* ponds, which may result in accumulation of sediments and sedimented nutrients in the fertilization pond. While algae density is thus important, so is algae composition; in practice, fertilization protocols aim at proliferation of green algae and diatoms (Hoa and Nhi, 2020; Hoa and Le, 2021).

Protocols for green water production have been developed empirically and thus also have some limitations:

- Green water is produced based on the local microalgae species entering the production system through the seawater inlet. Consequently, species suitable for *Artemia* production (e.g. diatoms) may be hard to be induced to proliferate.
- The algae composition is also affected by environmental factors, such as temperature, salinity, light, seasonality and nutrients in the soil. In adverse conditions, there is appearance of filamentous algae or algae of larger size (macroalgae), which are unsuitable as feed for *Artemia* and which will also compete with microalgae for available nutrients.
- Salinity in the fertilization pond is maintained at a low salinity (<50 g/L), which is suitable for microalgae to develop. However, at this relatively low salinity, *Artemia* predators such as shrimp and fish are often present (tilapia especially can grow well in the fertilization pond environment). Additionally, *Artemia* competitors (rotifera, copepoda, *Fabrea salina*, etc.) can grow and become abundant in the low salinity fertilization pond.
- The fertilization pond occupies part of the area that could be used for *Artemia* culture. In case of lower algal development, a larger area to be used as a fertilization pond is required, thus reducing the area in which *Artemia* are grown.

Direct fertilization into the *Artemia* pond (and not using a fertilization pond) does not have some of the above disadvantages: The *Artemia* production area can be maximized, and the higher salinity in the *Artemia* ponds (>80 g/L) limits the invasion of predators and competitors. However, these conditions also present some limitations: Fertilization at high salinity is less effective, as only a few types of microalgae can adapt to highly saline water (often the microalgae production does not meet the needs of the *Artemia* population). Moreover, direct fertilization in the *Artemia* ponds leads to accumulation of organic substances in these ponds, necessitating substantial labour costs for frequent raking (Plate 27) to keep organic particles in suspension as food for *Artemia*. The more transparent the water, the more intensive raking will be needed. An effective approach is generally when: (1) inorganic fertilizers are applied in the fertilization pond or its inlet canal; and (2) organic fertilizers are applied directly in the fertilization pond or the *Artemia* culture ponds. Supplying green water to the *Artemia* ponds not only provides food but also replenishes the leaked and evaporated water. In the Vinh Chau *Artemia* ponds, green water is usually supplied every two days, increasing the water level a few centimetres (corresponding with 10–15 percent of the *Artemia* pond volume), depending on the culture conditions.

PLATE 27
Mixing the water column and resuspending settled particles by raking the *Artemia* pond

Measurement of algal density and determination of algal species composition

The easiest way to estimate algal abundance is through the measurement of turbidity, which can be readily measured using a Secchi disc (Plate 28). The disc is lowered in the water up to

PLATE 28
Use of Secchi disc to measure turbidity

the point where the contrast between the white and black (or red) fields disappears, and the depth is recorded. After lowering the disc a little more, it is then brought up slowly until the contrast is again visible, and this depth is again recorded; the average of both recorded depth values is the turbidity measured. The turbidity also fluctuates during the day and is generally highest in the afternoon. Wind (concentration of algae in the downwind corners) as well as suspended solids (e.g. clay particles) can affect turbidity readings. In *Artemia* ponds, turbidity readings between 25 and 35 cm are optimal. At lower turbidity levels (= higher depth values measured with the Secchi disc), extra pumping of nutrient-rich water is needed. At higher turbidity (= lower values), there is an increased risk of oxygen depletion, especially at dawn. Overfeeding linked with higher turbidity may be a problem, especially for naupliar and early juvenile stages, resulting in low survival and growth; thanks to their more efficient filtering capacity, it is less of a problem for adult stages.

If time and equipment are available, algal density can also be estimated by analysis of the chlorophyll concentration (Takahashi, 2019; Jing-Yan, Li-Hua and Zhen-Hui, 2021; Solovchenko *et al.*, 2022) or by direct counting using a blood cell counting chamber (e.g. Bürker). These methods, however, are only valid if combined with a proper sampling programme. As algae populations are seldom homogeneously distributed over the pond, recommendations as given for *Artemia* sampling should be followed (see Section 2.2.5.4).

Additionally, the colour of the water (Table 8) can give useful indications concerning the type and concentration of particles and microalgae present in the culture ponds. If problems with *Artemia* performance are encountered, more thorough analysis of the algae samples is recommended.

Green colour typically indicates the presence of Chlorophyta (green algae), and bluish colour Cyanophyta (blue-green algae). Brownish colours are indicative of Bacillariophyta (diatoms), whereas red colour is typical for highly saline water with the microalga *Dunalliela salina* or with halophilic archaea such as *Halobacterium* (Hoa and Hong Van, 2019). The composition of algae not only affects *Artemia* growth and reproduction, but also affects the nutritional value of biomass and cysts (e.g. fatty acid profile). In Viet Nam, diatoms belonging to the genus *Nitzschia,*

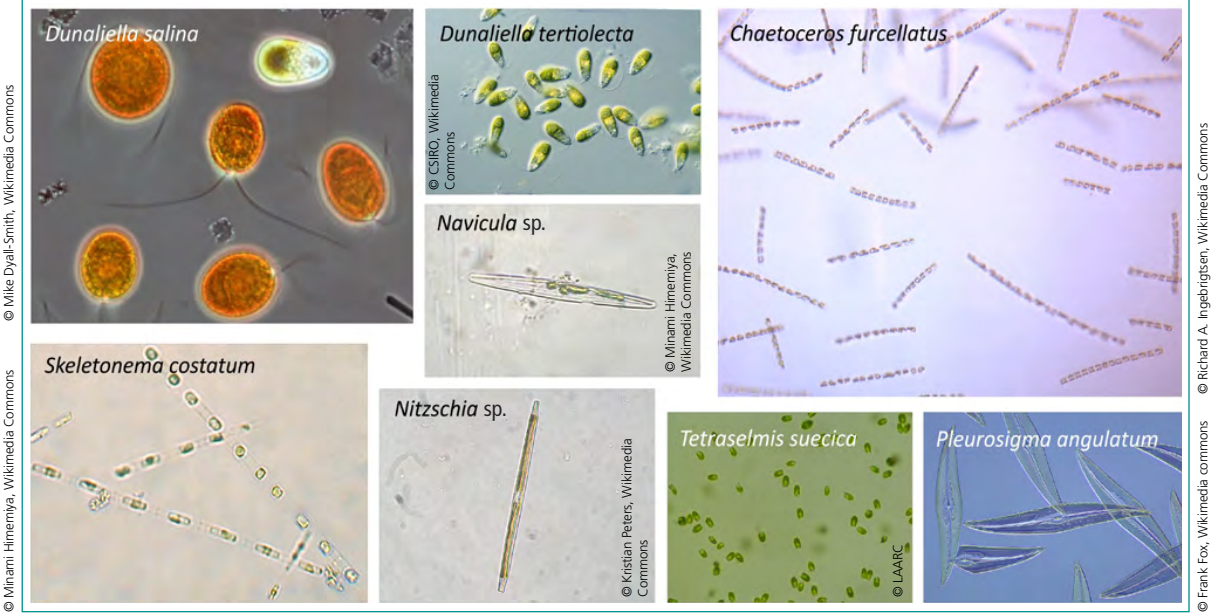
TABLE 8
Water colour corresponding with different types of suspended particles

Colour	Types of particles and microalgae expected
Clear	Few organisms present/low nutrient level.
Grey/white	High amount of suspended matter, probably clay/gypsum, especially after rain.
Green	Algae, probably green algae. When familiar with the site, different shades of green can be associated with different green algae.
Brown	Algae, possibly diatoms.
Red	If combined with high salinity, *Dunalliela* sp. or *Halobacterium* sp.

Source: Authors' own elaboration (LAARC).

Chaetoceros, Skeletonema, Navicula and *Pleurosigma* have been shown beneficial for *Artemia* production, such as the green algae genera *Chlamydomonas*, *Tetraselmis* and *Dunaliella* (Plate 29). Pond tests in Viet Nam have also tried to upscale the production of Chaetoceros in separate plastic-lined ponds of 15 m³, aiming to produce an outdoor culture of this species, eventually to be used as green water rich in *Chaetoceros*. Other species such as *Nannochloropsis* and *Chlamydomonas* may be difficult to digest because of their thick cell walls. The algal composition in the fertilization pond depends on the natural algae population at the water inlet: Usually, the most dominant algae present at the water inlet will also be the most dominant in the fertilization pond. Nevertheless, there may be shifts in species abundance depending on the fertilization scheme and environmental conditions in the pond (Hoa and Hong Van, 2019).

PLATE 29
Suitable microalgae species for *Artemia* production



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Fertilizers and fertilization strategies

Fertilizers used to induce microalgae growth in the fertilization pond include two types: inorganic fertilizers and organic fertilizers.

(a) Inorganic fertilizers

Nitrogenous fertilizers – source of nitrogen (N)

Common inorganic nitrogenous fertilizers are:

- Ammonium fertilizer: $(NH_4)_2SO_4$ (contains 20 percent nitrogen);
- Calcium nitrate fertilizer (15–16 percent nitrogen): has a quick effect in inducing green water; and
- Urea and amide fertilizer (46 percent nitrogen): easily dissolves but has a slower effect.

Phosphate fertilizers – source of phosphorus (P)

Common phosphate fertilizers are:

- Superphosphate: $Ca(H_2PO_4)_2{\cdot}H_2O$ (16–20 percent P_2O_5), high solubility;
- Dicalcium phosphate: $CaHPO_4{\cdot}2H_2O$ (35–48 percent P_2O_5), low solubility;
- Triple superphosphate: $Ca(H_2PO_4)_2{\cdot}H_2O$ (42-48 percent P_2O_5), good solubility;
- Sodium polyphosphate: 46 percent P_2O_5, in liquid form; and
- Phosphoric acid: 54 percent P_2O_5, in liquid form.

A substantial amount of phosphate precipitates in saltwater, and phosphorus is also quickly adsorbed in the pond bottom (Khoi, Guong and Merckx, 2006a). Minimizing losses of phosphorus caused by precipitation or adsorption is thus important by using a suitable phosphorus fertilization strategy. For example, it is recommended to select phosphate fertilizers with fine particle size, highly soluble in water, and to apply them frequently in small quantities to maximize their effect (Hoa and Le, 2021).

The combination of phosphate and nitrogenous fertilizers is the basis to enhance the growth of algae. Variation of these two components will also affect the biochemical composition of the algae. Fertilizers should first be dissolved in freshwater, even when using a liquid fertilizer; this solution can then be spread over the pond. Dissolving in freshwater increases the solubility, and this procedure facilitates even distribution of the fertilizer throughout the pond. Fertilization should not take place on a cloudy day because algae development is limited by low light intensity. Fertilizers are more efficient when applied in water of lower salinity (<50 g/L); for this reason, inorganic fertilizers should not be applied in high salinity *Artemia* ponds. Finally, the conditions in the fertilization pond should be kept as stable as possible (salinity, water level, etc.) to favour algal growth. A few days after fertilizing, the water colour will become brown or green, leading to high water turbidity. Green water from the fertilization pond is then pumped into the *Artemia* ponds.

The quantities of fertilizers needed vary and depend on the natural conditions of the site and the local microalgae species composition (Hoa and Nhi, 2020; Hoa and Le, 2021). Therefore, the fertilizers need to be optimized locally. In the Mekong Delta, nitrogen is generally applied at 1 mg/L (for nutrient-rich water) to 10 mg/L (for water that is poor in nutrients). For example, to increase the nitrogen content in the water column with 1 mg/L, a pond volume of 1 000 m^3 requires 1 000 g of nitrogen, corresponding with 2 174 g urea (as urea contains 46 percent nitrogen). If after two days algae still do not develop, a second dose higher than 1 mg/L should be supplied until green water appears and the transparency reaches approximately 15–20 cm. Normally, the recommended N:P ratio (by weight) to stimulate the development of the desired green and diatom algae is 10:1 (Geider and La Roche, 2002). However, because phosphorus dissolves slower in saltwater and is adsorbed quickly by the pond bottom, a ratio of N:P = 3–5:1 (Khoi, Guong and Merckx, 2006a, 2006b) may be more appropriate in field conditions. The N:P ratio also plays an important role in the chemical composition of the algae: In laboratory tests, the N:P ratio influenced not only cell density, but also cell structure and biochemical composition of *Tisochrysis lutea* and *Nannochloropsis oculata*, with higher fatty acid contents at N:P 20:1 and 30:1 as compared to 5:1 and 10:1 (Rasdi and Qin, 2015). On the other hand, application of too much phosphate fertilizers, especially when combined with high temperature (>28 °C) and high water transparency (down to the pond bottom), will stimulate the growth of benthic algae (Cyanophyta). Growth of Cyanophyta such as *Lyngbya* and *Oscillatoria* is also promoted by high phosphorous content combined with low salinity. These algae are too large in size for *Artemia*, which may be trapped in the algal filaments, thus causing the "sticky thoracopods" phenomenon.

After algae populations have developed, the same fertilization protocol should be followed for at least once a week. Usually, fertilization twice a week is recommended when algae development is rather low. Regular water supply to increase the CO_2 content in the water is essential. Even when following a strict protocol, primary production may still be affected by environmental conditions, such as temperature, light and salinity, as well as by the species composition and abundance of the local algae population at the inlet.

(b) Organic fertilizers

Organic fertilizers are commonly used in aquaculture to stimulate the production of natural food in shrimp/fish culture, and the same applies for *Artemia* culture (Le *et al.*, 2019).

Chicken, quail and duck waste, cow dung, pig dung and goat dung can all be used; cow dung, however, is not preferred because it seems to stimulate the development of filamentous benthic algae. Recently, the frequent occurrence of avian flu (bird flu), and the related use of medical products which may remain in the faeces, have made the use of chicken dung, especially fresh product collected on the farm, not recommendable.

In *Artemia* ponds, a combination of organic and inorganic fertilizers is commonly used: The inorganic fertilizers stimulate quick development of algae and mineralization of organic fertilizers (lower C:N ratio), whereas the organic fertilizers either act as a direct food source for *Artemia* or maintain a certain level of available nutrients in the pond as they slowly release nutrients into the water column. Organic fertilizers contain not only nitrogen and phosphorus but also many trace nutrients, for which they have a positive effect on algae growth; they stimulate the growth of bacterial flocs, which also serve as food for *Artemia*. Wheat and rice flour, rice bran and other agricultural by-products are also used as supplementary feed for *Artemia* (Anh *et al*., 2009). When highly soluble products are used such as soybean meal, they should first be treated by adding water with strong aeration for 1–2 h to allow all soluble components to be dissolved (producing a turbid suspension). Then aeration should be turned off to allow non-dissolved particles to settle. This process can be repeated several times until turbidity has become minimal; the fine particles are collected and can be supplied into the *Artemia* culture. Rice bran should only used if there is a lack of food for the culture ponds because it is relatively expensive, has an inappropriate particle size (too large for the *Artemia* filter apparatus) and contains a lot of indigestible fibre, leading to higher accumulation on the pond bottom. In *Artemia* ponds, organic fertilizers and supplementary products should only be used in suitable amounts, preferentially in small but frequent doses, and not when the temperature is too high or when there is no wind. When applying organic fertilizer, the pond water can become turbid (due to the manure particles in suspension), but these particles settle quickly and thus stimulate benthic algae mats to develop in case pond raking is not done sufficiently frequently. Because organic fertilizers have a high phosphorous content, they may also stimulate the growth of blue-green algae, and bacterial growth also leads to increased oxygen consumption (and possibly increased bottom acidity). Overdosing with manure may thus disrupt the processes in the pond bottom, cause oxygen depletion, and consequently bring about mass mortality of the *Artemia* population, especially after a calm night.

In *Artemia* production in the Mekong Delta, the recommended amount of organic fertilizer is 0.5–1.25 tonne/ha at the beginning of the crop as initial fertilization, followed by a fertilization scheme at a rate of 100–200 kg/ha for 2–3 days. When using rice bran or soybean meal, the rate is typically at 10–20 kg/ha per day. However, the quality and characteristics of organic fertilizers are not stable (depending on the animal batch, the feed supplied to the animals), and the treatment and storage procedures also affect their quality. This makes it sometimes difficult to determine a suitable amount.

The most common problem encountered in shallow *Artemia* ponds is the presence of benthic algae (lab lab) and filamentous algae that are usually floating in the water column. As explained earlier, both types of algae are unsuitable food for *Artemia*. Their growth can be limited by maintaining the pond water turbid and keeping the water level high enough. In *Artemia* ponds, the bottom should be raked daily (Plate 27) to make the pond water turbid again by resuspending organic particles and to disturb lab lab formation. Additionally, lab lab may also be removed from the pond by raking. If filamentous algae or lab lab develop in the pond, these may spread quickly and will affect cyst harvesting. Frequent raking may take a lot of effort and cost. If, in spite of these efforts, the production (cyst/biomass) in the pond is reduced because of the occurrence of lab lab or filamentous algae, or if the *Artemia* population has difficulties in recovering after removing them, it is best to terminate the crop, empty and dry the pond, and start a new production cycle.

2.2.4.3. Artemia *pond culture using bioflocs*

Biofloc technology in aquaculture

Bioflocs are defined as a complex of organic matter and a variety of microorganisms, including microalgae, bacteria and small metazoans such as rotifers, protozoa and copepods, and are present in all aquatic ecosystems. Biofloc technology (BFT) is widely applied in the farming of several aquaculture species such as whiteleg shrimp and tilapia, as it is considered an environmentally friendly and a highly effective aquaculture technology to recycle nutrients, ideally with little or no water exchange (de Schryver *et al.*, 2008; Avnimelech, 2009; Hargreaves, 2013; Hoa *et al.*, 2017). BFT is based on the development of microorganisms (mainly heterotrophic bacteria) by adding a carbon source to increase the C:N ratio in the culture environment (typically above 10:1). These microorganisms, contained within the biofloc particles, have several advantages, as they maintain the water quality by assimilating nitrogen compounds, thus producing "onsite" bacterial protein. Consequently, they increase the efficiency of aquaculture operations by improving the feed conversion ratio and lowering feed cost. Bioflocs provide a variety of nutrients for the cultured animals and also have been shown to have immunostimulatory properties (de Schryver *et al.*, 2012a, 2012b).

Laboratory studies have shown that bacteria can be used as a suitable supplemental feed for *Artemia*, especially when microalgae supply is low (Toi *et al.*, 2013, 2014). However, whereas there are multiple studies on application of BFT for freshwater, brackish water and marine aquaculture animals, application of this technology at high salinity (80–100 g/L), such as in *Artemia* culture, has been less investigated, especially in pond conditions (Sui *et al.*, 2013; Ronald *et al.*, 2013; Luo *et al.*, 2017; Gao *et al.*, 2015).

Biofloc technology in Artemia *culture*

In biofloc technology, a cheap carbon source (e.g. rice bran or wheat flour) is added into the fertilization pond or the *Artemia* ponds at an amount to maintain a C:N ratio ≥10:1 (considering the amount of nitrogen supplied through the fertilizers). Elevated C:N ratios in the water column stimulate production of heterotrophic bacteria and thus biofloc production. In *Artemia* pond production, the use of inorganic and organic fertilizers, the supplementary feed as well as *Artemia* metabolites lead to accumulation of nutrients in the water. Just as in other aquaculture applications, biofloc technology is seen as a promising tool to reduce this nutrient load and as a source of additional feed for *Artemia*, based on the outcome of experimental work in the laboratory and to a certain extent also in ponds (Hoa *et al.*, 2017; Van Stappen *et al.*, 2019). Laboratory experiments have shown that also at higher salinities (in the range 60–100 g/L) *Artemia* can be grown on bioflocs for a few weeks at least, resulting in similar survival, growth and fecundity (cyst production) as when fed exclusively with live microalgae, although the culture success may vary depending on the biofloc production and *Artemia* culture conditions (Hoa and Nam, 2019).

Also in high salinity ponds, biofloc production experiments have been conducted by stimulating biofloc production over a period of 3 weeks at various salinities within the range of 35–100 g/L, using chicken manure (at 1.5 kg/pond of 150 m^2 or 100 kg/ha over 3 days) and wheat flour (0.3 kg/pond of 150 m^2 or 20 kg/ha over 3 days) as carbon sources/fertilizers and maintaining a C:N ratio >10:1. It is important to mention, though, that many countries have warned against using chicken manure because of the risk of contamination with antibiotics.

These experiments resulted in the following conclusions on the effect of salinity and of the culture period on microalgae and biofloc production (Hoa and Nam, 2019):

- Microalgae species diversity decreased as salinity increased, and species diversity decreased in general towards the end of the culture period.

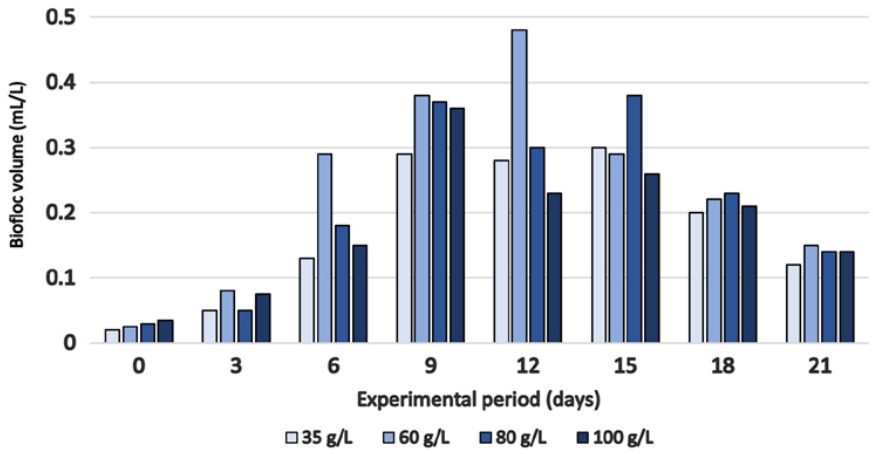
- The size of bioflocs may show high variations in any production condition and may also show differences over time. However, the biofloc particle size tends to be smaller – with a higher fraction within the range of uptake by *Artemia* adults, i.e. <50 µm – at higher (80–100 g/L) than at lower (35–60 g/L) salinity (Table 9).
- The amounts of bioflocs produced, expressed as total volume (Figure 25), total suspended solids or volatile suspended solids, may show changes over time: Typically, there is first a gradual increase in biofloc production, followed by a slight decrease. Ambient salinity, however, seems to have no clear effect on the total biofloc volume produced.
- The nutrient composition of the bioflocs may show slight differences over the production period, but protein content is generally low (e.g. 8–18 percent), whereas ash content is high (70–85 percent). As salinity increases, protein content tends to decrease and ash content tends to increase (Table 10).

TABLE 9
Size fluctuation (width-length) of biofloc particles (average ± standard deviation, µm) produced over a 3-week culture period in ponds at different salinities

	Salinity							
	35 g/L		60 g/L		80 g/L		100 g/L	
Day	Width	Length	Width	Length	Width	Length	Width	Length
7	62.6 ± 30.2	93.6 ± 30.4	59.8 ± 25.9	76.7 ± 27.4	51.6 ± 28.8	81.4 ± 46.6	39.7 ± 11.4	74.5 ± 30.5
14	71.0 ± 20.4	105.3 ± 30.9	57.5 ± 22.3	102.6 ± 36.4	61.5 ± 30.5	97.9 ± 51.4	37.1 ± 26.1	61.3 ± 36.8
21	52.3 ± 18.7	98.2 ± 55.7	55.3 ± 19.4	80.6 ± 41.6	32.5 ± 19.6	66.3 ± 28.3	33.8 ± 15.8	49.7 ± 22.2

Source: Hoa, V.N. & Nam, T.N.H. 2019. *Application of bio-floc technology for culture of Artemia*. N.V. Hoa, H.T. Hieu, N.A. Khoa & D.T.T. Uyen, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2981-6. 163 pp.

FIGURE 25
Fluctuations of biofloc volume produced over a 3-week culture period in ponds of different salinities



Source: Hoa, V.N. & Nam, T.N.H. 2019. *Application of bio-floc technology for culture of Artemia*. N.V. Hoa, H.T. Hieu, N.A. Khoa & D.T.T. Uyen, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2981-6. 163 pp.

TABLE 10
Proximate composition of bioflocs (percent dry weight) produced over a 3-week culture period in ponds of different salinities

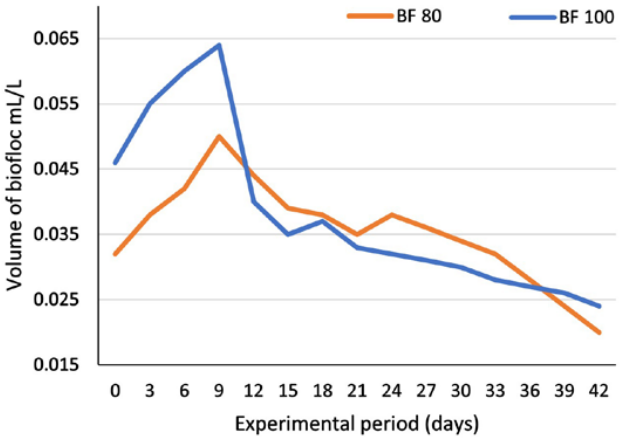
Salinity (g/L)	Day 7			Day 14			Day 21		
	Protein	Lipid	Ash	Protein	Lipid	Ash	Protein	Lipid	Ash
25	12.2	0.80	69.9	17.4	1.05	67.1	9.3	0.65	86.4
60	10.5	0.89	76.2	16.4	0.92	70.4	12.4	1.02	74.8
80	9.8	0.77	80.6	15.6	1.08	69.9	10.4	0.83	81.0
100	8.5	0.70	82.2	15.9	0.94	71.7	9.6	0.91	78.9

Source: Hoa, V.N. & Nam, T.N.H. 2019. *Application of bio-floc technology for culture of Artemia*. N.V. Hoa, H.T. Hieu, N.A. Khoa & D.T.T. Uyen, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2981-6. 163 pp.

Similar observations were found when biofloc production was stimulated using the same methodology in ponds at 80 g/L and 100 g/L where *Artemia* was cultured for 6 weeks, with ponds where only microalgae production was stimulated as control (Hoa and Nam, 2019).

- The biofloc volume initially gradually increased in all treatments, and then gradually decreased again until the end of the experiment (Figure 26).
- Female fecundity (number of cysts/brood) was higher in the biofloc treatments, resulting in a cyst yield 30–50 percent higher when using BFT as compared to the conventional green water culture system (Figure 27).

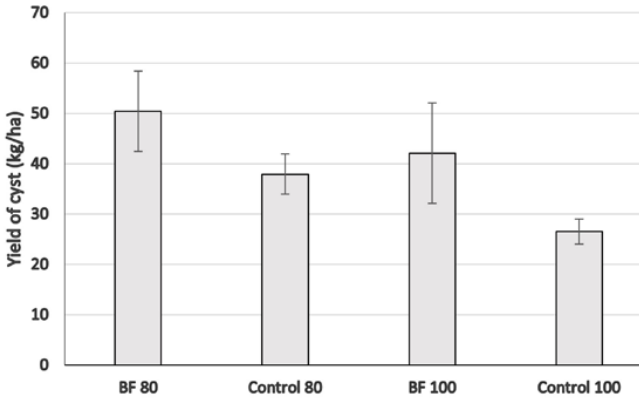
FIGURE 26
Fluctuations of biofloc volume (ml/L) produced over a 6-week culture period in *Artemia* ponds of different salinities (80 g/L and 100 g/L)



Source: Hoa, V.N. & Nam, T.N.H. 2019. *Application of bio-floc technology for culture of Artemia*. N.V. Hoa, H.T. Hieu, N.A. Khoa & D.T.T. Uyen, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2981-6. 163 pp.

Additionally, the experimental work in ponds showed that to obtain a maximal feeding effect of both microalgae and bioflocs, it is necessary to frequently measure and continuously manipulate both the N:P ratio (for algae production) and C:N ratio (for bioflocs) in the ponds to which the fertilization products are supplied in order to favour the simultaneous and balanced development of microalgae and bioflocs. Maintaining the density of algae and biofloc volume within a suitable range not only increases *Artemia* production but also maintains stable environmental conditions. As in green water production, raking of the pond bottom is important for the formation and distribution of biofloc particles in suspension: Regular raking prevents biofloc particles from settling down on the pond bottom, making them more available for filtration by *Artemia*.

FIGURE 27
Yield of cysts (kg/ha) produced over a 6-week culture period in *Artemia* ponds of different salinities (80 g/L and 100 g/L) using bioflocs or green water (control)



Source: Hoa, V.N. & Nam, T.N.H. 2019. *Application of bio-floc technology for culture of Artemia*. N.V. Hoa, H.T. Hieu, N.A. Khoa & D.T.T. Uyen, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2981-6. 163 pp.

2.2.4.4. Use of formulated Artemia feed

Researchers at Can Tho University in Viet Nam, recently have developed a commercial feed for *Artemia* using different feed ingredients (Table 11). This formulated feed can

TABLE 11
Proximate composition (percent dry weight) of ingredients used for commercial Can Tho University *Artemia* feed

Compound	Fishmeal	Soybean flour	Rice bran	Wheat flour
Crude protein	60.04	47.18	15.11	1.96
Crude lipid	7.82	1.24	14.63	0.20
Ash	27.68	7.12	9.17	0.34
Fibre	0.47	2.35	7.24	0.14
Carbohydrate	3.99	42.10	53.84	97.36
Water content (%)	9.89	10.03	11.59	11.76

Source: Authors' own elaboration (CTU, Viet Nam).

be used both for small-scale production purposes (e.g. laboratory culture) and for large-scale tank or pond production (Le *et al.*, 2019; Van Stappen *et al.*, 2019).

Using these and supplementary ingredients (squid oil, lecithin, vitamin premix), different experimental feeds with protein levels in the range of 20–45 percent and lipid levels in the range of 5–13 percent have been formulated and tested in laboratory conditions. Within these ranges, feeds showing a combination of intermediate protein levels (30–35 percent) and 9 percent lipid level resulted in the best survival, growth and female fecundity (Table 12 and Table 13 show, respectively, ingredient and proximate composition of these experimental diets).

The formulated feed is produced by first mechanically grinding all ingredients to a particle size below 50 µm. Then these ground materials are weighed and mixed in their correct proportions, after which the carboxymethyl cellulose is added as a binder. Water is added at 30–50 percent of the mixture, after which the paste is pressed through a pelletizer and left to dry. These dry pellets are finely ground with a grinder to particles below 50 µm. The end product is packed and stored in the refrigerator.

TABLE 12
Ingredient composition (percent dry weight) of various experimental types of commercial Can Tho University *Artemia* feed

Ingredient	30% protein feed	35% protein feed	9% lipid feed
Ca Mau fishmeal	29.00	35.43	29.00
Soybean flour	18.45	22.54	18.45
Rice bran	22.45	18.06	22.74
Wheat flour	24.93	18.71	22.68
Oil*	1.17	1.27	3.13
Vitamin premix**	2.00	2.00	2.00
CMC***	2.00	2.00	2.00
Total	100	100	100

* Squid oil and lecithin: ratio of 1:1.

** Vitamin premix – internations units (IU) or g per kg: vitamin A (400 000 IU); vitamin D3 (80 000 IU); vitamin E (1 g); vitamin K3 (2.4 g); vitamin B1 (1.6 g); vitamin B2 (3 g); vitamin B6 (1 g); niacin (1 g); vitamin B9 (0.8 g); vitamin B12 (0.004 g); folic acid (0.032 g); biotin (0.17 g); vitamin C (60 g); choline (4.8 g); inositol (1.5 g); ethoxyquin (20.8 g); copper (10 g); ferrous sulphate (20 g); magnesium (16.6 g); manganese (2 g); zinc (11 g).

*** *Carboxymethyl cellulose or cellulose gum.*

Source: Authors' own elaboration (CTU, Viet Nam).

TABLE 13
Proximate composition (percent dry weight) of various experimental types of formulated *Artemia* feed

Compound	Control*	30% protein feed	35% protein feed	9% lipid feed
Crude protein	42.12	30.58	35.52	31.14
Crude lipid	6.88	7.06	7.22	8.96
Ash	13.57	12.80	13.34	12.80
Fibre	2.24	2.10	2.11	2.12
Carbohydrate	35.19	47.46	41.81	44.99
Energy (kcal/g)**	4.50	4.39	4.45	4.31
Water content (%)	9.58	10.83	10.68	9.04

* Commercial *Penaeus monodon* feed.

** Crude energy is calculated based on the following values (kcal/g): protein = 5.65; lipid = 9.45; carbohydrate = 4.20.

Source: Authors' own elaboration (CTU, Viet Nam).

2.2.5. Monitoring and managing the culture system

2.2.5.1. Artemia *inoculation*

Artemia strain selection

As mentioned in Section 1.4, *Artemia* strains differ widely in ecological tolerance range and characteristics for use in aquaculture. Therefore, the selection of the

strain best adapted to the particular ecological conditions of the site and/or most suitable for its later application in aquaculture is very important. The introduction of a non-local *Artemia* strain should be considered very carefully, especially in those environments where it will result in the establishment of a permanent population, as in the saltworks in northeastern Brazil (see Box 5); and the inoculation of allochthonous strains in conditions where they may outcompete local strains and thus threaten local biodiversity should be strongly discommended. Strain selection can be based on the literature data for growth, reproductive characteristics and especially temperature/salinity tolerance (Vanhaecke, Siddall and Sorgeloos, 1984). Summarizing, a strain exhibiting maximal growth and having a high reproductive output at the prevailing temperature/salinity regime in the ponds should be selected. Usually, strains producing small cysts and nauplii are to be preferred unless production of biomass is the main objective (Baert *et al.*, 2002). In the latter case, selecting a fast-growing strain having a dominant ovoviviparous reproduction is recommended.

Developmental stage used for inoculation and hatching procedures

Stocking or inoculating *Artemia* in the instar I nauplius stage is essential, as stocking older stages (metanauplii, juveniles, adults) may result in lower survival within the first 24 h owing to salinity shock, resulting from the big difference in salinity between the hatching incubation environment (generally 20–35 g/L) and pond salinity (at least 80 g/L). Because of the presence of a salt gland, instar I nauplii generally survive the direct transfer from low to high salinity, which is not the case for any later stages, in which mouth and anus are open. Temperature shock may also occur to later nauplii stages. Therefore, synchronously hatching cysts should be used, which will result in a homogeneous instar I population after a standard hatching incubation time. If cysts of this quality are not available, the *Artemia* to be stocked should gradually be acclimated to pond salinity and temperature prior to stocking.

For hatching, standard procedures should be followed as much as possible, as described in Section 3.1.5. As hatching conditions under field situations are often suboptimal, at least the following directions should be observed:

- Hatching containers should be placed in shaded areas to prevent excessive heating by direct sunlight.
- Hatching water should be filtered, preferably using a 1 µm filter bag.
- If water remains turbid after filtration, the salinity of the hatching medium should be lowered to 20 g/L and cysts should be hatched at a density of maximally 1 g/L.
- Sufficient aeration and illumination should be provided to the hatching container(s), especially when cysts are incubated in the late afternoon or evening.

The quantity of cysts needed to obtain the number of nauplii required for inoculation (see further), and taking into account a 30 percent mortality at the time of stocking, is calculated from the pond volume and the hatching efficiency of the selected batch. Moreover, as hatching conditions may be suboptimal, hatching might be lower than expected (often only 75 percent of the hatching efficiency of the batch used).

Inoculation density

Pond stocking density is determined by nutritional conditions (water colour and turbidity caused by algae development) and by the temperature in the culture ponds. For example, a stocking density of 100 individuals/L is recommended at a pond turbidity of 15–25 cm. When stocking at higher density, the oxygen content may become limited, especially when water temperature is high and after a calm night. At higher transparency (>25 cm) – corresponding with lower food availability – the stocking density should be reduced to 50–70 individuals/L.

Based on empirical observations, high density culture is thought to favour cyst production in females and is thus preferred. However, high density culture should not

result in food limitation, as this leads to overall low production due to slower growth and maturation and lower fecundity. Especially the combination of high density with food limitation, high temperature and oxygen stress are thus to be avoided. Stocking at lower density, on the other hand, seems to promote nauplii production in females (i.e. ovoviviparity), but this may lead to a fast population density increase and fast maturation in the presence of sufficient food. Therefore, low initial stocking density can help to save on the amount of cysts needed as inoculum, but more time will be required (i.e. the time needed for the subsequent generations to reach adulthood) to increase the population density before high productivity can be achieved.

2.2.5.2. Overview of management procedures

From the time of inoculation onwards, active and daily *Artemia* pond management is needed throughout the production phase (the "crop"). At the start of the crop and immediately after stocking, *Artemia* ponds will usually show the phenomenon of "water bloom" during the first 7–10 days, caused by available nutrients from the water source or by nutrients released by mineralization from the pond bottom. In this initial period, *Artemia* has not yet reached the adult stage; the filtering apparatus is not very effective yet, and food demand is relatively low. Consequently, the *Artemia* population is not yet able to control algae development in the pond. Therefore, it is recommended not to fertilize in this period, or very sparsely and carefully, or to conduct fertilization only in the fertilization pond, as its green water is ready for use only a few days later.

After pond inoculation, routine activities include water level management; salinity and temperature control; control of water colour and food availability; and monitoring age composition, behaviour, appearance and nutritional status of the population (i.e. observing if the guts are completely full and dark versus empty or discontinuously filled and/or pale; Plate 30). Also, the reproductive status of the females should be regularly checked, e.g. the number of egg phases present in the reproductive process. Eggs should be concurrently present in the ovaries, the oviduct and the brood sac (= three phases), and if not, this may be the result of the feeding conditions or aging of the population. Additionally, the overall number of cyst-bearing versus nauplii-bearing females should be monitored.

In parallel, the green water pond should be monitored and maintained to ensure the availability of sufficient amounts of green water with the appropriate cocktail of algae for *Artemia* feeding. Salinity in the green water pond is also critical, not only to ensure maximal algae development, but also because too low salinity of the green water inflow could cause the salinity in *Artemia* ponds to decrease. Because pond culture is an open culture, abnormal or unforeseen weather conditions may have a negative effect on *Artemia* survival and development. For example, at the start of the *Artemia* season, abnormal rain, low temperature and low sunshine intensity may have a negative impact, as is also the case for high temperature, early rainfall, high salinity or proliferation of Lab–Lab toward the end of the season. Anticipating the negative interference of these conditions will help to manage the pond and to ensure *Artemia* survival and development at sufficiently low production costs and sufficiently high profit margin.

PLATE 30
Daily observation of the *Artemia* population

In the Vinh Chau conditions, and when working with Vinh Chau *Artemia franciscana*, normally the first riding couples can be observed around 7–10 days after inoculation; reproduction may start from day 10–15 onward. Generally, the first few broods are ovoviparously produced (i.e. nauplii production), but after a while part of the population switches into cyst production (i.e. oviparous mode). When cyst production is the aim, cyst harvesting may start when *Artemia* are 15–20 days old and when cyst material appears in the downwind corner. In biomass production, the biomass harvesting could start from day 12–15 onward. From the third week onward (i.e. about 20 days after stocking), reproductive activity stabilizes at a high level if the population is well managed: The keys for successful *Artemia* productivity are a stable nutritional condition and water temperature. Other factors such as salinity and oxygen, or occurrence of Lab–Lab, can also affect productivity but their impact is less decisive: Even when these factors are suboptimal, cyst/biomass yields will be stable or even increase if the nutrient levels and temperature are well managed.

2.2.5.3. Management of abiotic parameters

Control of salinity and water depth

As described above, saltworks operate with low water levels (approximately 15–20 cm) to facilitate the evaporation process. To convert a saltwork into an *Artemia* culture system, therefore, adaptations need to be done (see Section 2.2.3); through these adaptations, it is possible to convert a salt field into an *Artemia* farming system with a strong, compacted and heightened dyke system and maximal ability to hold the water.

Artemia is found to occur in a wide salinity range in nature; in pond culture, however, salinity is managed throughout the crop at a level that the *Artemia* population can grow and multiply (generally 80–120 g/L) but that is prohibitive for its predators and competitors. Too high salinity (>250 g/L) can be deadly to *Artemia* as well. When the salinity is too high, it is necessary to dilute the water with brackish water or even freshwater, sometimes after draining the saline water before adding low salinity water. Dilution should not be done too abruptly in order to avoid a salinity shock to the *Artemia* population (i.e. 20–30 g/L a day maximally). The amount of water to be supplied, as a function of the salinity of the culture pond and the salinity of the supply water, can be calculated by the following formula:

$$S = \frac{(V_1 \times S_1) + (V_2 \times S_2)}{(V_1 + V_2)}$$

With:
S = salinity in the pond after dilution (g/L)
V_1; S_1 = volume (m^3); salinity (g/L) in the pond before dilution
V_2; S_2 = volume (m^3); salinity (g/L) of the water supplied for dilution

Salinity is best measured with a refractometer that can be corrected for different temperatures. As algal concentration and other suspended materials influence the refractive index, it is recommended to filter the sample before measurement.

As mentioned in Section 2.2.3.7, due to the low salinity of seawater along the coast of the Soc Trang and Bac Lieu areas towards the end of the rainy season, i.e. in November and December, intake salinity at the start of the *Artemia* production season is generally only 12–15 g/L. Therefore, the preparation process to reach saline water of about 80 g/L lasts about a month (but sometimes more). This phase can become longer when cold winds and/or abnormal rains occur. To shorten the time for saline water preparation, farmers can add crude salt or brine stored from the previous year to the evaporation and/or *Artemia* ponds. The evaporation process may also be accelerated by frequent creation of water turbulence (e.g. by raking).

Given these considerations and to maximize the duration of the production period, farmers may begin to inoculate when the pond water level is at around 10 cm from the platform (the bottom), which is at least 40 cm in the peripheral ditches, and salinity ranges approximately 60–70 g/L. Then, the inoculated ponds are supplied with saline water to gradually increase the water level to 20–30 cm (i.e. at least 50 cm in the ditches), which is maintained until the end of the crop. Because of evaporation, salinity will meanwhile increase up to 80–100 g/L. However, stocking when the water level is still shallow will cause a significant difference of water temperature between day and night. Moreover, when inoculating shallow waters, nauplii can be blown by wind and caught in swarms in the pond corner, causing significant loss to the initial stocking population. Shallow water also makes it easier for waterfowl to predate on *Artemia*, especially because brine shrimp have the habit of swimming slowly, floating on the water surface, particularly after a calm night, making them an easy prey for water birds. A threat linked to stocking at salinity less than 80 g/L is the presence of copepods, which swiftly invade the ponds and outcompete *Artemia*, other zooplankton organisms (e.g. rotifers, the ciliate protozoan *Fabrea salina*) and fish (mostly tilapia) that compete for food (see Section 2.2.3.6).

After inoculation, pond salinity will continue to increase due to evaporation above 80 g/L; the higher temperatures towards the end of the dry season also play a crucial role in enhancing evaporation, at a rate of 2–5 g/L per day. In specific conditions (e.g. limited availability of lower salinity water as a result of the tidal cycle, intensive sunshine, windless conditions), salinity can thus increase up to 150–180 g/L with a water temperature exceeding 40 °C. This becomes a lethal situation for the *Artemia* population, and also has a negative effect on the density and species composition of the microalgae in the green water pond. The supply of green water into the *Artemia* ponds is often insufficient in these conditions. Moreover, in shallow ponds sunlight easily penetrates to the bottom and stimulates the growth of filamentous algae (Lab-Lab) that compete with microalgae for nutrients and that develop into algal masses rapidly covering the pond bottom when sufficient nutrients and sunlight are available. After reaching a plateau growth phase, these algae will die off and are broken up into smaller pieces that float on the water surface trapped with air bubbles. These can be blown by wind action and accumulate in the pond corners, where they may settle again on the bottom, decompose under anaerobic conditions and release toxic gases (e.g. H_2S, methane (CH_4), nitrogen dioxide (NO_2)) into the water column. These toxic conditions may cause mass mortality in the *Artemia* population. Furthermore, Lab–Lab appearing in the water column acts as a trap to the *Artemia* individuals and the cysts, which get attached to the algae threads and mucus, thus complicating cyst harvesting and leading to reduced yields.

In summary, salinity management, coupled with water depth, is very important in the *Artemia* culture process, as too low salinity (in an early phase) or too high salinity (towards the end of the crop) both have a negative impact on *Artemia* survival, growth and reproduction, and thus on harvesting. Proper management of salinity in the pond will contribute significantly to prolonging the crop, thereby increasing productivity.

Temperature control

Temperature affects the *Artemia* culture directly through population development and reproduction or indirectly through evaporation rate, salinity, water quality and algae growth (Vanhaecke *et al*., 1984; Vanhaecke and Sorgeloos, 1989). Therefore, pond temperature needs to be maintained at a suitable level depending on the *Artemia* strain used. Fluctuation of water temperature is obviously influenced by salinity, depth, transparency, wind speed and seasonal conditions in general. Over the 24 h cycle, water temperatures in the Soc Trang and Bac Lieu areas are in the range of 20–40 °C, while for example the optimal temperature for growth of the Vinh Chau type *Artemia* is

within the range of 28–32 °C. In March and April, in ponds with shallow water levels (less than 20 cm from the pond bottom), the highest water temperature is found in the afternoon and varies usually in the range of 37–38 °C. Water temperatures exceeding 35 °C are not suitable for optimal *Artemia* culture and can cause mass mortality as temperature further increases. Moreover, a thermocline may develop in a deep pond, especially after a calm night when the temperature in the bottom layers of the water column is higher with lower oxygen concentration. In case of a dense *Artemia* population, this may also lead to mass mortality. Excessive temperatures can be avoided by: (1) avoiding pond salinity to exceed 120 g/L; (2) keeping sufficient water depth by deepening the peripheral ditches and/or raising the water level; and (3) maintaining stable turbidity (i.e. higher transparency favours temperature increase). Regular raking of the pond bottom to mix the water column properly will help to lessen the impact of a thermocline. Moreover, farmers can also use local cheap materials such as palm leaves to partially cover the pond surface, providing shelter for *Artemia* during the hottest hours of the day. As temperature gradually increases towards the end of the dry season, attention should be paid to the water temperature when stocking *Artemia* in the pond, e.g. to start an additional crop late in the season: Whereas early in the season the temperature is still relatively low and stocking can thus be performed at a water level of about 10–15 cm (from the pond bottom), a gradual adjustment needs to be done when stocking towards the end of season (e.g. March–April). In this case, a depth of at least 20 cm is needed, followed by a continuous further increase of the water level as the dry season proceeds.

Control of dissolved oxygen

Dissolved oxygen levels in *Artemia* culture ponds should be higher than 2.5 mg/L; levels lower than 2 mg/L have a negative effect on *Artemia* production. The oxygen content is usually higher in the superficial water layer, especially when ponds are stratified. Oxygen content is often related to wind action and algae density and also exhibits daily cycles. Concentrations are the lowest at dawn (algal respiration), especially after a calm night, and the highest in the afternoon (algal photosynthesis). If problems with oxygen are anticipated, measurements should be made at dawn, and when encountering lower oxygen levels, it is necessary to supply new water and to mix the water column. Additional pumping, lowering the algae concentration, interrupting fertilization (especially with manure) or circulating the water in the pond will also increase oxygen levels.

Oxygen is measured with a portable oxygen meter. As oxygen levels change very quickly once the sample is taken, this parameter should be measured immediately after collection of the sample or ideally directly in the pond. While measuring, the probe should be moved constantly. The colour and behaviour of the *Artemia* are good indicators: When the animals are experiencing oxygen stress, they turn red, swim slowly, start surface swimming and growth is retarded. Extreme oxygen depletion may cause mass mortality.

Control of pH

In their natural habitat, *Artemia* are mostly found in a pH range between 7.8 and 8.2, which is often given as the optimal range, although pH tolerance is also strain–specific and some strains are adapted to alkaline conditions (see Section 1.2). For the rest, the effects of pH on growth and reproduction have not been studied in great detail.

Algal blooms can affect the pH (consumption of CO_2 increases pH). Therefore, in general, the highest pH is reached in the afternoon, while the lowest pH occurs near dawn. As seawater is usually well buffered, problems with pH are rare, except in areas with acid sulphate soils.

2.2.5.4. Population management and seasonal implications

The status of the *Artemia* population can only be assessed by using an appropriate sampling protocol.

Twice a week a number of samples (e.g. 10 samples/ha) must be collected in the different culture ponds. Samples should be collected at fixed sampling stations located in as many different "strata" as possible. A habitat can be divided in different strata, each stratum having slightly different environmental characteristics and consequently different *Artemia* densities: For example, in a pond with a peripheral ditch, the pond platform, the ditch and the corners can be considered as three different strata, as temperature and algae abundance may differ at these three places. Moreover, younger developmental stages tend to accumulate in the downwind section of the pond, whereas older stages may be found more in calm, upwind places. By sampling at different locations, the risk of not finding *Artemia*, although present in the pond, is reduced. Generally, it is recommended to sample in the early morning, when the population is distributed more homogenously. The following two sampling methods can be recommended:

- Per sample site, 5–10 L water is filtered over a sieve (100 µm).
- A conical net is dragged over a certain distance through the water. Drags can be horizontal or vertical. However, mesh size and diameter of the sampling net depend on the volume of water to be sampled, which in turn depends on the population density in the pond. If population density is high, nets with a diameter of 30–50 cm and mesh size of 100 µm can be used. In large ponds where population density is low, larger nets (diameter up to 1 m) are dragged over a longer distance. To prevent clogging, only the distal part of the net has a small mesh size (100 µm). The remainder of the net can have a mesh size of 300–500 µm.

Samples are fixed with formalin (4 percent) and carefully examined, dividing animals in groups. After inoculation of the instar I stage, *Artemia* undergoes 15 moultings to become an adult. Typically, the following stages are distinguished: nauplii (absence of thoracopods), juveniles (developing thoracopods clearly visible), subadults, adults (sexual differentiation apparent) and cysts.

The relative presence of each stage is given a score as follows:

\- = absent
\+ = present
++ = present at high density

Depending on the frequency of occurrence of different developmental stages, the population can be classified into different successive stages (Table 14).

The scores for each life stage of all samples taken in one pond are summed and plotted in time (Figure 28). Although such estimates are not accurate (i.e. they do not give the exact number of animals per litre), they are precise (i.e. they reflect correctly the variations in abundance). Such graphs show how a population evolves and allows for adaptation of the management procedures. Additionally, evaluation of the population also includes the reproductive characteristics of the females, i.e. for example, whether the females are carrying cysts or nauplii, female fecundity (number of embryos per female), presence of any females with empty ovary, and sex ratio. In practice, the relative proportion of different age classes may fluctuate over time according to the culture conditions and the population's status, but when different age classes show a stable proportion, the *Artemia* culture can be considered in a stable phase, which is generally linked with the highest productivity of cysts and/or biomass (Hoa, 2002). Persistence of a healthy stable state of the population depends on a number of factors, such as predator control or prevention by maintaining a certain salinity, and avoidance of lethal temperatures. The food factor, especially, plays a major role in increasing the

TABLE 14
Evolution of *Artemia* population composition in culture ponds

Population status	Population composition					Remarks
	Nauplii	Juveniles	Subadults	Adults	Cysts	
A	++	-	-	-	-	Population at stocking.
B	-	-	+	++	-	Population developing to subadult and adult stage.
C	++	-	-	+	-	Population with nauplii producing females.
D	+	+	+	+	-	Continuation of C; pond is in good condition.
E	+	-	-	++	-	Period after food shortage; population becomes distressed and will quickly change to F (in case of food shortage, the population has only adults and younger stages die off quickly).
F	-	-	-	++	-	Population does not have enough food for adults.
G	-	-	-	++	+	Most females switch to cyst production and population ceases to increase in density.
H	+	+	+	+	+	When the pond is optimally fertilized, the population shows all age classes, and the females also produce cysts.

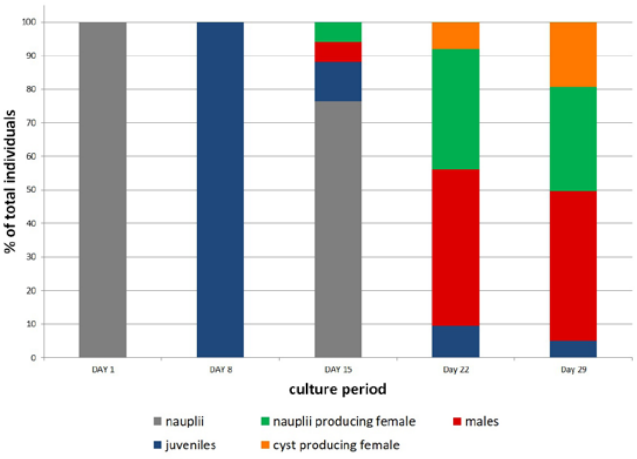
Notes: - = absent; + = present; ++ = present at high density.

Source: Authors' own elaboration (LAARC).

population and keeping it in a stable state. On the other hand, when food is lacking, the population may gradually decrease but does not disappear completely.

Evidence from the literature, largely based on laboratory culture of *Artemia*, shows that females tend to release nauplii for the first few broods, followed by cyst production for the rest of their reproductive life (Lavens and Sorgeloos, 1984, 1987a). However, this is much less straightforward in field conditions. In pond culture, females tend to release cysts immediately at high population density. Towards the end of the dry season when temperature and salinity are high, females tend to release more nauplii. Trials have been done to control the female reproduction mode by managing salinity and food conditions, showing 80–120 g/L generally to favour cyst production and lower salinity combined with the presence of sufficient food to stimulate biomass production.

FIGURE 28
Example of evolution of age class composition of an *Artemia* population after inoculation in the pond



Source: Hoa, V.N. 2002. *Seasonal farming of the brine shrimp Artemia franciscana in artisanal salt ponds in Viet Nam: effects of temperature and salinity*. Ghent University, Ghent, Belgium. PhD dissertation. 184 pp.

In summary, *Artemia* culture management should focus on maximal and continuous production, and this depends on the age of the population, available food and environmental factors, including weather conditions. Whereas the status of the *Artemia* population constantly fluctuates as a function of these factors, pond management should focus on a stable density of *Artemia* adults, with female ovaries always being filled with offspring. Because of the weather variability in recent years, the duration of the production season in the Soc Trang and Bac Lieu areas has declined, despite efforts of the farmers to speed up the population to enter into cyst production and to maintain it in this status. The peak period for cyst production in this region is nowadays narrowed to January, February and March (i.e. maximum 60–70 days in the whole season). In the remaining period of the dry season (April up to June), with unfavourably high temperatures, farmers now generally only harvest biomass. If there is no market for this biomass, it is recommended to terminate the crop to avoid

waste of costs if further maintaining the *Artemia* population, and to prepare the pond for culture of other aquatic species such as fish and crustaceans as the rainy season approaches.

2.2.5.5. Management problems and troubleshooting

Algal blooms

The soil of ponds in the Soc Trang and Bac Lieu saltworks is generally rich in biodegradable nitrogen and organic matter, high in calcium and manganese, but low in iron. Studies have shown that high levels of biodegradable nitrogen are correlated with nitrogen mineralization levels (Khoi, Guong and Merckx, 2006b; Hoa and Hong Van, 2019). Therefore, the determination of biodegradable nitrogen is an indicator to assess the degree of mineralization of nitrogen in the soil in the initial period when saline water is supplied into the ponds. The higher the biodegradable nitrogen, the more likely it is for algal blooms to occur, but excessive growth of microalgae in the ponds at an early stage will inhibit *Artemia* growth. In general, the level of biodegradable nitrogen in the soil is a criterion to adjust the doses of both inorganic and organic fertilizers throughout the culture process and to avoid excessive and/or untimely algal blooms.

PLATE 31
Lab–Lab appearing on the pond bottom and in the water column

© CTU

Occurrence of Lab-Lab (benthic algae)

Lab-Lab occurs in shallow ponds with nutrient–rich bottom layers or when excessive fertilization has taken place (Plate 31) (see Section 2.2.4.2). When the density of microalgae is high enough or when frequent raking maintains sufficient water turbidity, sunlight cannot penetrate to the bottom layer, which prevents proliferation of filamentous algae on the bottom. When water transparency increases because of the uptake of microalgae by *Artemia* and insufficient water mixing, Lab–Lab can develop and cover the pond bottom within 10–15 days in the presence of sufficient nutrients (nitrogen and phosphorus). Manual removal of the Lab–Lab (e.g. by raking) is labour-intensive and time-consuming. Meanwhile, the *Artemia* population is subjected to suboptimal conditions and may need a week or more to recover, consequently lowering productivity. Moreover, Lab-Lab may die after a while (e.g. when salinity increases or nutrients become limiting), and its decomposition process may further create suboptimal conditions, causing *Artemia* mortality and eventually complete disappearance of the *Artemia* population. Therefore, in addition to preventing Lab–Lab proliferation by maintaining water turbidity, it is important to remove Lab–Lab when its density is still low. In case of excessive growth, the pond should be drained and the bottom left to dry until the soil is cracking. Then a new production cycle can be started.

Clay turbidity phenomenon

Sometimes it is necessary to prevent possible turbidity caused by suspended clay particles so that light will penetrate deep enough into the pond for phytoplankton growth. Sodium levels exceeding the levels of potassium, calcium and magnesium in soil and water, combined with high salinity, will make clay particles disperse and float in the water column. On the other hand, the amount of organic substances in the soil

profile is also a factor affecting turbidity because organic substances can bind to clay particles, forming larger aggregates that will therefore settle quickly (Hoa and Hong Van, 2019). Prevention of clay turbidity in *Artemia* culture ponds can thus be done by increasing the amount of organic matter on the pond bottom through application of organic fertilizers. Also supplying green water from the fertilization pond, rich in organic matter with high algae density, into the *Artemia* culture ponds will increase the amount of dissolved organic matter.

Common diseases in Artemia *pond culture*

- ***Leucothrix* sp.:** is a flagellate bacterium with colonies having the shape of a cotton seed pod when showing excessive growth. These colonies fix on the *Artemia* exoskeleton, by preference on the thoracopods, and only become visible from instar V–VI stage onwards. The brine shrimp suffer physically, as the movements of their thoracopods become affected and filtration rates consequently are reduced. In case of excessive contamination, growth and moulting are arrested, and the population may collapse. This phenomenon usually occurs when the culture environment is heavily loaded with organic substances. In this case, regular renewal of the pond water and partial removal of organic matter are recommended.
- **Black spot disease**: This disorder occurs when the population is malnourished by lack of food or poor food quality (although the actual causative factors are not entirely known), and black spots (necrosis) develop on the extremities (i.e. thoracopods, antennae) (Plate 32) by detachment of the epidermis from the cuticula. Supplying high-quality feed does not save the affected animals but appears to avoid further losses.
- **Tailing pellet phenomenon**: In this condition, *Artemia* carries a long faecal thread. This phenomenon occurs when *Artemia* is fed too much food or food with low digestibility; it usually occurs when the green water supplied to the *Artemia* culture pond contains too many microalgae with a thick cell wall or filamentous algae. The condition can be remediated by providing another source of green water with more suitable algae species or by providing another direct food source.
- **White tail disease**: The *Artemia* abdomen colour is turned pale when observed by the naked eye. When looking under the microscope, it is clear that no food is retained in the last section of the intestine (Plate 33). This disorder may be related to dietary problems, but the

PLATE 32
Black spot disease on *Artemia* thoracopods

© LAARC

PLATE 33
***Artemia* juvenile showing white tail disease**

© LAARC

exact causes are not known. When the symptoms are observed in the population, mass mortality can occur within a few days. When encountering this phenomenon in the pond, it is recommended to supply green water from different sources, while sometimes also partial renewal of pond water may limit or completely overcome white tail disease.

2.3. TANK PRODUCTION AND USE OF ONGROWN *ARTEMIA* (by *Yeong Yik Sung, Thirukanthan Chandra Segaran, Khor Waiho, Tan Min Pau, Asmidar Mohamad, Krishnnappriyaa Gopi & Patrick Sorgeloos*)

2.3.1 Tank production of *Artemia* biomass

2.3.1.1. Overview of Artemia *biomass production*

Different stages of *Artemia* are utilized in aquaculture. *Artemia* biomass, particularly juveniles and adults, is primarily used as a food source for ornamental and food fish in both freshwater and marine environments, as well as feed for larvae of crustaceans such as shrimp, lobster and freshwater prawns. The application of ongrown *Artemia* has been shown to enhance the energy balance, which might lead to higher growth, to a quicker developmental rate, and/or a better physiological condition in aquaculture organisms (Choi *et al.*, 2021). In the instance of penaeid shrimp postlarvae culture, it has also been shown that adding *Artemia* biomass to the rearing tank can reduce cannibalism, boosting survival by 30-40 percent (Anh *et al.*, 2011).

Because of the scarcity of live or frozen biomass, its high cost and inconsistency in quality, ongrown *Artemia* applications were never developed in the past. However, with increased demand for *Artemia* biomass associated with the expansion of several aquaculture sectors, lower cyst prices, technological advancements and improvements in culture techniques, intensive pond and super-intensive tank brine shrimp production systems in or near aquaculture farms have sparked interest in *Artemia* biomass production.

The aquarium pet store business provides excellent marketing potential for live *Artemia* biomass generated in local culture systems. Several *Artemia* biomass production facilities have recently been established in Malaysia to assist the growth of the ornamental fish business, all of which use a stagnant batch culture system (see further). Today, most of the *Artemia* biomass produced in this sector is sold frozen since it is obtained from a limited number of resources and live transportation to other continents is too expensive.

2.3.1.2. Advantages of tank production and tank-produced biomass

Although tank-produced *Artemia* biomass is significantly more costly than pond-produced biomass, its application benefits are numerous. Regardless of environment or seasonal factors, ongrown *Artemia* may be cultivated and collected all year round. Hatching may be synchronized using good quality cysts, and *Artemia* of uniform size can be produced with adequate feeding and culture procedures. Specific stages (juveniles, subadults and adults) or uniformly sized prey can thus be collected according to the predator's size preferences. Tank production ensures a more consistent harvest in terms of quantity and quality, as well as improved nutritional profiles. Tank-produced *Artemia* can be disease-free (i.e. free from *Vibrio*) if the rearing method is well managed.

Super-intensive culture techniques offer two main advantages compared to pond production techniques. First, there are no constraints related to production site or time: The culture technique does not necessitate highly saline water or specific climatological conditions. However, it is recommended that the production site be in a place with a suitable seawater source because *Artemia*, as other aquaculture species, requires good quality and clean water to be reared to adult. Second, regular production may

be accomplished with extremely high brine shrimp densities (up to several thousand animals per litre), compared to a maximum of a few hundred individuals per litre in outdoor culture ponds. As a result, tank-based rearing systems may achieve very high output yields per volume of culture medium.

Production costs are estimated to be USD 1.5/kg to USD 12/kg live weight of *Artemia*, with wholesale prices for *Artemia* cysts ranging from USD 25/kg to USD 80/kg, depending on culture method and site facilities (Nguyen *et al.*, 2019). In practice, when setting up an *Artemia* culture, one should start by making an inventory of prevailing culture conditions and available infrastructure. The abiotic and biotic factors relevant for *Artemia* culture are listed in Table 15 and are further described in the following sections (Lavens and Sorgeloos, 1991).

TABLE 15
Overview of abiotic and biotic factors in *Artemia* biomass production

Factors		
Biotic	*Artemia*	*Artemia* strain selection
		Artemia culture density
Abiotic	Physico-chemical culture conditions	Salinity and ionic composition of the culture medium
		Temperature
		pH
		Oxygen concentration
		Water quality
	Infrastructure	Tank and aeration design
		Heating and lighting
	Culture techniques	Open flow-through system
		Recirculation type
		Stagnant culture
	Feeding strategies and diets	Diet selection
		Feeding strategies

Sources:
Modified from Lavens and Sorgeloos, 1991.

Lavens, P. & Sorgeloos, P. 1991. Chapter XIII: Production of *Artemia* in culture tanks. In: R.A. Browne, P. Sorgeloos & C.N.A. Trotman, eds. *Artemia Biology*, pp. 317-350. Boca Raton, Florida, USA, CRC Press, Inc.

2.3.1.3. Biotic factors

Artemia *strain selection*

Performance of different *Artemia* strains in tank culture has been scarcely studied, thus limiting our knowledge on the strain(s) to be selected. Previous studies were limited to the *Artemia franciscana* strain from the San Francisco Bay brand, which has been reported to be a better quality than the brands from China, the Philippines, Singapore and Thailand, than *A. salina* and several other non-specified *Artemia* strains/species (Islam, Kibria and Bhuyan, 2019).

Artemia *culture density*

High densities of *Artemia* can be cultured without impacting survival. Inoculation densities of up to 5 000 nauplii/L for batch culture, 10 000/L for closed flow-through culture, and 18 000/L for open flow through culture may be maintained without adverse effect (Table 16). These very high densities in themselves have no apparent effect on *Artemia* behaviour. Of course, each culture has a limited carrying capacity: At certain densities, culture conditions deteriorate (water quality deterioration, decreased individual food availability), and growth and survival decrease. Although crowding seems to have no effect on survival, it appears to have an impact on ingestion rate and growth. In stagnant systems, there was a noticeable drop in growth rate with increasing

animal density, since the maintenance of water quality requires a substantially lower individual feeding rate at high animal densities (Dhont, Lavens and Sorgeloos, 1993).

The cost-effectiveness of a culture definitely increases as *Artemia* density rises. In an open flow-through system, maximum densities are limited by feeding rate, whereas in a recirculating and stagnant system, water quality preservation determines a safe feeding level, which in turn determines the animal density at which the individual feed amount still allows a satisfactory growth rate.

An overview of maximal *Artemia* densities, as reported using different culture technologies, is given in Table 16.

TABLE 16
Maximal *Artemia* densities employed under different culture conditions

Culture system	Artemia/L	Culture period	Growth	Reference
Open flow-through	18 000	To adult	High	Tobias *et al*., 1980
Closed flow-through	>10 000	To adult	Moderate	Lavens *et al*., 1986
	5 000–10 000	To adult	High	
Stagnant	5 000	7 days	High	Dhont, Lavens and Sorgeloos, 1993
	20 000	7 days	Low	

Sources:

Tobias, W.L., Sorgeloos, P., Roels, O.A. & Sharfstein, B.A. 1980. International study on *Artemia*. XIII. A comparison of production data of 17 geographical strains of Artemia in the St. Croix artificial upwelling mariculture system. In: G. Persoone, P. Sorgeloos, O. Roels & E. Jaspers, eds. *The brine Shrimp Artemia*, pp. 384–392. Vol. 3. *Ecology, culturing, use in aquaculture*. Wetteren, Belgium, Universa Press.

Lavens, P., Baert, P., De Meulemeester, A., Van Ballaer, E. & Sorgeloos, P. 1986. New developments in the high-density flow-through culturing of brine shrimp *Artemia*. *Journal of the World Mariculture Society*, 16: 498–508. https://doi.org/10.1111/j.1749-7345.1985.tb00228.x

Dhont, J., Lavens, P. & Sorgeloos, P. 1993. Preparation and use of *Artemia* as food for shrimp and prawn larvae. In: J.V. Mc Vey, ed. *CRC handbook of mariculture*, pp. 61–93. 2nd Edition. Vol 1. *Crustacean culture*. Boca Raton, Florida, USA, CRC Press, Inc.

2.3.1.4. Abiotic factors

Physico-chemical conditions

Salinity and ionic composition of the culture medium

Although *Artemia* in nature is only found in high salinity waters (usually higher than 100 g/L), brine shrimp can thrive in natural seawater, and its optimal physiological performance, in terms of growth rate and food conversion efficiency, occurs at salinity levels that are considerably lower (i.e. from 32 to to 65 g/L). For *Artemia* cultivation, natural seawater (about 30-35 g/L) is the most practical solution. Small salinity adjustments can be made by adding brine or by diluting with tap water with low levels of chlorine. To prevent undissolved salts from remaining in the tanks, one should avoid adding sea salt directly to the culture and instead maintain a supply of brine for increasing the salinity as needed. In addition to natural seawater or diluted brine, several artificial media with different ionic composition have been successfully used in indoor brine shrimp production installations. Although the production of this artificial seawater is costly and labour-intensive, under certain conditions it may be cost-effective (Vanhaecke, Siddall and Sorgeloos, 1984).

Temperature, pH and oxygen concentration

For most strains, a common temperature range of preference is 25–28 °C. It follows that temperature must be maintained within the specific optimal range of the selected *Artemia* strain. Several methods for heating seawater are discussed below. According to published information, it is generally accepted that the pH tolerance for *Artemia* ranges from 6.5 to 8.0 (Vanhaecke, Siddall and Sorgeloos, 1984). The pH tends to decrease during the culture period as a result of denitrification processes. When the pH

falls below 7.5, small amounts of $NaHCO_3$ (technical grade) should be added in order to increase the buffer capacity of the culture water. The pH is commonly measured using a portable pH meter or with simple analytic laboratory kits. In the latter case, the instructions should be read carefully in order to make sure whether the employed chemical reaction is compatible with seawater.

With regard to oxygen (O_2), only very low concentrations of less than 2 mg O_2/L will limit the production of biomass. For optimal production, however, O_2 concentrations higher than 2.5 mg/L are suggested. Maintaining oxygen levels continuously higher than 5 mg/L, on the other hand, will result in the production of pale animals (low in the respiratory pigment haemoglobin), which may therefore be less perceptible and attractive for the predators. A dark red coloration (high haemoglobin content) is easily obtained by applying regular but short (few minutes) oxygen stresses (by switching off the aeration) a few days before harvesting. Oxygen levels should be checked regularly as they may drop significantly, especially after feeding. Oxygen is conveniently measured with a portable oxygen electrode. When oxygen occasionally drops below 30 percent saturation (i.e. 2.5 mg O_2/L in seawater of 32 g/L salinity at 27 °C), aeration intensity should be increased temporarily or air stones should be added. If oxygen levels remain low, the aeration capacity should be increased. It is important to note that for a given airflow, the oxygen level is more effectively increased by small air bubbles compared to big ones. Too small air bubbles, on the other hand, may get trapped between the thoracopods and consequently skim off the animals to the surface (Vanhaecke, Siddall and Sorgeloos, 1984).

Water quality

The quality of the culture medium is first affected by excess particles as well as by soluble waste products such as nitrogen compounds. High levels of suspended solids will affect production characteristics, either by their interference with uptake of food particles and propulsion by *Artemia* or by inducing bacterial growth that will compete for oxygen and eventually infest the culture tank. Information on harmful particle levels is not available since no practical method for their measurement has been developed. However, problems caused by excess particles can be detected through microscopic observation of the animals: Thoracopods should be unclogged, and the gut should be uniformly filled and unobstructed. With some experience, acceptable particle loads can be estimated on sight by holding up an aliquot of the culture in a transparent beaker against a light source. Soluble waste products, on the other hand, give rise to toxic nitrogen compounds (e.g. NH_3-N, NO_2--N, NO_3--N). Levels of nitrogen components can be measured with appropriate laboratory kits (Vanhaecke, Siddall and Sorgeloos, 1984).

Infrastructure

Tank and aeration design

Artemia may be grown in any type of container as long as the aeration system provided allows optimum oxygenation and enough mixing of feed and animals throughout the complete culture volume. However, too strong aeration should be avoided. Thus, aeration and tank design must be considered concurrently since the circulation pattern is influenced by the combination of both. A broad range of culture tanks have been shown to be acceptable, and materials can be composed of concrete, plastic or fibre, with the latter being the most durable. An example of a suitable tank design is shown in Figure 29.

Rectangular tanks are the most convenient for cultures up to 1 m^3; however, round tanks are also commonly used. They can be aerated using an air-water-lift (AWL) system, an aeration collar installed around a central standpipe, or perforated PVC tubes attached to the tank's bottom. For bigger quantities (>1 m^3), concrete tanks

FIGURE 29
Schematic layout of a 1 000 L *Artemia* culture tank

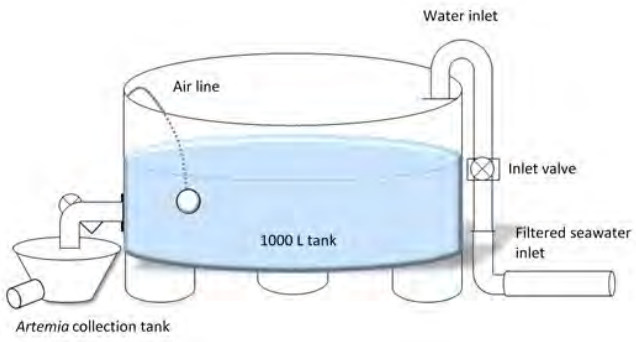


PLATE 34
Lighting set-up for *Artemia* culture tanks

walled on the interior with impermeable plastic sheets or coated with special paint are preferable. Large fibre tanks are also suitable, but they may be more expensive. These massive tanks have typically been used as raceway systems.

Heating and lighting

When *Artemia* is cultivated in tropical climates, heating is typically unnecessary. However, when the ambient temperature falls below the ideal culture values (25–28 °C), heating is required. Electric thermo-regulated resistors are the most practical way to heat small water volumes (up to 1 m^3). A heat exchanger consisting of a thermostatic-controlled boiler with copper tubing under or on the bottom of the culture tank is recommended for bigger volumes. Heat losses may be reduced by insulating the tanks with Styrofoam and covering the surface with plastic sheets. Another option is to build facilities that are entirely indoors using appropriate insulating materials, although this is expensive and only essential in temperate climates. On a practical level, heating should be minimized in order to reduce production costs.

Lighting may be added to increase *Artemia* growth; it has been observed that adding light to provide constant illumination can boost feeding rates, with growth more than 10–20 percent faster than when grown with ambient light (12 h). This effect may be accomplished by mounting any light source on top of the culture tank, preferably 1 m above the water level. LED lights may be used to save on electricity (Plate 34).

Culture techniques

Depending upon the objectives and the opportunities, different culture procedures for super-intensive *Artemia* production may be applied. The final selection of one or the other type of installation will be subject to local conditions, production needs and investment possibilities. However, two basic options should be considered: should water be renewed (open flow-through) or not? Furthermore, in the latter case, should a particular water treatment be applied (closed flow-through) or not (stagnant or batch system)? Obviously, all kinds of transition types exist, ranging from open flow-through with 0 percent recirculation to closed flow-through with 100 percent recirculation. In practice, even in case of complete recirculation, a small part of the culture water must be regularly renewed.

The culture system should be designed in such a way that the water quality can be maintained optimally. This means that the concentration of particles and soluble metabolites should remain minimal to prevent toxicity problems, proliferation of microorganisms and interferences with the filter-feeding apparatus of the brine shrimp.

Stagnant systems

A simplified and reliable technique for the intensive culture of *Artemia* biomass for use as a nursery diet for fish and shrimp has been developed at Universiti Malaysia Terengganu, in Kuala Nerus, Malaysia. The goal was to create a versatile culture process that can be used to produce *Artemia* of various sizes up to 9 mm. In this system, cultures are carried out in 1 000 L round fibre tanks (Figure 29). Four air stones attached to the bottom provide continual aeration. Cysts are hatched, and the nauplii are counted and transferred to the culture tanks at 20 animals/ml, with a 14-day culture time. The animals are given extract made from micronized palm kernel expeller (see Box 7). The feed is mixed in seawater daily, incubated for 24 h and then fed to the *Artemia*. The daily feed ratios are set to keep the culture turbidity between 15 cm and 20 cm. The feed preparation is highlighted in Annex 2. Nonetheless, each culture needs constant adjustment of the ratios based on the level of transparency.

Open flow-through system

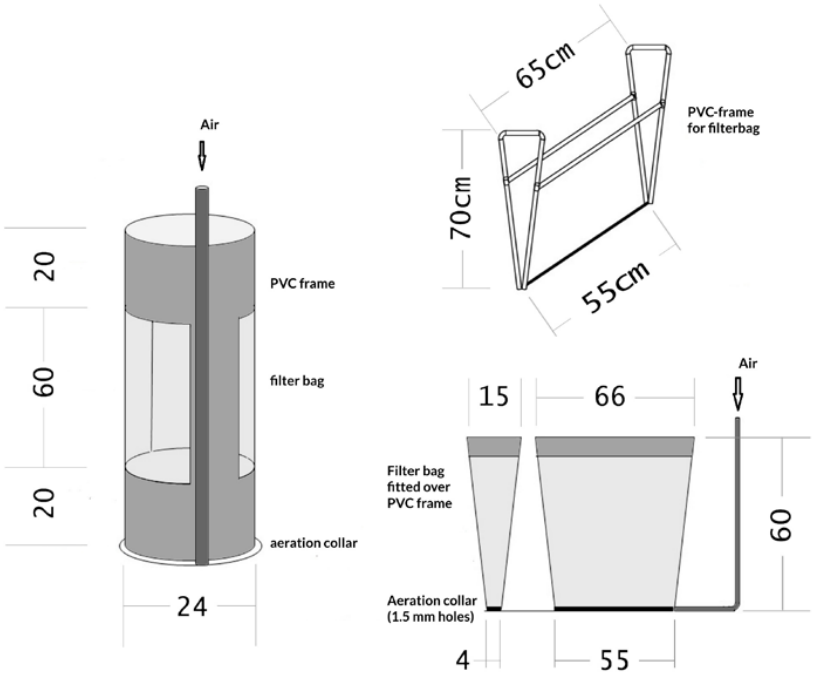
It is obvious that a discontinuous or continuous renewal of culture water by clean seawater, with consequent dilution of particulate and dissolved metabolites, will result in the best possible culture conditions and highest production capacities. Application of an open flow-through culture technique, however, is limited to those situations where large volumes of sufficiently warm seawater (or brine) are available at relatively low cost, or where large quantities of algal food are available (for example, effluents from artificial upwelling projects, tertiary treatment systems and intensive shrimp grow-out ponds).

The most important and critical equipment in flow-through culturing is the filter used for efficient evacuation of excess culture water and metabolites without losing the brine shrimp from the culture tank. These filter units should be able to operate without clogging for at least 24 h in order to reduce risks of overflowing.

Initially, filters were constructed as a PVC-frame around which an interchangeable nylon screen was fixed. The aeration was positioned at the bottom of the filter and ensured a continuous friction of air bubbles against the sides of the filter screen, which resulted in an efficient reduction of filter-mesh clogging (Figure 30). The upper part

FIGURE 30

Schematic view and dimensions (in cm) of filter systems used in flow-through culturing of *Artemia*

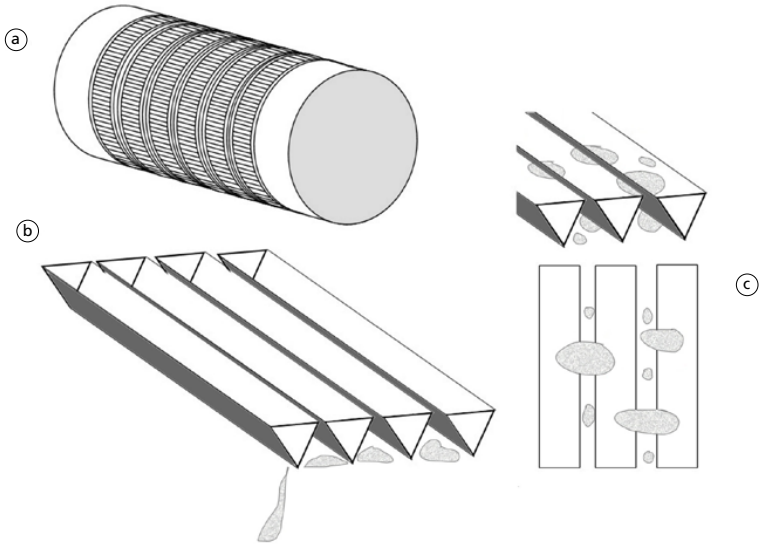


Sources:
Modified from Brisset *et al.*, 1982.

Brisset, P.J., Versichele, D., Bossuyt, E., De Ruyck, L. & Sorgeloos, P. 1982. High density flow-through culturing of brine shrimp *Artemia* on inert feeds - Preliminary results with a modified culture system. *Aquacultural Engineering*, 1(2): 115–119. https://doi.org/10.1016/0144-8609(82)90003-6

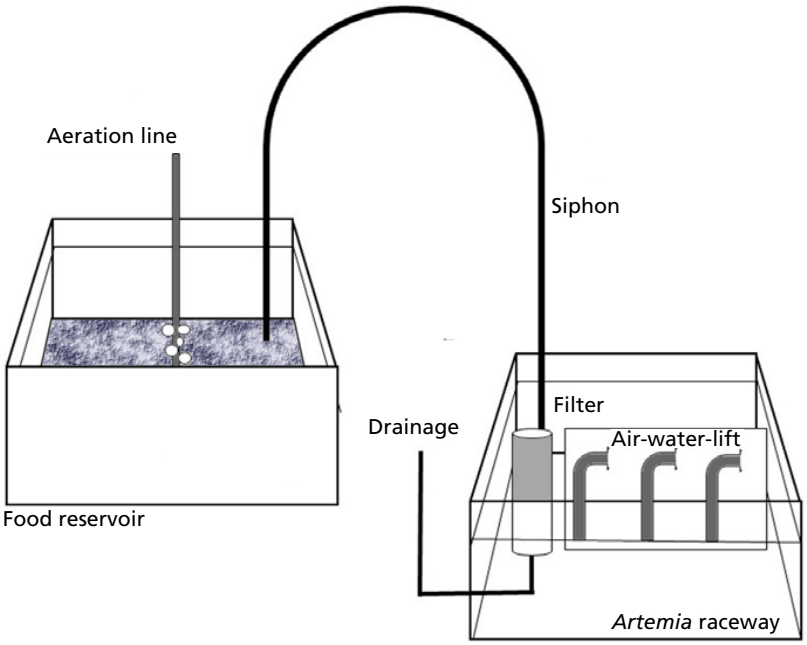
of the filter bag positioned just above and underneath the water level was made of smooth nylon cloth or plastic as to prevent any trapping of the brine shrimp that are foamed off by the effect of the aeration collar. Later, a new type of cylindrical filter system (Figure 31) was introduced. It consists of a welded-wedge screen cylinder made of stainless steel that is vertically placed in the centre of the culture tank (Figure 32). The base is closed by a PVC-ring and bears a flexible tube for the evacuation of the effluent. An aeration collar is fixed to the lower end of the filter.

FIGURE 31
Filter system based on a welded-wedge screen
(a) Stainless steel welded-wedge cylinder; (b) detail of welded-wedge screen; (c) schematic illustration of filter functioning



Sources: Dhont, J. & Lavens, P. 1996. Tank production and use of ongrown *Artemia*. In: P. Lavens & P. Sorgeloos, eds. *Manual on the production and use of live food for aquaculture*, pp. 164-195. FAO Fisheries Technical Paper 361.

FIGURE 32
Schematic view of a 1 000 L open flow-through system



Sources:
Modified from Dhert *et al.*, 1992.

Dhert, P., Bombeo, R.B., Lavens, P. & Sorgeloos, P. 1992. A simple semi flow-through culture technique for the controlled super-intensive production of *Artemia* juveniles and adults. *Aquacultural Engineering*, 11: 107-119. https://doi.org/10.1016/0144-8609(92)90013-N

This welded-wedge system has several advantages in comparison to the nylon mesh types:

- Oversized particles with an elongated shape can still be evacuated through the slit openings (Figure 31c).
- The specially designed V-shape of the slit openings creates specific hydrodynamic suction effects as a result of which particles that are hardly smaller than the slit opening are actively sucked through.
- Possible contact points between particles and filter are reduced to two instead of four mesh borders, which consequently reduces clogging probability.

This filter can be operated autonomously for much longer periods than traditional nylon mesh-filters. Therefore, proportionally smaller welded-wedge filters can be used, leaving more volume for the animals in the culture tank. Furthermore, they are cost-effective since they do not wear out.

As brine shrimp grow, the filter is regularly switched for one with larger mesh or slit openings in order to achieve a maximal evacuation of moults, faeces and other waste particles from the culture tanks. Before changing to a larger mesh, it should be checked whether animals can cross the larger mesh. If so, switching filters is still too early and the actual filter is returned after cleaning. A set of filters covering a 14-day culture period should consist of approximately six different slit or mesh openings ranging from 120 µm to 550 µm (Table 17).

TABLE 17
Example of food and water renewal management in a 300 L super-intensive *Artemia* culture system

Culture day	Slit opening of filter (µm)	Flow rate (L/h)	Retention time in culture tank (h)	Interval between feedings (min)	Daily food amount (g)
1	120	80	3.75	36	100
2	150	100	3	30	120
3–4	200	100	3	24	150
5–7	250	150	2	20	180
8–9	300	150	2	20	180
10–12	350	200	1.5	15	250
13–14	350	300	1	12	300

Sources:
Data compiled from Lavens and Sorgeloos, 1991.

Lavens, P. & Sorgeloos, P. 1991. Chapter XIII: Production of *Artemia* in culture tanks. In: R.A. Browne, P. Sorgeloos & C.N.A. Trotman, eds. *Artemia Biology*, pp. 317–350. Boca Raton, Florida, USA, CRC Press, Inc.

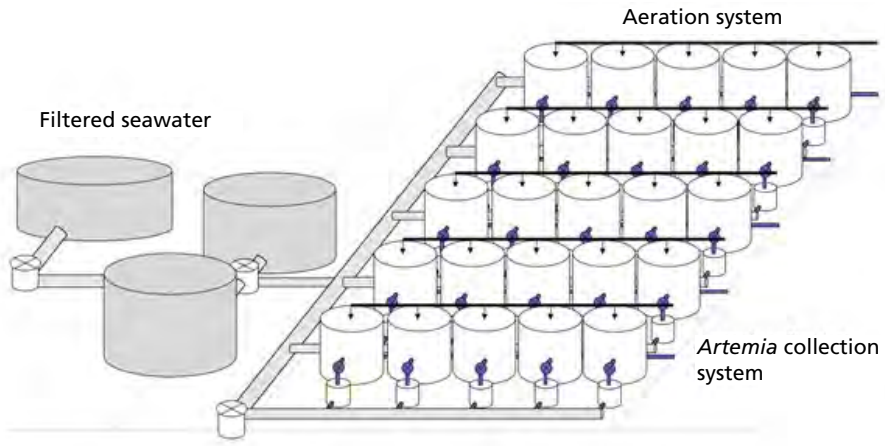
If the water circulation in the culture tank is optimal, the filter may be positioned anywhere in the tank. In cylindrical tanks, especially with conical bottoms, the filter is ideally placed in the centre.

Closed flow-through (recirculation) system

When only limited quantities of warm seawater are available, open flow-through systems cannot be considered. Yet, if one decides to operate at high animal densities and/or for prolonged culture periods, the accumulation of particles and soluble metabolites will reduce the water quality until good culture practices become impossible. Under these conditions, the high-density flow-through culturing of *Artemia* can be maintained only by recirculating the culture water over a water treatment unit. This unit should be designed to remove particles and decrease the levels of harmful nitrogen components.

Even though there have been significant research efforts to develop performing recirculation systems, the operation of practical recirculating systems for *Artemia* is still more of an art than a science. That is why no specific recirculation technology is recommended here. Figure 33 should therefore be considered as one example of an

FIGURE 33
Schematic layout of a closed flow-through system for culturing *Artemia*

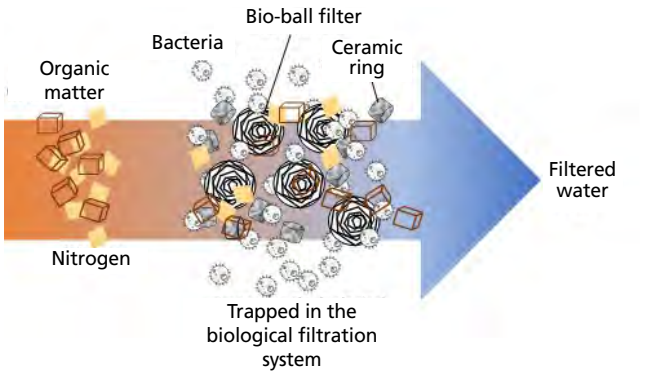


Source: IMB, Malaysia.

operational recirculating system for *Artemia* culture, as was developed at the *Artemia* Culture Hatchery at Universiti Malaysia Terengganu, in Kuala Nerus, Malaysia. The system consists of fibre tanks connected by a pipeline that delivers filtered seawater via gravitational flow. As shown in Plate 34, the systems are equipped with aeration and heating/lighting devices. Each tank has a diameter of 1.2 m and a depth of 1 m, and a rearing volume of approximately 1 000 L. Prior to discharge, all process effluent must be treated for solids removal, as the tanks operate on a flow-through basis with a single drain configuration. As depicted in Plate 34, tank drains are located on the bottom of the tank, allowing intermittent collection of *Artemia* biomass directly into a collection tank device.

For details of the recirculation system developed at the *Artemia* Reference Centre at Ghent University, Belgium, with rotating biological contactor or biodiscs as biological filter, refer to Dhont and Lavens, 1996. The biological filter, or biofilter, is the key component in the filtration section of a recirculating aquaculture system (RAS). A large surface area is supplied for microorganisms that reduce ammonia levels in recirculating water systems by physiologically oxidizing ammonia to relatively harmless nitrate. Ammonia is initially converted to nitrite by bacterial nitrification, and then nitrite is further oxidized to nitrate. Before stocking *Artemia*, a biofilter is normally conditioned for several weeks by adding ammonia and monitoring its breakdown to ensure bacterial populations are sufficient to remove ammonia and nitrite at rates required during operation. Oyster shell, gravel, nylon netting, plastic rings and sponge foam pads are some of the most common biofilter media options, but there are many others. The most important considerations for constructing biofilters are maximizing surface area for bacterial growth, ensuring high dissolved oxygen levels, ensuring uniform water flow through the filter, and providing enough empty space to prevent clogging (McGee and Cichra, 2000). The mechanism of action for consideration in designing a biological filter is depicted in Figure 34.

FIGURE 34
The mechanism of action of a biological filtration system



Source: IMB, Malaysia.

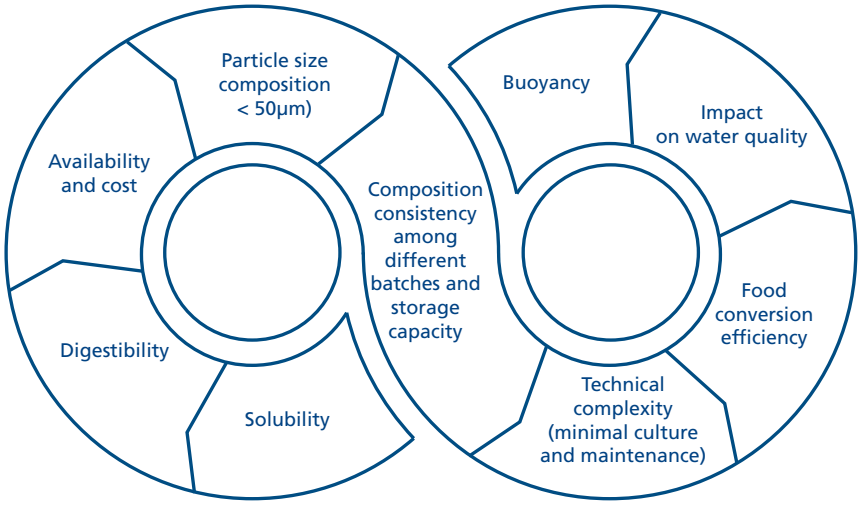
Feeding strategies and diets

Artemia's non-selective feeding habit makes them relatively easy to culture and mass produce. *Artemia*'s feeding behaviour can be affected by a variety of parameters, including the development stage, the food concentration and the culture conditions, all of which affect *Artemia*'s food filtration, ingestion and assimilation processes. When food is available in sufficient concentration to allow for adequate feeding, the swimming speed of adults can be reduced by 50 percent. When feeding formulated feed, amounts should be managed such as to ensure that good water quality is maintained at all times. In this context, it is critical to increase daily feeding frequency while giving smaller amounts of feed each time.

Diet selection

Artemia are not selective in their diet and consume food largely based on size (see Section 1.1). As the cyst reserves are depleted, exogenous nutrition plays a crucial role in ensuring that they develop and reproduce optimally. Although different food alternatives have varying nutritional contents, this variation does not have a significant impact on *Artemia* production in high density culture. Other important factors in selecting a proper dietary source are shown in Figure 35.

FIGURE 35
Factors that play a role in the selection of a suitable diet for *Artemia* culture



Source: IMB, Malaysia.

The following feeds can be considered for *Artemia* tank production:

Microalgae

Microalgae are the natural diet of *Artemia*. For example, *Dunaliella* sp. is grazed upon by *Artemia franciscana* in the Great Salt Lake (Utah, United States of America). To date, various microalgae species have shown great potential in feeding and enriching *Artemia* during aquaculture production, enhancing growth, survival and reproductive output; for example, *Isochrysis* sp., *Nannochloropsis* sp., *Chaetoceros* sp., *Tetraselmis* sp. and *Dunaliella* sp. have been used in *Artemia* culture. Although they bloom easily, maintaining a constant viable and optimal microalgae production is not easy, and the direct purchase of concentrated microalgae is costly. Additionally, mass culture of microalgae requires space, time and expertise, all of which would incur high costs. Another drawback is the time needed for microalgae to reach their maximum concentration. Although in general the most commonly used microalgae bloom within 4 to 7 days, the need to culture them for a designated period requires the farmer to run additional technical processes. Intensive *Artemia* culture requires sufficient quantities

of microalgae; supplying suboptimal algal concentrations therefore jeopardizes the optimal production of *Artemia*. Moreover, not all microalgae are suitable to be used as *Artemia* food. For example, the cell walls of some microalgae species (e.g. *Chlorella* and *Stichococcus*) are too thick to be digestible by *Artemia* (Dan *et al*., 2022).

Dried algae

Algal meals (e.g. dried form of *Spirulina* and *Scenedesmus*) contain almost similar nutritional composition as live algae and can be used as food for *Artemia*. However, some major drawbacks of using dried algae as *Artemia* diet include the high cost (between USD 10/kg and USD 20/kg) and the need for special attention to the water quality of the culture medium owing to the indigestible portion of dried algae.

Co-feeding of microalgae and bacteria/bioflocs

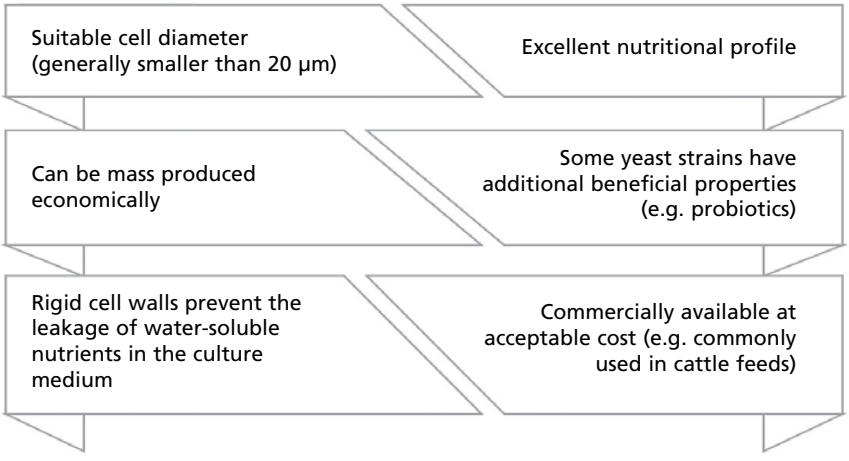
Co-feeding of microalgae and bacteria is considered as a viable alternative to improve nitrogen assimilation in *Artemia*. When presented together at specific concentrations, the presence of microalgae and bacteria has a synergistic effect in *Artemia* and provides a better nutrient source to sustain high density *Artemia* populations (Toi *et al*., 2014). However, co-feeding of microalgae and bacteria requires technical expertise, from the identification of suitable bacteria strains to the optimization of bacteria-microalgae proportions.

The current advancement in biofloc technology offers a viable alternative to feed *Artemia* while maintaining water quality. Biofloc technology involves the use of heterotrophic bacteria to convert unwanted nitrogenous compounds into microbial biomass that is valorized as a protein source for *Artemia* (Yao *et al*., 2018). This is especially effective in an integrated culture system involving the culture of *Artemia* and other aquaculture organisms such as crustacean or fish species, whereby the wastewater of the farmed animals could be used to produce bioflocs, to be subsequently used for *Artemia* feeding. This method greatly reduces the environmental impact from aquaculture. In addition, incorporating bioflocs into the *Artemia* diet is also a promising method to introduce healthy probiotics to the targeted aquaculture species (Liang *et al*., 2022).

Single-cell proteins

These are isolated from microorganisms (bacteria or yeast) with high protein content as purified proteins or dried cells. Single-cell proteins (SCP) have several characteristics that make them an interesting alternative for microalgae (see Figure 36). One added benefit of SCP is that they exhibit recombinant properties. This enables SCP to

FIGURE 36
Characteristics that make single-cell proteins a suitable alternative feed for *Artemia* culture



Source: IMB, Malaysia.

incorporate beneficial characteristics by the aid of genetic tools while using *Artemia* as a vehicle to deliver them to predators such as fish or shrimp larvae.

Food wastes and by-products

Artemia can ingest a variety of food wastes and/or by-products because of their non-selective feeding nature. Economically, the utilization of food wastes/by products is practical and desirable because of their immediate availability, ease of storage and low cost, especially in comparison to the laborious production of microalgae. Oatmeal, rice bran, corn bran, wheat bran and soymeal are some of the possible waste products that may be employed as a feed source for high-intensity *Artemia* culture. When food wastes/by products are employed, it is critical to evaluate their viability as a single diet or mixed diet by assessing *Artemia* growth and survival. In optimal conditions, food wastes/by-products can replace 90–100 percent of live algal food. By-products might also be supplemented with additional necessary nutrients, including fish oil, cholesterol and phospholipids (Vahdat and Oroujlou, 2021).

The ideal particle size for feeding by adult *Artemia* is maximally 50 µm (or much smaller for earlier stages; see Section 1.1). However, because bigger particle size products are often available, additional processing and treatment are frequently necessary to increase the fraction of particles smaller than this limit. Products with large-sized particles can be manually moistened with saltwater, homogenized using a standard electrical blender, and processed through a 50 µm filter if labour is inexpensive and readily available. Manual homogenization and filtration of products, on the other hand, must be done on a daily basis since the filtered feed suspension has low storability. It should be noted that the homogenization and filtration of high fibre products such as rice bran and wheat bran may not be appropriate since more than 90 percent of the product (in the form of fibre) may be filtered away. If cost is not an issue, additional mechanical homogenization procedures, for example micronization, can be employed for large-scale dry meal preparation for *Artemia* high density culture.

Most organic compounds that are not consumed by *Artemia* degrade in the culture medium and impact water quality by adding nitrogenous chemicals such as ammonia and nitrite, depending on the food source. This is especially true for diets high in soluble protein, such as soybean meal. It is advised that such diets be prewashed by vigorously aerating the feed suspension for 1–2 h to minimize the soluble fraction. Following sedimentation, the protein fraction found in foam form or remaining in the water column can be removed, leaving the sedimented particles with less soluble protein that may degrade water quality. The washing process can be repeated as needed.

More details on the use of agricultural wastes for *Artemia* farming can be found in the extended review by Jumalon Ogburn *et al.* (2022).

BOX 7

Formulated *Artemia* feed from raw palm kernel, PKC-Nutri+®

A palm kernel expeller-based extract has been formulated as a novel inert feed to boost growth and production of *Artemia* in tank systems at Universiti Malaysia Terengganu, in Kuala Nerus, Malaysia. A palm kernel expeller is a by product from palm oil production and is considered to be a cheap agricultural by-product. The formulated feed, termed as PKC-Nutri+® (see plate on page 94), generally contains 21 percent protein, 47 percent carbohydrate, 6 percent lipid, 22 percent fibre and 4 percent ash. Nutrient analysis performed on the *Artemia* biomass fed with palm-kernel expeller-based feed revealed 59 percent protein, 13 percent carbohydrate, 6 percent lipid, 14 percent fibre and 8 percent ash. Therefore, even though palm-kernel expeller-based feed primarily contains relatively low crude protein levels, *Artemia* accumulated protein levels equivalent to those when feeding on microalgae has demonstrated that brine shrimp are effective protein converters from the palm-kernel expeller-based feed's high carbohydrate sources and illustrated that

BOX 7 (CONTINUED)

this product is a suitable inert feed to replace the use of expensive microalgae, which is an advantage when considering operating costs.

Formulated *Artemia* feed from raw palm kernel, PKC-Nutri+®

Feeding strategies

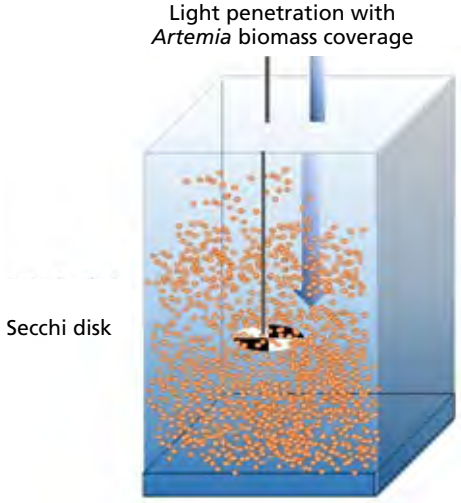
Because *Artemia* is a continuous filter-feeding organism, the highest growth and minimal deposition of unconsumed feed is achieved when feed is distributed as frequently as possible.

When feeding single-cell proteins, algal or yeast concentrations should be maintained above the critical minimum uptake concentration that is specific for the algal species and the developmental stage of *Artemia* (Abreu-Grobois *et al.*, 1991).

Since *Artemia* has a high clearance rate of microalgae, the algal concentration in the culture tank should be determined several times a day and the retention time adjusted so as to maintain levels well above the estimated minimal uptake concentration. If there are no data on the ingestion rate or optimal feed levels, different algal concentrations should be tried out and the feeding level should be estimated by microscopical observation. Well-fed animals have a completely filled gut and release compact faecal pellets. Underfed animals have an empty or barely filled gut and tend to release loose faecal pellets (see Section 2.2.4.1).

Levels of dry feeds, consisting of fragments and irregular particles, cannot be counted in the culture tank. Therefore, a correlation between optimal feed level and transparency of the culture water has been developed: The feed concentration in a culture tank is commonly determined by measuring the transparency of the water with a simplified Secchi disc (Figure 37). Experience has taught that optimal feed levels coincide with transparencies of 15 to 20 cm during the first culture week and 20 to 25 cm the following week (Lavens *et al.*, 1986). Once animals reach the adult stage, the best production yields are obtained when gradually switching from a transparency-controlled feed distribution to a feeding scheme of about 10 percent dry feed weight of the live weight of *Artemia* per L/day (Lavens and Sorgeloos, 1987a). A feeding scheme is given in Annex 2.

FIGURE 37
Feeding strategy with the aid of a Secchi disc to assess light penetration with *Artemia* biomass coverage



2.3.2. Artemia biomass harvesting techniques

Harvesting of high-density *Artemia* cultures can be facilitated by taking advantage of the animals' surface respiration behaviour. When the aeration in the culture tank as well as the flow-through and automatic feeding is interrupted, the oxygen level in the water drops rapidly and all waste particles sink to the bottom. After about 30 minutes, *Artemia* respond to oxygen depletion by concentrating at the water surface, where they perform surface respiration. The concentrated population, which is free of suspended particles, may be simply scooped up with a net with an appropriate mesh size.

When the culture water is free of particles, brine shrimp can also be harvested by draining the entire culture through a sieve that is partially submerged in a water recipient (Plate 35). *Artemia* should be properly cleaned in either freshwater or seawater. The collected *Artemia* can subsequently be supplied as a high-quality live food source for both freshwater and marine predators. Because *Artemia* is a hypo osmoregulator (i.e. its body fluids have a constant low salt content of about 9 g/L), the salinity of the predator culture water is of no significance. Brine shrimp can survive in saltwater for several days without feeding. When introduced to freshwater, *Artemia* will continue to swim for approximately another 5 h before dying as a result of osmoregulatory stress. Live brine shrimp can be transported in plastic bags containing chilled seawater and oxygen (see Section 2.4.3.2).

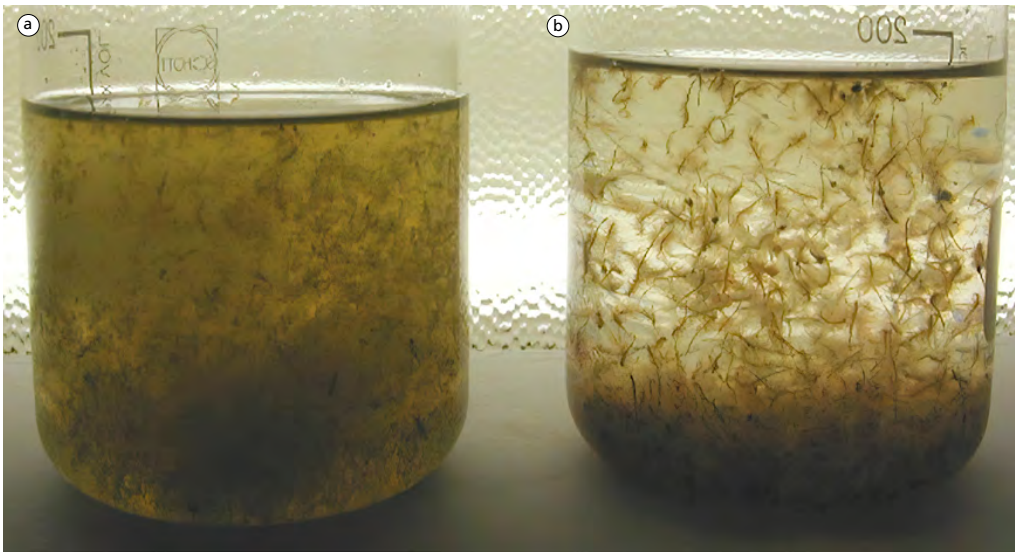
Artemia harvested but not consumed can be frozen or dried in flakes. To achieve the highest possible product quality, brine shrimp biomass must be frozen immediately after being thoroughly washed with freshwater while still alive. The biomass should be placed in plastic bags or ice trays in thin layers (0.5 cm) and quickly frozen (at least -25 °C). When the biomass is properly frozen, the adults' exoskeleton is not damaged. When the frozen *Artemia* are thawed, they produce intact animals that do not contaminate the water due to the leaching of body fluids (Plate 36).

PLATE 35

***Artemia* harvesting technique by draining the culture tank over a submerged sieve**

PLATE 36

Example of biomass frozen inadequately (a: turbid water with partially decomposed *Artemia*) and adequately (b: clear water with intact *Artemia*) after thawing

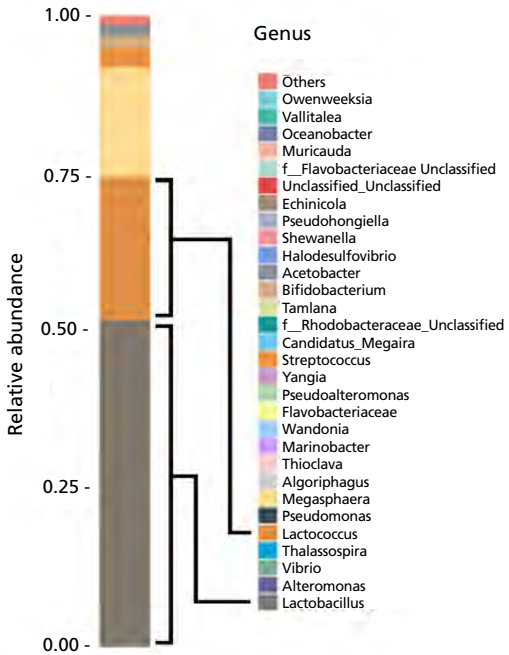


2.3.3. Microbiological aspects of *Artemia* biomass tank production

Infections with filamentous *Leucothrix* bacteria, which occur primarily in nutrient-rich environments, may cause substantial losses of subadults. The *Leucothrix* colonies attach to the exoskeleton, preferentially the thoracopods, and become visible only from the instar V/VI onwards (see Section 2.2.5.5). The brine shrimp suffer physically when the movements of their thoracopods are disrupted, resulting in lower filtration rates. Overfeeding of the tanks eventually stops development and moulting, leading to the collapse of the *Artemia* culture. Terramycin may be used as a cure; however, antibiotics cannot be administered in recirculation systems because they will impair the biological treatment unit. The most practicable option appears to be an increase in salinity from 35 g/L to 50–60 g/L along with a higher rate of water replenishment.

FIGURE 38

Composition of the microbial community (at genus level) in the palm-kernel expeller-based feed for *Artemia*



Source: IMB, Malaysia.

A second disease identified in *Artemia* cultures is the so-called "black spot disease" (Plate 32), in which parts of the animals display black spots, particularly at their extremities (i.e. thoracopods, antennae). This condition is characterized by the separation of the epidermis from the cuticula and is assumed to be caused by a nutritional deficit that disrupts lipid metabolism. Black spot disease is typically observed in high density culture of *Artemia* utilizing agricultural by-products as food supply when water quality deteriorates (possibly interfering with the make-up of the bacterial population) and/or when feeding rates are not appropriate. Improving these circumstances does not appear to save the affected animals, but it does appear

to prevent subsequent losses. In contrast to other agricultural by-products, the use of palm-kernel expeller-based feed as feed does not cause black spot disease to arise during *Artemia* culture. An examination of the microbial metagenomes (Figure 38) of palm-kernel expeller-based feed, against the top 30 most abundant classifications according to the Microflora Diversity Analysis using 16s rDNA amplicon sequencing, showed a high proportion of *Lactobacillus* and *Lactococcus* (approximately 75 percent), both of which are commonly known as probiotic or "positive bacteria".

2.3.4. Production figures and costs of *Artemia* biomass tank production

Estimating production costs for *Artemia* culture is difficult. While fixed costs may be generalized reasonably well, operational costs are wholly dependent on local variables. Local factors, for example, affect what type of operation is recommended: Open flow-through in locations with plentiful clean and warm seawater is one example. Nevertheless, significant additional expenses are involved when a recirculating unit is required. Feed should be chosen from among locally accessible options. Not only is price important in feed choices, but so are their processing costs. Furthermore, whether the feed should be mechanically micronized or manually squeezed through a filter bag will be determined by the cost of micronization in comparison to the cost of local labour. Finally, heating and pumping expenses are determined by a variety of local factors, such as power costs, ambient temperature and the position of the saltwater stock in relation to the culture tanks.

Plate 37 illustrates the interactions between the Universiti Malaysia Terengganu and Malaysian farmers for transferring the knowledge of *Artemia* culture and tank production to famers for improving *Artemia* production.

PLATE 37

Technology transfer of *Artemia* culture and tank production to Malaysian farmers

Many farmers, particularly those in the ornamental fish sector in Malaysia, have profited from the use of palm-kernel expeller-based feed as feed for *Artemia* (see Box 7). Unlike in some other parts of the world, live *Artemia* is difficult to obtain and costs between USD 20/kg and USD 30/kg in Malaysia, depending on availability, and farmers generally rely on imported live food (frozen *Artemia*, bloodworms, etc.), which happens to be of poorer quality owing to handling and transport. When practiced by local farmers at small or medium scale, an initial inoculum of 3 g/500 L (nauplii) in a stagnant batch culture system may yield 1 kg to 2 kg of adult *Artemia* biomass after 14 days of culture. The production costs, including feed, labour, utilities and others, may be lowered from USD 10/kg to USD 3/kg using the existing *Artemia* tank culture technique with palm-kernel expeller-based feed, the latter with the use of natural saltwater.

2.4. HARVESTING AND PROCESSING TECHNIQUES (by *Nguyen Van Hoa* and *Gilbert Van Stappen*)

2.4.1. Harvesting management

Section 2.4 focuses on harvesting and processing of *Artemia* produced through pond farming. The good practices described, however, are universally applicable, i.e. also for

Artemia harvested from natural salt lakes. *Artemia* pond farming may target to harvest two main products – either cysts or biomass, or both. Attention needs to be given to determining the suitable time for harvesting, the frequency of harvesting and the status of the raw products to ensure their best quality.

2.4.2. Harvesting and processing of cysts

2.4.2.1. Harvesting cysts in ponds

Depending on the weather conditions, pond management and development of the *Artemia* population in the culture pond, harvesting activity may take place as early as 2 weeks after stocking (e.g. in the Mekong Delta, Viet Nam). Cysts start to appear in the culture pond (visible as small particles with colour varying from light to dark yellow, or light to dark brown, and often attached to foam or debris in the downwind corner), first in the water column and later coming up to the pond surface where they aggregate in clusters. Thanks to the wind blowing on the pond surface, floating cysts will drift towards the pond shore and generally accumulate in the downwind corner behind the cyst barriers (Plate 38), where they can be harvested. As explained in Section 2.2.3.2, it is important to prevent cysts from being washed ashore because they may be exposed to high temperatures and repeated hydration/dehydration cycles that in turn may decrease the viability of the final product. Furthermore, cysts accumulated on the shore may also become airborne when dry. In case of strong winds, the cyst appearance on the water surface will be less since cysts will stay suspended in the water column; they will gather again on the surface when water turbulence diminishes. When the water is very agitated and much foam develops, cysts may also get trapped and are lost in the airborne foam. Therefore, cyst barriers (floating bamboo sticks or plant branches, for example) should be installed, as these act as wave breakers and reduce foam formation. On windless days, on the other hand, the cysts may not easily come together in the pond corner.

Freshly released cysts do not immediately develop into nauplii, even when the incubation conditions in the habitat are favourable. These cysts remain in a state of diapause, which means that all metabolic activity is reversibly interrupted. Only after deactivation of this diapause – which, for San Francisco Bay-type *Artemia franciscana* is the case after dehydration (Lavens and Sorgeloos, 1987b) – can the cysts resume their development when incubated under acceptable hatching conditions (see also Section 1.5.4).

Maximum guarantee for good quality, minimal loss of harvest and at the same time reduced contamination with impurities are ensured when cysts are harvested from the

PLATE 38

Cysts floating on the water surface, driven by the wind to the pond corner (a) and ready for harvesting (b)

Source: Hoa, V.N. & Nam, T.N.H. 2019. *Application of bio-floc technology for culture of Artemia*. N.V. Hoa, H.T. Hieu, N.A. Khoa & D.T.T. Uyen, eds. Ho Chi Minh City, Viet Nam, Agriculture Publishing House. ISBN 978-604-60-2981-6. 163 pp.

water surface on a regular basis. Therefore, cyst harvesting is usually carried out daily, sometimes twice a day (i.e. in the morning and late afternoon) or even more frequently. Cysts are easily harvested with a scoop net (i.e. a standard plankton net or a net made of fine fabric so that water can easily pass through) with a mesh size of 100–150 µm.

2.4.2.2. Overview of cyst processing procedures

For small-scale production, *Artemia* farmers generally sell their cyst product directly in raw form, after preliminary processing as described in Section 2.4.2.3, to the intermediary or processing plants. In this case, farmers may not engage into investment for a full-fledged processing unit.

In contrast, for larger-scale production, such as as cooperatives or larger-scale companies, the equipment for cyst processing and packing is required in order to obtain a clean, marketable product (e.g. packed in cans, featuring acceptable hatching parameters and shelf-life). The equipment includes a variety of tanks (for separation of cysts from debris), centrifuge(s), dryer unit(s) such as fluidized bed dryer (FBD) units, and equipment for screening and packaging (Figure 39; Plate 39). The scale of the processing plant and the investment needed depend on the scale of the farming area and thus the volumes of raw cysts produced (and/or purchased).

FIGURE 39

Diagram of the equipment in a cyst processing plant

(1) Brine storage and separation cone(s); (2) cyst separator cones (freshwater separation); (3) screens to remove external water and remaining debris; (4) centrifuge; (5) fluidized bed dryer unit; (6) screening/ separation machine; (7) vacuum and canning machine; (8) warehouse for temporary storage of final product

PLATE 39
Example of standard equipment items in cyst processing: Centrifuges (left, front); sets of sieves (right, front); tanks for freshwater density separation (back)

Prior to transfering to the plant where further processing will be done, cysts undergo primary processing (see Section 2.4.2.3). Summarizing, and as illustrated in Figure 39, the cysts, which have been stored and undergone a density separation (heavy debris sinks; cyst material floats) in saturated brine (1), are washed with fresh/tap water (2) to remove all saline water until the salinity has reached 0 g/L; this process should not take more than 10 minutes to avoid cyst hydration. Separation of empty cyst shells from full cysts may be done through a short freshwater incubation (full cysts sink, empty cysts float). Then, the wet cysts are collected in bags or screens (3) to remove most of the external water and separate the cysts from remaining debris, after which they are centrifuged (4) to remove the excess water. Now the wet cysts are ready for drying (5). Drying capacity depends on the dimensions of the drying chamber used: In a small-scale FBD unit, the drying capacity is up to 20–30 kg cysts (wet weight), while a larger-scale cyst drying system can process up to a few tens of kilograms of wet cysts per day. Dried cysts are then collected from the FBD, allowed to cool down and passed over a screen (6) to separate them from the last remaining impurities. These purified cysts are now ready for canning (7 and 8) (see Figure 39).

A more detailed description of the successive processing steps is given in the sections below.

2.4.2.3. Primary processing and storage of cysts

Harvested raw cysts are usually mixed with debris, algae and sometimes dead *Artemia*, so it is necessary to screen the cysts with pond water immediately after harvesting. This primary processing of raw cysts is performed through the following steps (Plate 40).

Step 1

Slowly pouring pond water over a net with a mesh size of 1 mm (net I), the cysts are cleaned from larger debris while ensuring that cysts also attached to the debris are

PLATE 40

Primary processing of *Artemia* cysts

(a, b) Cysts accumulating in pond corner; (c, d) rinsing cysts with pond water over screen; (e) squeezing cysts to remove excess water; (f) raw cysts after primary processing

recuperated through extra washing/spraying. This activity is generally done close to the culture ponds because the pond water that is used for rinsing has the same salinity as the water from where the cysts were collected. Using freshwater or lower-salinity water for rinsing cysts may lead to water absorption and initiate cyst metabolism (Lavens and Sorgeloos, 1987b), thus affecting cyst quality.

Step 2

Using a net with a mesh size of 400 µm (net II), cysts are cleaned from impurities larger than 400 µm.

Step 3

Cysts are collected on a 150 µm sieve while further rinsing them with pond water. The final product is a collection of raw clean *Artemia* cysts. The excess external water can gently be removed by squeezing the cyst mass.

Step 4

Raw (or wet) cysts washed through the steps are to be stored in saturated brine (250–300 g/L) where they will undergo partial dehydration. To avoid quality decrease, this incubation in brine should be done soon after harvesting. Daily, cysts are stirred up manually or by a mixer to expose all cysts homogenously to the brine. Dark sediment and odorous water that settle at the bottom of the container must be siphoned out, after which the container must be replenished with new saturated brine to maintain the salinity of the brine high enough for optimal storage. More salt must also be added frequently as new cysts are added because these partially hydrated cysts will lower salinity and thus lead to less efficient dehydration. Cysts having gone through this primary cyst processing will have lowered their water content to around 20–25 percent, and they can be stored for a couple of months under ambient temperature (see Plate 41), although storage in cool conditions is to be preferred. Depending on the strain, this dehydration will partially or completely deactivate diapause (Lavens and Sorgeloos, 1987b).

PLATE 41
***Artemia* cyst incubation in brine**

Step 5: Density separation in brine

Density separation should be done in freshwater and saline water, both aiming to clean the product in a different way. Saline separation will separate the sinking part of the debris (heavy substances) from the cysts: these float in the saline water. Freshwater separation will separate the floating debris (wood, feathers, empty shells) from the full cysts: These sink in freshwater.

Whereas many impurities of larger size than the cysts are removed through rinsing the cysts with pond water during primary processing (see above), removal of heavy debris in the same size range as the cysts is carried out through density separation in brine. Cysts submerged in brine float, while heavy debris (i.e. sand, gravel, heavy organic matter) sinks. This treatment is especially recommended when cysts have been harvested close to or on the shore, and thus contain a lot of heavy debris. If the equipment is available onsite, density separation is often performed near the production sites (owing to the availability of saturated brine) soon after harvesting.

As an alternative to incubation in brine, excess brine can also be removed (i.e. by hand squeezing), and the semi-moist product can be stored in bags made of cotton or jute; the remaining brine will further leak from the bags during storage. When stored as a semi-moist product over longer periods (>1 week) in areas of high air humidity, crude salt should be mixed with the cysts to prevent the rehydration of the highly hygroscopic cysts. Storage in bags also facilitates transport. Apart from diapause deactivation as a result of the dehydration process itself, the storage ("aging") in brine may further deactivate diapause in certain strains and batches (Lavens and Sorgeloos, 1987b).

Step 6: Density separation in freshwater

During the freshwater processing step, the cyst material is further cleaned through density separation and prepared for subsequent drying. As freshwater is used, the cysts will partially hydrate. If the cysts remain hydrated for too long a period under aerated conditions and sufficiently high temperature, the embryos will eventually reach an irreversible state of the hatching metabolism (i.e. cysts cannot be dehydrated

again without affecting the viability of the embryos) (Lavens and Sorgeloos, 1987b). Even when cysts are dehydrated before reaching the irreversible phase of metabolism, their energy reserves may have been depleted to levels that result in a decrease of hatchability. To prevent prolonged metabolism and consequent depletion of energy reserves, freshwater processing should ideally be limited to a maximum of 10 minutes and with cold water at a controlled temperature. The different activities in the freshwater processing step are as follows.

Removal of excess brine:

– Before density separation in freshwater, excess brine must be removed to prevent salinity (density) increase of the water and consequently suboptimal separation. This can be done by allowing the brine to leak from the cysts mass (when packed in textile bags), followed by thorough rinsing (5–10 minutes) over a screen using freshwater.

Density separation in freshwater:

– Cyst material submerged in freshwater will separate into a high-density (sinking) fraction and a low-density (floating) fraction. The sinking fraction contains mainly full cysts and some non-cyst material of similar density and size as the full cysts. The floating fraction contains mainly empty and cracked cysts shells and light-density non-cyst material of a similar size range. To reduce the bacterial load of the final cyst product, the cysts can be disinfected during the freshwater treatment by adding H_2O_2 or hypochlorite (OCl-; liquid bleach) to the freshwater separation tanks prior to adding the cyst material. The concentration of active chlorine in the freshwater of the separation tanks should be less than 200 µl/L (or ppm).

Removal of excess water:

– After collection of the sunk full cyst mass in bags, the bulk of the freshwater can be removed by a screen and by firmly squeezing the bags in which the cyst mass has been collected. If the cysts are to be dried, further removal of excess water can be achieved by centrifugation, which will significantly reduce the drying time and, consequently, the required energy inputs (Plate 42). Moreover, it will also reduce the stickiness of the product, which also facilitates the drying process.

PLATE 42
Removal of excess water by centrifugation

2.4.2.4. Cyst drying and packing

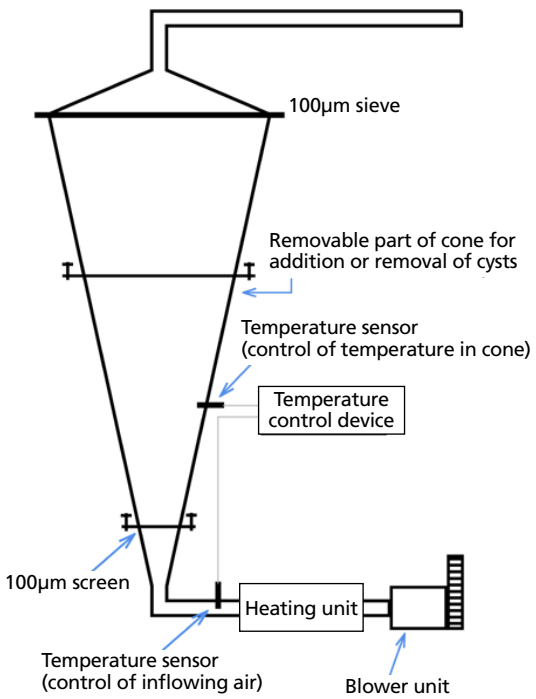
Drying process

Depending on the drying procedure, the hatching quality and shelf-life of the cysts can be affected. The following factors have an impact on the quality of the final dried product, and thus the drying process must be well managed.

- Targeted final water content of the finished product: After a freshwater treatment, the water content of the cysts should be reduced (see above) below the critical level of 10 percent as soon as possible in order to stop the metabolic activity and, consequently, to ensure a long shelf-life. Usually, a water content between 3 percent and 8 percent is aimed for.
- Optimal drying time: Best results are obtained when a water content of maximally 10 percent is reached within 8 h or less. Prolonged drying (e.g. >24 h) results in a decreased hatching percentage, caused by a decrease of energy reserves. Fast drying might also have a negative effect on the product because its temperature might become too high during the drying process, damaging the embryo inside the cysts (Lavens and Sorgeloos, 1987b).
- Drying temperature: The maximal drying temperature (air and product temperature) depends on the product (strain, moisture content) and the applied drying technology (quantity, drying technique). In general, a product temperature below 35 °C is usually safe.
- Homogenous drying is important. Uneven drying will result in some cysts drying very slowly and eventually not reaching a water content below 10 percent, possibly causing reduced hatching output and shelf-life.

The most efficient strategy is to aim at a relatively fast and homogenous drying process, resulting in a final water content below 10 percent; this can be obtained by a fluidized bed dryer (FBD). The basic design of an FBD, as presented in Figure 40, consists of a conical drying chamber, a blower, a heating unit with a temperature control device, and an exhaust filter/bag filter. In the "fluidization" process, hot air is introduced at high pressure through a perforated bed of moist solid particles (= cysts). The wet solids are lifted from the bottom and suspended in a stream of air (fluidized state). Heat transfer is accomplished by direct contact between the wet solids and hot air. The vaporized liquid is carried away by the drying air. Sometimes, to save energy, the exit (hot) air is partially recycled.

FIGURE 40
Schematic drawing of a fluidized bed dryer for *Artemia* cysts



Source: LAARC.

The conical shape of the drying chamber ensures optimal mixing of the cyst product throughout the drying process, which results in homogenous drying without excessive formation of cyst aggregations. Improved drying efficiency is further obtained by the heating unit. A first temperature sensor controls the inlet temperature (in certain cases as high as 90 °C). As the cysts become drier, evaporation decreases and the temperature in the drying chamber slowly increases. A second temperature sensor can be fitted inside the drying cone to avoid an increase of the cone temperature above critical levels (which are strain/batch–specific and should be tested) at the end of the drying process. Typically, and depending on the dimensions of the FBD and the amount of cysts, the drying process should last 3–5 h.

Although they are cheaper, alternative drying methods, such as open air or indoor drying, are not recommended because the drying process is not fast, not homogeneous and too difficult to standardize, especially in areas with high and/or highly fluctuating relative humidity. Moreover, with poor mixing, small aggregates (lumps) of cysts may be formed, which in turn affect the overall quality of the final product.

Companies processing large quantities of cysts (harvested from large salt lakes or coastal salt ponds) use various industrial dryers (Plate 43), based on the same principle of keeping cysts suspended in a drying chamber.

After cyst drying, so-called air classification may be applied to separate remaining empty and cracked shells that were not removed during freshwater separation (or when freshwater separation was not done). This fraction can be carried out by releasing the dried cyst material in a horizontal air stream in which heavy particles tend to fall down faster than lighter particles. Cyst material blown through a horizontal air stream with several collecting vessels underneath will thus separate different fractions: These are, successively, heavy particles (remaining non-cyst material or cyst aggregates), full cysts, and finally empty shells, cracked shells and light non-cyst material. Additional pre-packaging steps may also involve the mixing of different cyst batches and/or screening (to remove the last remaining impurities from the cysts; Plate 44) to ensure a marketable product of constant quality, as cyst quality of harvested batches may vary depending on production season/year or on specific harvesting location.

Packaging step

Before canning, samples of the dry cysts are generally taken for quality control; packaging and labelling of the end product are generally done in accordance with the commercial requirements for different quality grades. Quality control of dried cysts involves determination of quality parameters (Plate 45), such as hatching percentage, hatching efficiency,

PLATE 43
Industrial *Artemia* cyst dryers

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PLATE 44
Set of screens for *Artemia* cysts

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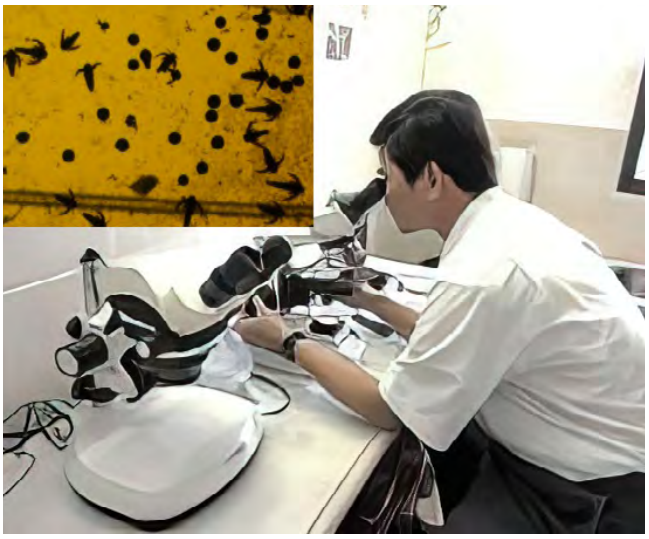
PLATE 45
Microscopic observation to measure hatching quality. Inset top left: Microscopic view of sample taken for determination of hatching quality



moisture content (%) and cyst diameter (µm) (see Section 3.2). Lipid composition, especially highly unsaturated fatty acid (HUFA) content, is also an important quality criterion. Additionally, depending on the customer (especially in relation to export markets), measurement of microbiological standards (i.e. bacterial/pathogen loads) may be required.

Cyst canning under vacuum or under nitrogen gas atmosphere allows storage for months or even years without major decrease in hatching. However, apart from being subject to the packaging conditions (air/vacuum/nitrogen), the shelf-life of dry cysts is usually also strain/batch specific. Immediately after drying, the cysts should be packed or in any case transferred to airtight containers or sealed polyethylene bags to prevent rehydration of the highly hygroscopic cysts; these cysts should also be kept in cool storage (below 10 °C) is recommended.

2.4.3. Harvesting and processing of biomass

2.4.3.1. Harvesting biomass in ponds

After 2–3 weeks of culture, *Artemia* (such as Vinh Chau *Artemia franciscana*) may reach the adult stage and start to reproduce, with the appearance of a new ovoviviparously produced generation; when the population becomes abundant enough, farmers will decide when to harvest. Different sizes of biomass, e.g. late juvenile, subadult and adult stages of *Artemia*, can be collected by using nets with an appropriate mesh size.

Adult *Artemia* biomass can be collected from large shallow ponds with conical nets mounted in front of a motorboat or pulled by farmers. In small ponds, dipnets are generally used (Plate 46). Alternatively, nets can be installed (temporarily) at the pond outlet and biomass is then collected automatically when the water flows (by pumping or gravity) to the next pond. In this case, nets should be large enough to facilitate harvesting: For example, for 100 kg of adult biomass, a filter mouth of 1 m by 2 m and a filter length of 3 m to 6 m should be used. A mesh of 100 µm should be fit to the end of the net where adults accumulate so as to prevent extrusion and hence damage the animals. The nets should be emptied every hour; *Artemia* biomass accumulating at the tip of the net is exposed to anaerobic conditions, which it can tolerate just temporarily. Because *Artemia* is rich in proteolytic enzymes, it is essential to harvest them alive.

In small ponds, the most popular method for biomass harvesting is to harvest partly by using a scoop net or driftnet to drag along the pond side or the areas where most *Artemia* accumulates. The total available biomass can be harvested (batch culture), or only part of it; the latter strategy corresponds with a continuous culture system. In practice, when harvesting biomass in the adult stage, a net with a mesh size of 1–2 mm should be used; if the biomass is juvenile or subadult, then a smaller mesh size (e.g. 500–750 µm) is needed. In Viet Nam, biomass harvesting is often conducted at noon time, as at higher temperature adult *Artemia* tend to accumulate near the water surface at the upwind side, which facilitates harvesting.

Experimental pond trials in Vinh Chau, Viet Nam, have shown that different harvesting frequencies applied over the production season lead to different amounts of total biomass harvested over the season (Anh, 2009). In the local conditions, maximal

PLATE 46

Biomass harvesting with a nylon net

Manually (a, b) or at the pond outlet (c, d); note paddle wheels (see arrow in d) and bamboo poles to help biomass concentrate in the harvesting net behind

biomass harvests over a 3-month culture period were thus achieved by following a strategy of harvesting once every 3 days (Table 18).

In Thailand *Artemia* farmers manage to produce biomass all year round and some reach very high yields (see Box 8).

TABLE 18

Total biomass (mean ± standard error; kilogram live biomass/ha) harvested over a three-month period using different harvesting frequencies

Harvesting cycle	1 day	3 days	6 days	9 days
Total biomass	1 323 ± 116	1 587 ± 128	1 091 ± 101	975 ± 112

Source: Anh, N.T.N. 2009. *Optimisation of Artemia biomass production in salt ponds in Vietnam and use as feed ingredient in local aquaculture*. Ghent University, Ghent, Belgium. PhD dissertation. ISBN 978-90-5989-308-5, 250 pp. https://biblio.ugent.be/publication/2064659

BOX 8

Artemia biomass soon to become a cost-effective alternative for fishmeal?

by *Patrick Sorgeloos*

Maybe the most successful *Artemia* biomass farm in the world, already in production for more than 15 years, is operated by the *Penaeus monodon* shrimp farmer Mr Banchong Nissagavanich in Chachoengsao Province, in Thailand, nearly 60 km east of Bangkok. Two ponds are in operation on a year-round basis, having a surface area of about 6 500 m² and a depth of 2 m, as to be able to drain the freshwater upon heavy rainfall during the rainy season: 110 g/L salinity at the start of the rainy season to still have 70–80 g/L at the end of the rainy season. Ami-ami, a waste product of monosodium glutamate production, and

BOX 8 (CONTINUED)

different wastes of plant and fruit processing, cow dung and even kitchen waste are used as organic fertilizers and added daily. Every other day, the pond bottom is raked with a heavy chain dragged by a boat over the pond bottom. Harvesting of the *Artemia* biomass that accumulates at the water surface in the morning hours, when low oxygen levels prevail, is done by pushing it into a harvesting net by a paddle wheel. Harvest yields range from 100 kg to 120 kg wet weight *Artemia* biomass per pond per day, or expressed in kilograms per hectare per month, approximately 4.5 tonnes. The local *Artemia franciscana* (San Francisco Bay strain was introduced in Thailand over 30 years ago) no longer produces cysts but is adapted to the local conditions.

Live biomass proves to be much more nutritious than frozen *Artemia* (different personal communications) and is used for feeding broodstock as well as for the nursery stages of different species of fish and crustaceans. Farming the *Artemia* at these high salinities apparently assures high biosecurity, biomass free from shrimp pathogens, as over all these years of feeding live biomass to his *P. monodon* broodstock Mr Banchong has never experienced a disease outbreak. In 2005, Kongkeo and Wilkinson had already reported this *Artemia* farm in their article *Recycling Water and Making Money*.

In his recent article *Microbially Derived Co-Products from Zero-Discharge Aquaculture*, Brune (2021) describes as a potential solution the use of filter-feeding aquatic organisms such as tilapia or brine shrimp in integrated aquaculture processes to harvest and convert waste microbial solids to higher value co products, as opposed to disposal of waste sludge. In super-intensive penaeid shrimp farming, brine shrimp co-culture offers potential to convert microbial solids to a fishmeal replacement product, at a potential value of about USD 1.5/kg dry weight.

Although the traditional use of *Artemia* biomass is as food source in aquaculture applications, there is an increased interest in considering *Artemia* as high-quality ingredient in human diets (see Box 9)

BOX 9

Brine shrimp *Artemia* as a direct human food

by *Nguyen Van Hoa* and *Patrick Sorgeloos*

In 1979, at the occasion of the First International Symposium on the Brine Shrimp *Artemia* in Corpus Christi, Texas, the United States of America, we published a short paragraph on "The potential use of *Artemia* as a protein source" (Sorgeloos, 1980):

> "*Besides an improved perspective for the use of Artemia in the aquaculture hatcheries, it becomes obvious that other applications show very high potential, even including direct use in human nutrition. Although the acceptability of brine shrimp as food for man might seem to be speculative or restricted to a few areas in the world, it is certainly worthwhile to be considered, not the least for developing countries. From an energetic point of view, brine shrimp production is a much more efficient way to produce animal protein than to culture carnivorous fish and crustaceans with Artemia and fishmeal as diet ingredients!*"

Direct consumption of brine shrimp by humans has been and continues to be practiced by indigenous tribes in the Americas and Africa: "… *Indians inhabiting this region used to collect large quantities of this crustacean which they dried and used as food*" (Jensen, 1918). The Dawada people of Libya consume(d) dried *Artemia* flakes as "… *a superb source of protein rich in* β*-carotene and riboflavin*" (Ghannudi and Tufail, 1978), and market(ed) these "*pains d'Artemia*" (*Artemia* bread) as a nutritious delicacy over a wide area (Oudney, 1828 in Bovili, 1968; Delga *et al.*, 1960; Monod, 1969). Taste-testing panels on *Artemia* conducted

BOX 9 (CONTINUED)

in Hawaii concluded that "… *the response to an experimental shrimp tempura prepared from frozen brine shrimp was quite favorable*" (Helfrich, 1973; Davidson, 1974). Although *Artemia* thrives in very high salinity waters, it is not too salty for human consumption because *Artemia* is a strong osmoregulator, maintaining its body fluids at a moderate brackish salinity.

We had to wait more than three decades to be able to announce the production and use of "*Artemia* omelet" as a new culinary item in the Mekong Delta of Viet Nam. Combined salt-cum-*Artemia* production has become a very lucrative business with major positive socioeconomic ramifications in the coastal area of Vinh Chau and Bac Lieu in the Mekong Delta. Over 500 families of salt farmers have improved their income with more than USD 5 000 per household per dry season with the production and sales of brine shrimp cysts (Hoa and Sorgeloos, 2015). Although the main interest has always been in cyst production, more recently *Artemia* farmers have started to harvest and market *Artemia* biomass for use in local aquaculture as nursery, grow-out and maturation feed for fish and shrimp, either live or frozen.

Similar *Artemia* projects are being set up in artisanal salt farms in different countries in Asia (e.g. Bangladesh, India, the Lao People's Democratic Republic and Myanmar). Although the main interest is to cater to the local aquaculture industry through local availability of high-quality *Artemia* cysts and biomass, there is also good potential to use *Artemia* biomass as a direct protein source for local community members, either under the form of the *Artemia* omelet as described here or as a partial replacement in local recipes for shrimp, crab or fish cakes.

"*Artemia* omelet/kebab" (b, c) produced from fresh biomass (a)

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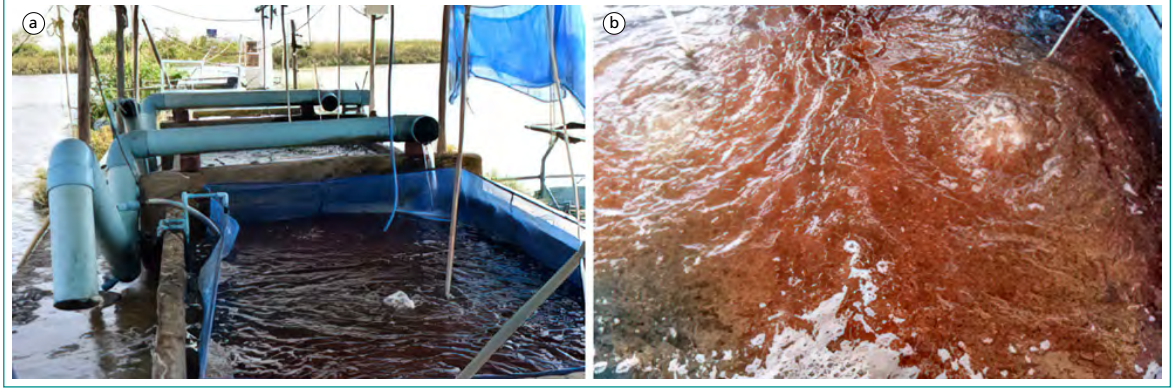
2.4.3.2. Biomass processing

Biomass harvested from ponds is usually mixed with other substances, such as debris and organic residues, so it needs to be cleaned/washed before it can be marketed. This can be done in a recirculation system with a protein skimmer (Plate 47) or by thorough

PLATE 47

Use of a recirculation system and protein skimmer (a) to clean the biomass (b)



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washing over 1 mm mesh nets with clean seawater. In the latter case, sufficient water and aeration should be provided, and biomass should always remain submerged to prevent physical damage to the animals (Plate 48).

PLATE 48
Storage of biomass in nets

Once cleaned from impurities, the biomass can be stored alive in nets or tanks at the same or lower salinity as in the production ponds, or further treated for different applications. The following storage methods are to be used, according to the needs:

- Short-term storage of biomass, to be subsequently used as live food or for freezing/drying (>90 percent survival).

  For this purpose, the rinsed biomass is transferred to containers with seawater and mixed with ice to cool to 5–10 °C under strong aeration. If the biomass is to be used or further processed within 1–3 h, a density maximum of 500 g wet weight (WW) biomass per litre of seawater can be maintained. For longer storage (up to maximally 12 h), the density should be lowered to a maximum of 300 g WW/L.

- Live transport for marketing as a live product (>90 percent survival after 24 h).

  In this case, a similar technique is to be used as for transport of live fish/shrimp larvae:
  – 10 L plastic bags are filled with 2–3 L of seawater.
  – *Artemia* is added at a density of 100 g WW/L.
  – The bag is inflated with compressed air or oxygen (depending on the duration of transport) and closed with a rubber band.
  – The filled bags are packed in Styrofoam boxes filled with ice.

PLATE 49
Transport of frozen *Artemia* biomass (Thailand)

In addition to live use, *Artemia* biomass can also be frozen (Plate 49 and Plate 50) for subsequent use as a food source in fish/shrimp hatcheries or for the pet market. Alternatively, biomass can be dried in thin layers and used as an ingredient for larval feeds (flakes or particulate diets). Because *Artemia* is rich in proteolytic enzymes, it is essential to

process the biomass alive. Therefore, the following considerations are important (see also Section 2.3.2):

- Freezing should be done as fast as possible (in thin layers of < 5 mm, down to -25 °C or -30 °C), as slow freezing will result in proteolytic activity and leaching of essential nutrients when used subsequently.
- When drying, the best quality biomass meal is obtained with freeze drying or spray drying. Acceptable quality can be obtained with drum drying (flaking) or oven drying. If the drying process is too slow (e.g. sun drying), excessive oxidation occurs (black colouring) and proteolytic activity will result in product losses.

Quality requirements may depend on the market; in Viet Nam, live biomass usually does not require any quality control measurements. However, for trading frozen biomass, information on biochemical and microbiological composition is generally required.

PLATE 50
Frozen *Artemia* biomass (Thailand)

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3. Use of cysts, nauplii and metanauplii

by *Gilbert Van Stappen, Geert Rombaut* and *Patrick Sorgeloos*

Artemia cysts are offered on the market in a number of brands, corresponding with a variety of quality criteria, among which hatching quality, cyst size (and hence naupliar biomass) and nutritional composition (especially related to highly unsaturated fatty acids) are among the most important. Practical aspects related to their daily use in the hatchery (for example, ease of nauplii harvesting) and microbiological aspects determine the quality of the product.

After the cysts are hatched, *Artemia* larvae, in general, are fed to (shell)fish larvae either in the freshly hatched instar I nauplius stage or, following an enrichment procedure to enhance their nutritional value, as metanauplii in the instar II stage (or subsequent instar stages, depending on the enrichment methodology). The choice to use freshly hatched versus enriched *Artemia* depends on the context of application, i.e. the type of *Artemia* used versus the species and stage of (shell) fish larvae to which it is fed. *Artemia* may also be offered as live food in the form of decapsulated cysts (cysts from which the outer cyst shell has been removed by a chemical process) or as umbrellae, but as compared to the widely spread use of (meta) nauplii, the use of these stages is restricted to specific applications.

Many techniques for proper use of *Artemia* have been worked out in the past and are described in this chapter. Still, research and development efforts continue to consolidate, expand and diversify our knowledge on how *Artemia* can be applied in aquaculture with maximal efficiency and convenience for the *Artemia* user. This chapter also describes these more recent insights and practical developments to automate and rationalize the daily procedures related to the use of cysts, nauplii and metanauplii.

3.1. OPTIMAL CONDITIONS AND EQUIPMENT FOR THE CORRECT USE OF CYST PRODUCTS IN HATCHERIES

3.1.1. Biosecurity

As explained below, "biosecurity" generally refers to the whole set of procedures and measures to minimize (or even rule out) the entrance and spreading of infectious pathogens into a population. As such, along with the development of aquaculture of the past decades, biosecurity has acquired enormous importance over the entire aquaculture production chain, but especially in the hatchery phase.

Handling and applying *Artemia* cysts, nauplii and metanauplii, which is the focus of Chapter 3, is only one aspect of all activities ongoing within a fish or shellfish hatchery. From a biosecurity perspective, it should be emphasized that proper procedures for *Artemia* use must be considered within the broader framework of proper hatchery procedures as a whole. In other words: Applying biosecure *Artemia* procedures does not reach their ultimate goal of contributing to hatchery biosecurity if these are not part of biosecure hatchery procedures in general. Consequently, Section 3.1.1 addresses principles and measures of biosecurity within overall hatchery organization and operation.

3.1.1.1. The biosecurity concept

Biosecurity is an important strategy to minimize the risk of occurrence of stressors and promote sustainable aquaculture development. It refers to the measures and methods adopted to secure a disease-free environment in all phases of aquaculture practices

(i.e. hatcheries, nurseries, grow-out farms) by reducing the risk of introduction, establishment and spread of pathogenic agents from and within an operational aquaculture unit. The importance of the implementation of biosecurity in commercial hatcheries is a prerequisite for harvesting high-quality larvae and to ensure a sustainable and profitable growth of the shrimp and fish industry (FAO, 2003, 2007). This requires that people adopt a set of attitudes and behaviours in their daily practices. As the production of live food is one of the critical steps in the production of fish and shellfish larvae, a good follow-up of the standard operating procedures (SOPs), supported by an adequate amount of scientific knowledge, is one of the driving forces for good manufacturing practices and management.

3.1.1.2. Biosecurity programme and plan

Biosecurity involves strict management protocols to prevent specific pathogens from entering the system. The basic elements of a good biosecurity plan are to:

- identify hazards by understanding disease transmission and risk factors;
- assess and prevent risk factors;
- determine appropriate biosecurity measures to be undertaken on priority basis; and
- audit the implementation of the biosecurity plan.

The biosecurity measures determined in the biosecurity programme and plan must be an integral part of the SOPs. They must be constantly reviewed, updated and adapted to any new conditions. A document containing the SOPs should be available to each staff member and in the different operating areas. The staff should be trained, challenged and audited on the importance of these procedures.

3.1.1.3. Basic principles of biosecurity

Segregation

Segregation is the foremost important element of biosecurity. It involves keeping potentially infected animals and materials away from uninfected animals. If a pathogen does not enter a holding area, no infection can take place. Segregation measures include controlling the entry of animals, people and equipment from outside the hatchery; fencing the hatchery area and department of production and controlling access for people and pets; providing footwear and clothing to be worn only in the hatchery; and implementing an all-in-all-out management system.

Cleaning

Cleaning is the second most effective step in biosecurity, i.e. removing all foreign materials and organic matter from the aquaculture system (followed by disinfection to remove all pathogenic microorganisms if any; see further). Any materials that must pass through the segregation barrier (in either direction) should be thoroughly cleaned. There should be no visible dirt on the surface of the materials. Soap, water and a brush are adequate for small objects, but a high-pressure washer is needed for large surfaces or vehicles.

Disinfection

Disinfection is the application, after thorough cleaning, of procedures intended to destroy the pathogenic or parasitic agents, including zoonoses. This applies to premises, buildings, floors, vehicles and different objects that may have been directly or indirectly contaminated. The effectiveness of disinfection depends on the contact time and concentration of the disinfectant.

The recommended sanitation and disinfection protocol to prevent or reduce the pathogen load in the system and hatching tanks includes the following steps:

- Proper cleaning protocol of tanks and hatching room:
  - Remove all visible debris and dirt.
  - Use soap and water to wash, and rinse and dry.
  - Apply a disinfectant solution (at proper concentration).
  - Neutralize any chlorine residues with sodium thiosulphate ($Na_2S_2O_3$).
  - Rinse.

- Overall system maintenance and cleanliness:
  - Backwash and treatment of filters as needed to reduce organic loading in hatcheries.
  - Wash and disinfect air and water pumps/lines in hatcheries.
  - Flush sediment out of water lines as needed and disinfect them.
  - Maintain proper sanitation-disinfection strategies.
  - Keep nets and other equipment off the floor to control contamination and keep them sterile.
  - Pull out dead and moribund culture organisms as soon as possible and dispose appropriately.
  - Avoid cross-contamination of equipment or water from one system to another.
  - Use disinfectants for equipment, including nets and footbaths (placed at strategic locations around the facility, e.g. at the entrance and exit of quarantine buildings, hatcheries, farms and other systems).

Proper attention to *Artemia* products: Ensure proper storage (in a cool, dry location) and usage (follow manufacturer recommendations/expiry date) of *Artemia* cysts to prevent loss and contamination by microorganisms, pathogens or toxins.

3.1.1.4. Biosecurity measures

Water treatment

The choice of the water source and its treatment are of utmost importance as water is one of the points of entry of pathogenic agents in the hatchery.

The access to the right source of water with an optimal water quality is highly determined by the site:

- Seawater taken from the ocean typically has 34–36 g/L salinity and has an overall stable chemical composition and quality. The microbial quality greatly depends on the location of the hatchery and the season.
- Brine water is a high-concentration solution of salt in water. In diverse contexts, brine may refer to the salt solutions ranging from about 3.5 percent or 35 g/L (a typical concentration of seawater, on the lower end of that of solutions used for brining foods) up to about 26 percent or 260 g/L (a typical near-saturated solution, depending on temperature). For hatchery purposes, the concentration of the brine solution should be between 90 g/L and 120 g/L. The correct salinity is subsequently obtained by adjusting the salinity of the brine to 28–30 g/L by diluting it with freshwater. This diluted brine water may be used as a water source by hatcheries that are located far from the sea.
- Deep wellwater is obtained from wells of 40–60 m in depth. This type of water contains a high concentration of iron (Fe) and manganese (Mn), which must be oxidized and then removed through mechanical filtration. It generally has a constant temperature and is considered to meet high microbial quality standards.

In Table 19, the preferred range for the chemical and physical characteristics of typical hatching water is given. Making sure that your water is within this range for each of the parameters is a key step in ensuring and maintaining good performance of the hatchery.

TABLE 19
Recommended water parameter values for water used in a hatchery

Water quality parameter	Unit	Range
Salinity	g/L	10–35
pH		7.5–8.5
Dissolved oxygen	mg/L	>4
Alkalinity	mg/L	>120
Total hardness (as $CaCO_3$)	mg/L	>150
Calcium hardness (as $CaCO_3$)	mg/L	>100
Magnesium hardness (as $CaCO_3$)	mg/L	>50
Total ammonia nitrogen	mg/L	<1.0
Nitrite (NO_2^-)	mg/L	<1
Nitrate (NO_3^-)	mg/L	<60
Hydrogen sulphide (H_2S)	mg/L	<0.002
Total dissolved solids	mg/L	10–50
Total suspended solids	mg/L	10–50
Heavy metals	mg/L	<0.01
Pesticides	mg/L	Undetectable by gas liquid chromatography

Sources:
Venkateswarlu, V., Seshaiah, P.V., Arun, P. & Behra, P.C. 2019. A study on water quality parameters in shrimp L. vannamei semi-intensive grow out culture farms in coastal districts of Andhra Pradesh, India. *International Journal of Fisheries and Aquatic Studies*, 7.4: 394–399. https://www.fisheriesjournal.com/archives/2019/vol7issue4/PartF/7-4-64-509.pdf

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Regular monitoring of water quality can identify possible problems before they cause suboptimal hatching, growth reductions or mortalities in the larvae. Therefore, it is recommended to measure the water quality parameters on a frequent basis (Table 20), which will allow to analyse problematic incidents and to correct and adjust when needed.

TABLE 20
Recommended frequency of monitoring water parameters

Water quality parameter	Minimum frequency
Temperature	4 times per day
Dissolved oxygen	4 times per day
Salinity	1 time per day
pH	2 times per day
Alkalinity	1 time per day
Total ammonia nitrogen, NO_2^-, NO_3^-	1 time per day
Settleable solids	1 time per day

Note: Total ammonia nitrogen = NH_3 and NH_4^+; NO_2^- = Nitrite; NO_3^- = Nitrate

Sources: Global Seafood Alliance. 2022. *Hatchery Standard 2.0. Best Aquaculture Practices Certification Standard*. 76 pp.

Electronic meters and probes tend to give the most accurate results, but waterproof types tend to be expensive and, unless they are used properly and calibrated frequently, they can give serious errors. For some parameters such as ammonia, suitable probes may be prohibitively expensive. However, if taken care of and used properly, this equipment can be cheaper in the long run than test kits, and also promote more frequent measurements because no reagents are used up.

The alternative to meters are test kits made by a variety of manufacturers and for many different water parameters. While these are less accurate owing to human objectivity in deciding on colours or shades, they are a useful tool in the hatchery and avoid the initial purchasing cost of meters and the cost of repairs or damage. Although apparently simple, these kits often depend on quite complex chemical reactions to develop the colours, and it is vital that the appropriate kits are chosen in the correct range of measurements and that they are used properly. Some are not suitable for seawater; some measure in different ranges, and when measuring ammonia (or ammonia nitrogen) for example, it is essential to understand exactly what is being measured and how it affects the *Artemia* hatching process, *Artemia* quality and larvae.

NOTE: It is highly recommended to calibrate all measuring devices again at the beginning of the day to prevent errors, which may result in misinterpretations and loss of production.

The recommended water treatment to prevent or reduce the pathogen load in an aquaculture system should include the following steps and components.

Steps of a typical water treatment:

- Intake of the water.
- Mechanical filtration of the seawater via sand filtration to remove particles > 50 µm.
- Storage of the filtered water in a settling reservoir. In the settling tank, 0.5–2 mg/L of potassium permanganate ($KMnO_4$) can be added to aid settlement and disinfection.
- Oxidation of dissolved organic matter and heavy metals in oxidizer reservoir.
- Subsequent removal of dissolved organic matter in a protein skimmer using ozone to improve oxidation. The recommended oxidation reduction potential for optimal performance is 550–600 mV.
- Transfer of the pretreated water to a second reservoir and removal of particles via mechanical filtration (>1 µm).
- Conditioning and improvement of water parameters (alkalinity, minerals, pH, etc.).
- Application of ethylenediaminetetraacetic acid (EDTA) to remove dissolved metals (5–10 mg/L).
- Application of strong aeration.
- Transfer of the water to the production areas via a water treatment system equipped with a UV system, a filter with active coal, and cartridge filters to remove particles >0.5–1 µm or an ultrafiltration system to remove particles >0.03 µm.
- Adjustment of temperature when needed.

Components of the water treatment system (Table 21; Plate 51):

TABLE 21
Components of a water treatment system

Item	Purpose
Seawater pump pit	Water suction
Sub sand pipe	Water intake
Settling reservoir	Settling of dirt and debris and organic material
Oxidizer reservoir	Oxidation of organic matter
Protein skimmer	Removal of dissolved organic matter
Ozone generator	Oxidation of organic matter and disinfection of water
Reservoir to disinfect the water	Chlorine or ozone disinfection
Reservoir to condition the water	Adjustment of alkalinity, minerals and application of EDTA
Mechanical filter (pressurized sand filter)	Removing particles >50 µm
Mechanical filter (cartridge filter)	Removing particles >1 µm
Mechanical filter (ultrafiltration)	Removing particles >0.03 µm
UV treatment	Sterilization
Blower	Aeration and removing ozone (O_3)
Heat exchanger	Adjustment of temperature
Chiller	Adjustment of temperature

Source: Authors' own elaboration (INVE, Belgium).

PLATE 51
Components of a water treatment system
(a) mechanical ultrafiltration system (0.03 µm); (b) venturi injection of ozone; and (c) protein skimmer

The effectiveness of the water treatment system must be validated by microbiological and chemical tests.

Disinfection of water and air distribution system
The disinfection of the air and water distribution systems is highly recommended and should be planned on a frequent basis. Chemicals that are used as disinfectant for PVC pipe systems are (FAO, 2003):

- Sanocare PUR®, VIRKON®: Prepare a 1 percent Sanocare® PUR solution for disinfection of walls and tank bottom. Around 300 ml of Sanocare® PUR solution is required per m² surface.
- Sodium hypochlorite (NaOCl): 20–30 mg active chlorine/L.
- Calcium hypochlorite ($Ca(OCl)_2$): 20–30 mg active chlorine/L.
- Muriatic acid (37 percent) (hydrochloric acid (HCl), aqueous): 10 percent solution.

A recommended treatment to prevent microbial growth in seawater and air distribution systems includes the following steps:

- Determine the total volume of water that is contained in the seawater distribution system.
- Fill the tank or reservoir with the required amount of seawater.
- Dissolve the recommended dose of the selected disinfectant and mix.
- Remove the remaining water from the water distribution system and replace with the disinfectant solution.
- Check the system and open and close all valves to ensure that the disinfectant is everywhere present in the water distribution system.
- Leave the disinfectant for a minimum of 2 h in the water distribution system.
- Remove the disinfectant solution from the water distribution system.
- Rinse with normal filtered seawater for 20 minutes.
- Check the presence of remaining and residual disinfectant.

NOTE: During production, the process needs to be coordinated to avoid that the disinfectant is transferred to the production tanks.

Water and tank disinfection during production
Complementary to the primary water treatment, the use of a disinfectant to disinfect the water prior to its use in the tank itself is recommended; this process will kill all residual microorganisms in and on the surfaces of the tank.

- Disinfect by immersion in a chlorine solution (150 mg active chlorine/L).
- Clean the tank with soap and rinse with clean and disinfected water.
- Clean the tank with a disinfectant (e.g. hypochlorite solution).
- Rinse again extensively with clean and disinfected water.
- Let the tank dry out.

- Validate the cleaning and disinfection procedure via plating on thiosulphate–citrate–bile salts–sucrose (TCBS) agar (see Section 3.2.4.1). Fill the tank with filtered and disinfected seawater. Make sure that all organic material (for example, in *Artemia* hatching, the cysts and cyst shells) is removed, such as the material remaining in the outlet and in the valves of the tank.
- Disinfect the hatching water with, for example, 10 mg active chlorine/L and aerate gently for ±1 h.
- Deactivate any remaining chlorine by adding 8 mg/L sodium thiosulphate ($Na_2S_2O_3$).

Facilities

The production area of the hatchery should be kept clean and disinfected. All equipment in use must be removed, cleaned, and dried and disinfected before proper storage for later use.

From a biosecurity perspective, it is crucial that the *Artemia* cyst hatching room(s) is/are clearly spatially separated from the other rooms and facilities of the (shell) fish hatchery. Cleaning and disinfection of the hatching room should be done as follows:

- Remove dust and waste present on the floor, and in the tanks and gutters.
- Clean and disinfect the floor and the gutters according to the hatchery SOPs.
- Thoroughly clean all equipment, such as sampling tubs, trays and nets. Rinse with clean water and then disinfect. Do not share equipment between facilities and tanks unless necessary. ln these cases, disinfect all equipment prior to being reused.
- Allow the facilities to dry.

Fences or walls should surround the hatchery site to prevent the entry of animals and people who could be potential disease carriers.

Restriction of movement of personnel

Personnel will carry a variety of bacteria on their clothes, boots and skin. It is essential to reduce the transfer of these bacteria from one area to another and from one hatchery to another by the visits of local colleagues and other persons. It must be strictly forbidden to enter the production area without the authorized protective clothing. As part of biosecurity procedures, visitor policies should be implemented to restrict the movement of visitors in the different areas of production of the hatchery.

It is important to manage the personnel of the hatchery so that transfer of people between areas is limited as much as possible. Each area must be separated by footbaths, and the use of rubber boots in the hatchery should be compulsory. Each area should also have a handwash facility with a commercial disinfectant that is renewed regularly. It should be enforced that personnel use these facilities and do not step over footbaths or fail to wash their hands when entering the hatchery or moving from one area to another.

Separate boots and overalls should be provided for the broodstock buildings; in large hatcheries shirts or overalls of different colours (a colour code) can be provided to identify staff of specific areas and to help reduce the transfer of personnel outside their area of work.

If a practical demonstration is required to convince personnel of the importance of biosecurity, take a sterile piece of cotton wool and wipe it over the sole of a rubber boot and then wipe it onto the surface of an agar bacteria culture plate. Alternatively, press the hand of a hatchery worker onto the surface of an agar plate. Incubate the plates for 48 h at 28 °C. There should be a substantial number of colonies present, demonstrating the contamination of boots and hands.

Hands and boot disinfection

For the same reason as expressed above, all staff should wash their hands before starting work, after breaks, and when changing work activities. All visitors to the site should be required to wash their hands before entering any of the production units. To enable this, soap, a disinfectant and a sink must be available next to the entrance door of each production unit.

Wastewater discharge management

The discharge of wastewater from the different production areas can carry considerable amounts of organic matter, faeces, moults, feed residuals, algae, bacteria, etc., that must be managed properly to avoid cross-contamination (facilities, and intake of seawater).

The recommended way to discharge wastewater includes the following steps:

- The wastewater discharge must be pumped into the main drain that transfers the water to different wastewater reservoirs.
- The oxidation pond is divided in four compartments allowing for rotation and weekly use of another compartment.
- Before discharge into a compartment, the water is filtered to collect live organisms and to avoid the introduction of these organisms into the pond.
- When the maximum volume of the compartment is reached, calcium hypochlorite $Ca(OCl)_2$ is dosed at 100 mg/L.
- The wastewater is drained when no residual chlorine is detected.

Standard operating procedures

A document containing the standard operating procedures (SOPs) for each procedure in the hatchery should be drawn up, given to each member of staff and be readily available in a communal staff area. Staff should be trained in the importance of following the procedures with emphasis on the critical points; staff should also sign a document saying that they have read and understood the procedures.

3.1.2. Cyst storage

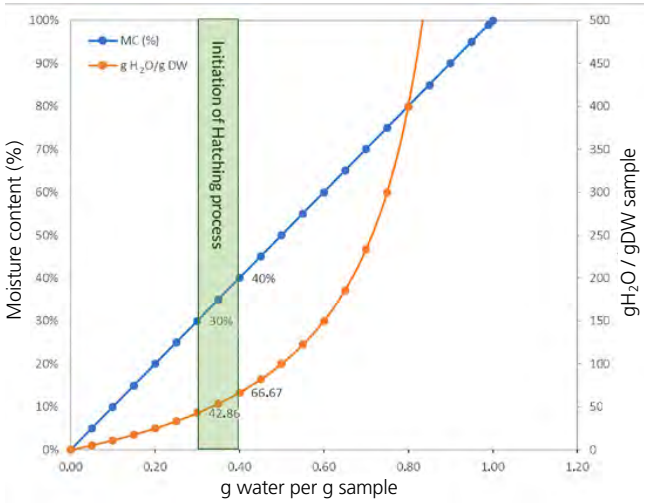
The dry encysted embryos of *Artemia* (*Artemia* cysts) are very resistant to severe stress and appear to tolerate high doses of UV and ionizing radiation (Tanguay, Reyes and Clegg, 2004), surviving years of continuous anoxia while hydrated at physiological temperature, also surviving thermal extremes and desiccation–hydration cycles (reviewed by Clegg and Conte, 1980; MacRae, Bagshaw and Warner, 1989; Warner, MacRae and Bagshaw, 1989; Liang and MacRae,1999; Clegg, 2001; Clegg and Trotman, 2002). Different biochemical adaptation mechanisms present in the encysted embryo (presence of trehalose, large guanine nucleotide pool, small heat shock proteins [p26, artemin] and late embryogenis abundant proteins) are critical to the stress tolerance of the cysts. The chorion, the tough shell of the cysts, plays an important role in protecting the embryo from mechanical damage and UV radiation (Clegg, 2005; Liu *et al*., 2009; Hibshman, Clegg and Goldstein, 2020).

Hydrated cysts are far more sensitive towards temperature and handling stress than the dried product. The viability of the hydrated product is already affected below -18 °C and above +40 °C; a reversible interruption of the metabolism (= viability not affected) occurs 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. Water levels in the range of 30–65 percent initiate metabolic activities, eventually reducing the energy contents down to levels insufficient to reach the state of emergence even under optimal hatching conditions (Figure 41). The water content of cysts can easily be determined by a standard procedure (see Annex 9). A depletion of the energy reserves is furthermore attained when the cysts undergo successive dehydration/hydration cycles. Long–term storage of such material may result in a substantial decrease of the hatching outcome.

Cysts exposed to water levels exceeding 65 percent for too long a period will have completed their pre emergence embryonic development; subsequent dehydration of these cysts will, in the worst case, result in mortality of the differentiated embryos.

As a result of the metabolic characteristics of hydrated cysts, a number of recommendations can be formulated with regard to their storage. When cysts (both decapsulated and non-decapsulated) are stored for a long time, some precautions must 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 the effects of dehydration or combined dehydration/hydration. For diapausing cysts (see Section 1.5.4), 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.

FIGURE 41
Relationship between moisture content (MC) and g water per g dry cysts
The green bar indicates the range in which the hatching process is initiated when dry cysts are hydrated (i.e. ± 30–40 percent moisture content or ± 42.86–66.67 g H_2O per 100 g dry cysts)



Source: Clegg, J.S., Zettlemoyer, A.C. & Hsing, H.H. 1978. On the residual water content of dried but viable cells. Experientia, 34: 734–735. https://doi.org/10.1007/BF01947290

Although diapausing and quiescent cysts contain what seems to be a complete set of components needed for desiccation and temperature tolerance, they are thus still sensitive for specific processing and storage conditions. Hatching quality in stored cysts is slowly decreasing when cysts contain water levels from 10 percent to 35 percent water (H_2O). This process may, however, be retarded when the cysts are stored frozen or cooled, i.e. at maximally +4 °C. Even properly packed dehydrated cysts only keep their viability when stored in a dry place and at maximally +4 °C, as higher temperatures can reduce the quality of the product. When frozen, the cysts should be acclimated for one week at room temperature before hatching. The exact optimal water level within the cyst is not known, although it has been stated that a too severe dehydration (down to 1–2 percent) results in a drop in viability. In this case, the viability may not only be affected by the low water level within the cysts, but might also be affected by the drying method applied on the cysts. It is important that during the drying process (see Section 2.4.2.4) the rate of dehydration is well managed, as rapid water loss can significantly reduce the quality and the hatching potential of the cysts. In fact, in the case of *Artemia* cysts, it seems that there is no lower limit to desiccation on condition that proper methods are used that do not involve very high (damaging) temperatures. For example, treatments designed to remove all cyst water (based on the Burnauer–Emmett–Teller gas bombardment technique) had no effect on viability: In that case, virtually all water was removed, and all the embryos survived (Clegg, Zettlemoyer and Hsing, 1978).

3.1.3. Cyst disinfection

A major problem in the early rearing of marine fish and shrimp is the susceptibility of the larvae to microbial infections. Dry *Artemia* cysts have a very low number of associated bacteria, but the water in which cysts hatch is rapidly colonized by bacterial species. Glycerol is released at hydration of the cysts (see Section 3.2.3.4) and offers an

ideal culture medium for bacteria, such as *Vibrio* species. Most *Vibrio* are opportunistic bacteria, which can cause disease and mortality outbreaks in larval rearing, especially when animals are stressed or not reared under optimal conditions.

Unless commercial cysts are used with a pathogen-free certificate, it is recommended to apply a routine cyst disinfection procedure. For this purpose, although other products are sometimes used (e.g. iodine or formaldehyde), it is proposed to use hypochlorite (bleach), which is easily and widely commercially available, either in crystalline form or in liquid solution (see Annex 3); it is widely used in aquaculture because of its spectrum of activity and cost-effective pricing. Drawbacks of bleach, however, include its caustic nature in concentrated liquid and gas forms as well as its decreasing effectiveness in sunlight. Moreover, hypochlorite treatment will not kill all germs present in the alveolar and cortical layer of the outer shell. Therefore, disinfection of cysts is an issue that must be considered within the wider context of biosecurity measures in *Artemia* cyst hatching and in live food procedures in the hatchery in general (see Section 3.1.1) Complete sterilization can be achieved through cyst decapsulation, as described in Section 3.1.4.

3.1.4. Cyst decapsulation

3.1.4.1. Procedure and general considerations

The hard shell that encysts the dormant *Artemia* embryo can be completely removed by short-term exposure to a hypochlorite solution (bleach), a process leaving the outer cuticular membrane surrounding the unhatched brine shrimp embryo intact. This procedure is called decapsulation. The decapsulation procedure involves successively the hydration of the cysts (as complete removal of the shell can only be performed when the cysts are spherical), removal of the outer shell in a hypochlorite solution, and washing and deactivation with the remaining hypochlorite. As the chemical decapsulation reaction is fastest at higher pH, generally sodium hydroxide (NaOH) is added (Sorgeloos *et al.*, 1977).

The chemical reaction produced by the hypochlorite is exothermic, meaning that heat is produced, and especially at higher ambient conditions (e.g. in tropical countries) this can lead to the temperature of the decapsulation solution increasing up to above 35 °C, which provokes damage to the hydrated embryo. Consequently, measures must be taken to lower the temperature of the solution (see Annex 4). Commercial bleach solutions generally indicate the concentration of active chlorine (which is needed to calculate the amount of bleach needed), but a titrimetric method also can be used for the determination of active chlorine in hypochlorite solutions (see Annex 5).

Once widely applied because decapsulated cysts offer some advantages compared to the non decapsulated ones, the use of decapsulation as a practical hatchery routine has nowadays to be discommended for the following reasons:

- The use of hypochlorite is hazardous for workers (as chlorine gas is being released), especially at big scale.
- The effluents of the decapsulation process contain toxic compounds such as hypochlorite, which should not be drained into the environment (the reason that increasing restrictions are imposed by authorities on the discharge of effluents).

Furthermore, the technique also has some additional practical disadvantages:

- Sufficient expertise is needed in the correct application of this technique to avoid prolonged exposure of the decapsulated embryo to the toxic hypochlorite, especially when applying decapsulation at larger scale.
- Application modalities may need slight amendment when using various *Artemia* strains, which may differ in the characteristics of their chorion.
- The procedure is time-consuming.

Decapsulation was mainly used (and may still be used in specific conditions, e.g. within a research and development context) for the following practical and nutritional reasons (relative to feeding instar I nauplii or a microdiet):

- When hatching "normal" (= non-decapsulated) cysts, the complete separation of *Artemia* nauplii from their shells is not always possible. Unhatched cysts and empty shells, introduced into the larval tanks, can cause deleterious effects when they are ingested by the predator: They cannot be digested and may obstruct the gut (Herald and Rackowicz, 1951; Morris, 1956; Rosenthal, 1969; J.E. Shelboume, cited by Provasoli, 1969; Stults, 1974). This may result in a higher mortality rate. This problem is more likely to occur for certain larval aquaculture organisms (e.g. larval fish) than for others (e.g. larval crustaceans) because of their specific feeding behaviour.
- Nauplii that are hatched out of decapsulated cysts have a higher energy content and individual weight (30–55 percent depending on strain) than regular instar I nauplii because they need to spend less energy for the hatching process. In some cases where cysts have a relatively low energy content, the hatchability might thus be improved by decapsulation because of the lower energy requirement to break out of a decapsulated cyst (Vanhaecke, Lavens and Sorgeloos, 1983),
- Decapsulation results in an initial disinfection of the cyst material; this effect is, however, transient since *Vibrio* may still proliferate in the hatching tank as glycerol is being released (see Section 3.2.3.4).
- For those applications where direct use of decapsulated cysts as live food is possible (see below), their use eliminates the need for hatching facilities and for the daily labour-intensive production of nauplii.
- For the development of the bacteria-free gnotobiotic *Artemia* rearing system (see Box 10)

BOX 10

The gnotobiotic *Artemia* toolbox

Artemia cysts are characterized by the presence of a chorion or cyst shell. As described in Section 3.1.4, this cyst shell may be removed by a decapsulation procedure that involves the use of a strong oxidant such as hypochlorite. During the decapsulation procedure, cysts also get sterilized. Hence, if the decapsulation is performed in a sterile environment and if that sterile environment is maintained during the subsequent hatching phase (for example, by applying filter-sterilized air; sterile hatching medium), axenic nauplii can be obtained: Such nauplii are free of bacteria. These axenic nauplii can subsequently be used to study, in a very rigorous way, how microorganisms interact with these nauplii. It is possible to set up an experimental environment in which the nauplii interact with one or several known microbial species, which are purposely added to the axenic environment. In this way, a so-called "gnotobiotic" experimental environment is built, in which every living creature in the experiment is known. This Gnotobiotic ARTemia rearing system is sometimes, for convenience, referred to as GART.

GART can be used for many different applications. For instance, by measuring the growth rate of *Artemia* in mono-association with a particular microbial food item (bacteria species or strain, or axenic algae), the nutritional value of these items can be assessed. Axenic *Artemia* can also be brought into contact with pathogenic *Vibrio* strains, allowing to verify how virulent these strains are when they are in mono association with *Artemia*. From some *Vibrio* strains, mutants are available, incapable of performing certain functions. These strains allow to verify in vivo how important that particular function is for in vivo virulence, for example. The GART system also allows to verify if certain probiotic strains are active in vivo or if certain compounds such as glucans (immunostimulants) can protect *Artemia* against virulent *Vibrio*. In addition, as the *Artemia* genome is now known (see Box 1), the GART system allows to verify in detail how *Artemia* reacts to a certain treatment in a very rigorously controlled environment. Observations made with the GART system can accelerate scientific discovery and can be the basis for the set-up of validation experiments with, for instance, larval stages of aquaculture shrimp species.

Decapsulated cysts can be directly hatched into nauplii or fed as such, depending on the feeding behaviour and digestion capability of the predator concerned. 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 a few months), the decapsulated cysts can be dehydrated in saturated brine and stored for later hatching or for direct feeding. During overnight dehydration (with aeration to maintain a homogenous suspension), cysts usually release over 80 percent of their cellular water, and upon interruption of the aeration, the now coffee-bean shaped decapsulated cysts settle out. After harvesting of these cysts with 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.

As the need to obtain a maximal separation of nauplii from unhatched cysts and empty cyst shells, especially in fish larviculture, used to be one of the main drivers to apply decapsulation, alternative procedures and technologies have been developed, allowing for easier or even nearly complete physical separation of the nauplii from these other fractions without the use of decapsulation (see Section 3.1.6).

3.1.4.2. Direct use of decapsulated cysts

Decapsulated or "shell-free" *Artemia* cysts can be used as a direct energy-rich food source for fish and shrimp, for those fish and crustacean larvae that can ingest and digest and that accept this non swimming type of live food as a potential prey. The cysts have the appearance and the practical advantages of a dry feed. The embryo of a decapsulated cyst has a smaller size (200–250 µm) than freshly hatched *Artemia* (450–500 µm), which is more suitable for predators with a small mouth size. If they have been dried before application, decapsulated cysts have a high floating capacity in seawater and sink only slowly to the bottom of the culture tank. Leaching of nutritional components does not occur (in contrast with artificial diets) since the outer cuticular membrane acts as a barrier for larger molecules.

Nevertheless, the direct use of decapsulated cysts is much more limited in larviculture of fish and shrimp compared to the use of *Artemia* nauplii. Dried decapsulated *Artemia* cysts have proven to be an appropriate feed for larval rearing of various species, such as freshwater catfish (Clarias gariepinus) (Verreth, Storch and Segner, 1987; Bardócz *et al*., 1999) and carp (Cyprinus carpio) (Vanhaecke *et al*., 1990; Sui, L., 2000); marine penaeid shrimp (Stael *et al*.,1995; Ribeiro, F.A.L.T. and Jones, 1998); milkfish (*Chanos chanos*) (De Los Santos *et al*., 1980); and a wide variety of tropical aquarium fish (Lim *et al*., 2002). In addition, decapsulated cysts are also used as a food source in the mass culture of predating bugs and beetles that are used as biological control agents against crop pests (Vandekerkhove *et al.*, 2009); their use for this purpose often proves to be more cost-efficient than more conventional diets, such as the eggs of the flour moth (Ephestia kuehniella). From a nutritional point of view, the gross chemical composition of decapsulated cysts is comparable to freshly hatched nauplii. 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, when 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, predators that are mainly or entirely bottom feeders (such as older penaeid larvae) do not encounter this problem. Finally, these cysts may be more difficult to digest by some predators (e.g. larvae of mud crab *Scylla* sp.), as the decapsulation process only removes the outer alveolar layer of the cyst shell, leaving the underlying membranes intact.

3.1.5. *Artemia* hatching

Although hatching of small quantities of *Artemia* cysts is basically very simple, several parameters need to be taken into consideration for the successful hatching of large

(i.e. kilograms) quantities of cysts, which is a common daily practice within large hatcheries (Plate 52, Plate 53, Plate 54 and Annex 6). These parameters are primarily:

Aeration	**pH**
Temperature	**Cyst density**
Salinity	**Illumination**

For routine operation, it is most efficient and economical to work in standardized conditions to allow predictable and maximal production of a homogeneous instar I population after a fixed incubation time.

PLATE 52
Lifted hatching tanks (for easier drainage and harvest) in a temperature-controlled room; illumination provided by sunroof and artificial lights

© Viet Uc Shrimp hatchery, Bac Lieu

PLATE 53
Hatching tank with sufficient aeration

© Viet Uc Shrimp hatchery, Bac Lieu

Aeration

Best hatching results are achieved in containers with a conical bottom, aerated from the bottom with airlines. Cylindrical or square-bottomed tanks will have dead spots in which *Artemia* cysts and nauplii accumulate and suffer from oxygen depletion. Transparent or translucent containers will facilitate inspection of the hatching suspension, especially when harvesting. Adequate aeration should be provided to maintain dissolved oxygen levels. The aeration intensity must be sufficient to maintain oxygen levels above 2 mg/L, preferentially > 4 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 (see Section 3.1.3) and/or by the addition of a water conditioner.

Aeration

Although optimal temperature for hatching may show slight differences among strains (see Section 1.4.4), the temperature of the hatching medium is preferentially kept constant in the range of 25–30 °C (using heaters with thermostat or a climatized room) to ensure standardization of the hatching process (= the time needed to harvest a maximum of instar I stage individuals). Below 25 °C, cysts hatch more slowly, and above 33 °C the cyst metabolism is irreversibly stopped (see Section 3.1.2). However, the effect of temperatures outside this range on the hatching output is largely strain-specific.

Salinity

The quantitative effects of the incubation salinity on cyst hatching are, in the first place, related with the hydration level that can be reached in the cysts. Above a threshold salinity, insufficient quantities of water can be taken up by the cysts; this threshold value varies from strain to strain but is approximately 85–90 g/L for most *Artemia*

PLATE 54

Different types and sizes of cylindroconical hatching tanks in different hatcheries around the world, from small conical tanks of a few 100 L up to 2 500 L

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strains. In the second place, the incubation salinity will interfere with the amount of glycerol that needs to be built up to reach the critical osmotic pressure within the outer cuticular membrane of the cysts to result in breaking. The fastest hatching rates will thus be noted at the lowest salinity levels since it will take less time to reach this breaking point. Overall, optimal hatching can be obtained in the range of 10–35 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 needs to be built up. For some sources of cysts, hatching them 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.

pH

The bicarbonate buffering system of seawater normally provides sufficient buffer capacity to the hatching medium to ensure that its pH remains above 8 during the hatching process, a measure required for optimal functioning of the hatching enzyme as long as all hatchable cysts have not yet hatched. If needed, that is, when low salinity water is used, the buffer capacity of the water should be increased by adding up to 1 g of $NaHCO_3$/L. Increased buffer capacities, by adding bicarbonate or NaOH, are essential when high densities of cysts are hatched (resulting in higher CO_2 production, which may lower the pH).

Cyst density

Cyst density may interfere with the other abiotic factors that are essential for hatching, such as pH, oxygen and illumination. A standard density of 2 g/L is recommended. It can be higher, however (even ≥5 g/L), but then oxygen, pH and light are more critical factors to control.

Illumination

Strong illumination is essential, at least during the first hours after complete hydration (see Section 1.5.2), to trigger/start embryonic development and to ensure better hatching synchrony. Measurement of light, defined as the portion of the electromagnetic spectrum that can be perceived by the human eye, is complex, depending on which aspect of light (electromagnetic waves) is being measured. Light "illuminance" is one of the conventional ways of expressing how light brightness is perceived by the eye, and this aspect of light is often expressed in "lux" units. For optimal hatching, illuminance at the water surface of the hatching tank should be at least 2 000 lux. Especially when working at higher cyst density and/or bigger hatching containers, it is important to ensure that insufficient illumination is not a limiting factor for the hatching process: Depending on the actual set–up and tank dimensions, light intensity within the hatching tank itself may be much lower than at the water surface. Although a sufficient level of illumination is mostly attained during daytime in transparent tanks that are set up outdoors in the shade, it is advisable to keep the hatching tanks indoors and to provide artificial illumination so as to ensure good standardization of the hatching process.

3.1.6. Separation and harvesting of *Artemia* nauplii

As with hatching procedures, separation and harvesting procedures should aim at harvesting instar I nauplii and not any older larval *Artemia* stages: First for nutritional reasons (see Section 3.1.7), but also from a biosecurity point of view, as once *Artemia* larvae have moulted to the instar II stage, they may ingest the *Vibrio* present in the hatching medium. As shown through analytical data obtained from a major shrimp hatchery, total *Vibrio* loads in the *Artemia* harvested after hatching may range from values in the order of 10^3 colony forming units (CFUs) per gram (see Annex 11) up to values as high as 107 CFUs/g even after proper washing – dependent on the share

of instar II individuals in the harvested population – because *Vibrio* ingested by these instar II *Artemia* are not removed by the washing process.

After hatching and before feeding the instar I nauplii to fish/crustacean larvae, the *Artemia* instar I nauplii should be separated from empty cyst shells, unhatched cysts (both having the rigid chitinous non-digestible chorion), hatching membranes, debris and the hatching medium itself, and they should be cleaned with clean and disinfected seawater to guarantee high-quality live food.

As explained in Section 3.1.4, the chemical removal of the shells makes shell separation redundant, but its practice can compromise human health and raise environmental concerns. If no decapsulation is applied or any alternative technology for separation is used (e.g. magnetic separation of cyst shells; see below), nauplii harvesting should be done as described in Annex 7. As one of the main factors, it is crucial that the harvesting nets remain submerged at all times to prevent mechanical damage of the nauplii, which may promote *Vibrio* proliferation during further storage and use of the nauplii. Applying this harvesting protocol in a correct way requires sufficient practical expertise and often remains a challenge for routine application in hatcheries.

The initial separation of nauplii in the hatching container by flotation of the empty cyst shells and by sinking of the non-hatched full cysts, resulting in a physical separation, are sometimes followed by further separation using the "double–sieving method". Double sieving requires two superimposed, fine-meshed nets that function as a two-layered filter to separate the cysts from the *Artemia* nauplii, forcing the nauplii to pass through the mesh while the cysts are retained within. This filtration process is time-consuming and laborious; the success of separation depends on the skills of the operator and is often highly variable. Moreover, the process is almost certain to result in physical damage of the nauplii. Damaged and dead nauplii are a less attractive feed and leak nutrients to the medium, favouring unwanted development of bacteria such as *Vibrio* and increasing biosecurity risks in the hatchery. Especially for the latter reason, the double–sieving method is highly discommended.

To avoid the time–consuming mechanical separation of nauplii and its shortcomings, and the environmental issues linked to the use of decapsulated cysts, alternative technologies have been developed. In one of these techniques, separation is facilitated by using chemicals that release hydrogen peroxide when dissolved in water, e.g. sodium percarbonate ($2\ Na_2CO_3 \cdot 3\ H_2O_2$). The product is added to the hatching tank after the standard hatching incubation and allowed to mix for a short while. After that, aeration is terminated and the nauplii are given time to separate from the cyst shells, after which harvesting and rinsing are done in the standard way.

PLATE 55
Application of SEP–Art® technology: Magnetic rods (left) submerged in the concentrator/rinser tank (right) attract cyst shells and unhatched cysts

Another commercialized, alternative technology to facilitate nauplii separation is the SEP–Art® technology. This technology separates nauplii from their cysts using magnetism (Plate 55): SEP–Art® cysts are coated with a non-toxic layer of magnetic material that does not affect the overall hatching characteristics of the cysts. The SEP–Art® separation tool magnets thus solely attract the non-hatched cysts and empty shells, resulting in their separation from the *Artemia* nauplii during the process. Once the cysts attach to the magnets, they can be removed easily from the *Artemia* nauplii suspension. As opposed to the

conventional separation methods, SEP–Art® does not compromise the vitality of the nauplii, as it does not employ physical force or a chemical reaction. Furthermore, it maximizes the recovery of the hatching output and speeds up the harvest and collection of the nauplii. Compared to the decapsulation procedure that requires sufficient expertise for a correct application, SEP–Art® tools are easy to use, and hence accessible to untrained workers. The process does not produce chemical waste, making it more environmentally friendly even at large scale.

Before hatching into instar-I nauplii umbrella-stage *Artemia* can also be offered as food source to different fish and crustacean larvae (see Box 11). This practice has gained more attention since its introduction in the commercial larval rearing of mud crab in the Mekong delta in Viet Nam (Hai *et al*., 2020).

BOX 11

Use of umbrella-stage *Artemia* as live food

by *Nguyen Van Hoa* and *Patrick Sorgeloos*

The umbrella is the first non-swimming larval stage of *Artemia* after the breaking of the cyst shell: It is the embryo, still surrounded by the hatching membrane. Nowadays, umbrella-stage *Artemia* is sometimes used to feed larvae of (shell) fish species, such as penaeid shrimp and cobia fish (*Rachycentron canadum*). But especially in larviculture of mud crab, the use of umbrella-stage *Artemia* from the Vinh Chau strain (the smallest *Artemia* strain on the market) as starter food for larvae of mud crab (*Scylla paramamosain*) instead of rotifers is the main reason for the successful development of a mud crab hatchery industry in the Mekong Delta, Viet Nam.

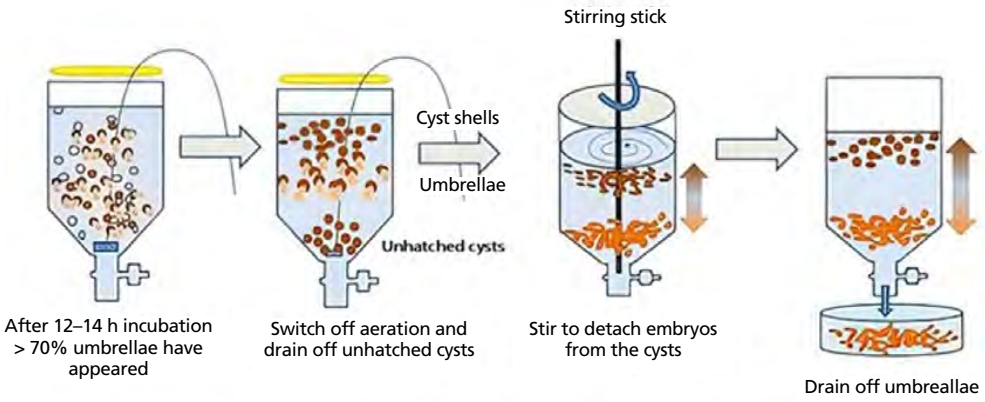
Umbrella-stage *Artemia* can be collected during the hatching process of *Artemia* cysts (see Figure on page 138), i.e. upon breaking of the cyst shell or chorion, the pre-nauplius larva or umbrella, still surrounded by its hatching membrane, protrudes from the shell and as a result of its specific buoyancy characteristics it hangs underneath the empty shell. This cyst "breaking" process ends when the hatching membrane ruptures and the free-swimming instar I nauplius larva emerges (see Section 1.1).

The use of umbrellae can open several opportunities:

- Some studies have explored the possibility to feed umbrella–stage *Artemia* for further shortening and possibly complete substitution of rotifer start feeding in predators other than mud crab larvae (Van Can Nhu, 2009), and thus to avoid the complexity of the production procedures of this type of live food. Umbrellae may also be used as a relatively easily available "back–up" live food in case of sudden crashes or shortage of rotifer production.
- Umbrellae provide a more energy-rich food with lower bacterial load in comparison with rotifers, and also compared to hatched nauplii, as no energy is lost for the development up to the free swimming nauplius and for its swimming activities.
- The immobile umbrella stage can also be a good alternative for live *Artemia* nauplii when fed to poor hunters (e.g. shrimp larvae at zoea stages) because the vigorously swimming nauplii may result in an inefficient food uptake by the predator (Cobo *et al*., 2015). Using heat–killed, blended or frozen *Artemia* nauplii can facilitate capture by the predator, but this affects the nutritional quality of the prey and may deteriorate rearing water quality. On the other hand, because of their immobility, feeding umbrellae should be done through drip feeding, so that there is a continuous supply of live food. If not, non-consumed umbrellae will sink to the bottom of the larval tank, where they are not or less available for uptake. There they will eventually hatch into the nauplius stage that becomes available in the water column, but that is (e.g. in the case of mud crab larvae) too large for the predator to ingest.

BOX 11 (CONTINUED)

Production of "umbrella" stage *Artemia*



Source: Authors' own elaboration (CTU, Viet Nam).

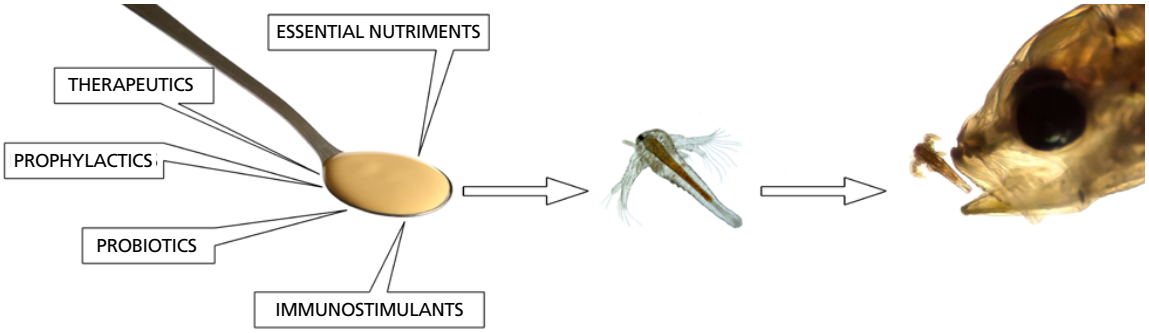
3.1.7. Enrichment of nauplii

Soon after *Artemia* became a popular live food item in the early days of aquaculture, it was realized that many *Artemia* strains were deficient in certain essential nutrients – with deficiencies being different for *Artemia* from various geographic origins – and therefore did not satisfy all the requirements of the (especially marine) predator larvae to which it was fed (Léger *et al*., 1987; Navarro, Amat and Sargent, 1992, 1993). Many studies since have focused on the role of highly unsaturated fatty acids (HUFAs; i.e. polyunsaturated fatty acids with 20 or more carbon atoms and three or more double bonds) in the development of fish and shellfish larvae and their presence (or absence) in *Artemia*.

Linoleic acid (LA, 18:2ω6), together with alpha-linolenic acid (ALA, 18:3ω3), can be synthesized in plants but not in animal tissues. However, both fatty acids are required for cellular processes and for the production of other vital fatty acids, such as omega-6 (ω6) and omega-3 (ω3) fatty acids. Therefore, since they must be taken in through the diet, they are considered as essential fatty acids (EFAs). Whereas ω6 and ω3 HUFAs are usually formed in the body of most animals from their parent compounds, the levels produced are variable compared to those needed for maintaining optimal health. Linolenic acid is readily converted to eicosapentaenoic acid (EPA, 20:5ω3) in freshwater fish, but its conversion to EPA, and subsequently to docosahexaenoic acid (DHA, 22:6ω3), is limited or even absent in most marine fish. Consequently, this implies that marine fish must be given these essential nutrients in their diet and in sufficient quantities.

The ω3 HUFAs especially play a significant role in the development of marine fish larvae. Initially, much attention was given to boosting EPA levels in *Artemia*, but in the late 1980s focus expanded to DHA as well (Léger, Naessens-Foucquaert and Sorgeloos, 1987). The yolk of most wild marine fish eggs has a DHA:EPA ratio of about 2, which implies that at least a 2:1 ratio of DHA to EPA needs to be observed in first-feeding larvae. Sufficiently high DHA:EPA ratios were found to be vital in boosting growth, stress tolerance, as well as pigmentation in marine fish larvae. Marine fish have large quantities of DHA along with EPA in the phospholipids of their cellular membranes, where they enhance membrane permeability, particularly in the visual and neural membranes. Lack of these EFAs has the potential to cause stunted physiological growth and altered behaviour, such as poor vision in low light intensities, reduced stress tolerance and impaired pigmentation. Studies have also shown that other than DHA and EPA, the ω6 HUFA arachidonic acid (ARA, 20:4ω-6) may equally be essential for marine fish larvae (Bell and Sargent, 2003). As with ω3 HUFAs, marine fish species

FIGURE 42
Schematic diagram of the use of *Artemia* as a vector for transfer of specific components into the cultured larvae



Source: Authors' own elaboration (LAARC).

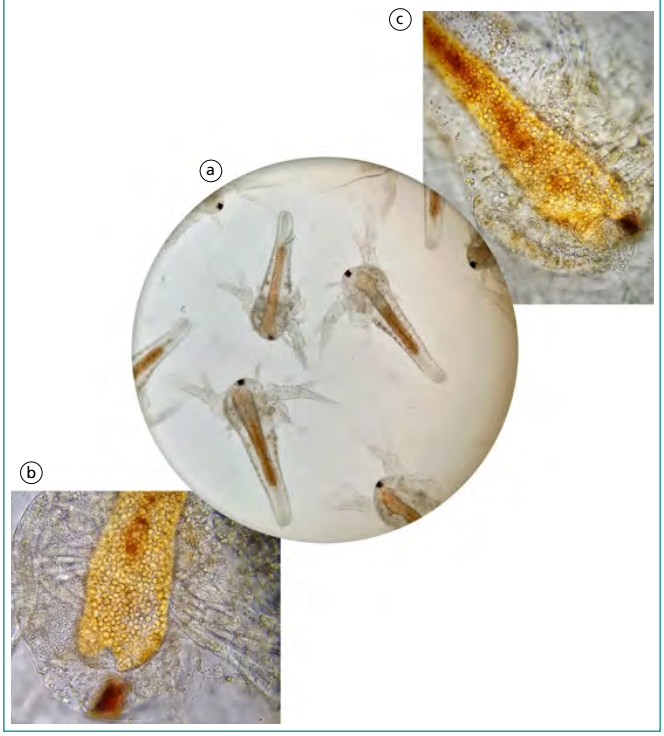
are unable to synthesize ARA from its parent compound. ARA is a precursor to the production of eicosanoids, which are a group of immunological compounds, vital for the fish immune system. However, studies indicate that the requirement of ARA in fish appears to rely on the fish species and larval development and thus care needs to be taken when providing this nutrient.

In view of the deficiency of fatty acids, as found in many *Artemia* strains – albeit with substantial differences between strains (see Section 1.4.6) – research has been conducted to improve *Artemia*'s lipid profile. Because of its feeding characteristics, *Artemia* allows a very convenient way to manipulate its biochemical composition. Since *Artemia*, after moulting to the second larval stage (i.e. about 8 h following hatching, depending on ambient conditions), is non-selective in taking up particulate matter (see Section 1.1), methods have been developed to incorporate lipid products into the brine shrimp nauplii prior to offering them as a prey to the predator larvae (Figure 42). Originally developed for enrichment with HUFAs, the methodology was soon adopted for other compounds (phospholipids, immunostimulants, vitamins, carotenoids, etc.) on condition that these can be offered in a particle small enough for uptake by *Artemia*. This method of bioencapsulation, also called *Artemia* enrichment, is routinely applied in many marine fish and crustacean hatcheries for enhancing the nutritional value of *Artemia* with essential fatty acids. It has had a major impact on larviculture output not only in terms of survival, growth and success of metamorphosis of larval aquaculture animals but also with regard to their quality, for example, reduced malformations, improved pigmentation and increased stress resistance. Nonetheless, for several marine fish species, the optimal dietary levels of ω3 HUFAs are still not met by enriched *Artemia*.

Different enrichment products and procedures have been developed based on selected microalgae, yeasts, probiotics, microencapsulated and microparticulate products, emulsified products and self emulsifying concentrates or combinations thereof. The highest enrichment levels are obtained when using emulsified concentrates. The emulsion is a self-dispersing complex of selected marine oil sources, often supplemented with other nutritional compounds such as fat-soluble vitamins and carotenoids. Upon dilution in seawater, finely dispersed stable microglobules are formed, which are readily ingested by *Artemia*. The high enrichment levels are the result not only of an optimal product composition and manufacturing process but also of proper enrichment procedures: The freshly hatched nauplii are transferred to an enrichment tank at a density of maximum 500 *Artemia* per ml, with the enrichment medium consisting of disinfected seawater (see Section 3.1.1) maintained at 25–30 °C. According to the instructions of use, the enrichment product is added in one or two rations; a strong aeration (using air stones) or addition of pure oxygen is required so as to

PLATE 56

Enriched *Artemia* metanauplii (a); in the gut (b, c), the small oil droplets ingested from the enrichment product are visible



maintain dissolved oxygen levels above 4 mg/L. The latter is necessary to avoid suboptimal conditions that may lead to significant mortalities. Peroxidation of the lipids may also increase oxygen demand and contribute to these suboptimal conditions. The enriched metanauplii (Plate 56) are harvested after 22 h, thoroughly rinsed (see Section 3.1.6. and Annex 7) and then fed directly or stored at below 10 °C before administration, thus assuring high nutritional value (by minimizing metabolization of the HUFAs within *Artemia*; see Figure 43) and also to control biosecurity.

By using these enrichment techniques very high incorporation levels of HUFAs can be obtained that are well above the maximal concentrations found in natural strains (see Table 22).

To date, modern commercial enrichment products not only boost essential fatty acid levels but also aim to result in a more generally balanced live food in terms of protein, phospholipid and energy content. Moreover, the products generally include a wide range of essential or beneficial (micro-) nutrients, such as vitamins, pigments, sterols, antioxidants and enzymes. A trend in the development of commercial enrichment products is to include "natural" ingredients such as microalgae or special yeasts in the formulation, as these are thought to have, for example, additional nutritional or immune-stimulating action that cannot be provided through individual nutrients. The enrichment process can thus also be linked to the hygiene and disease control procedures in hatcheries through incorporating bacteriostatic products or serving as a vehicle for delivery of immune–stimulants, probiotics, vaccines, and antimicrobial peptides, to name a few. In terms of physical appearance, dry powdered enrichment products have acquired their own market position, as they might be less perishable, can be more easily stored and are more convenient in use (e.g. dosage). New developments also include products that allow obtaining the same nutrient enrichment levels in a shorter time period or that allow enrichment at higher *Artemia* density, thus saving on time, space and labour requirements in hatcheries.

FIGURE 43

Evolution of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)

Levels in mg/g dry weight (DW (with DHA catabolism)) during storage of DHA- and EPA-enriched Great Salt Lake *Artemia franciscana* at 27 °C and 16 °C. Note: E16 = stored at 16 °C; E27 = stored at 27 °C; NE = non-enriched

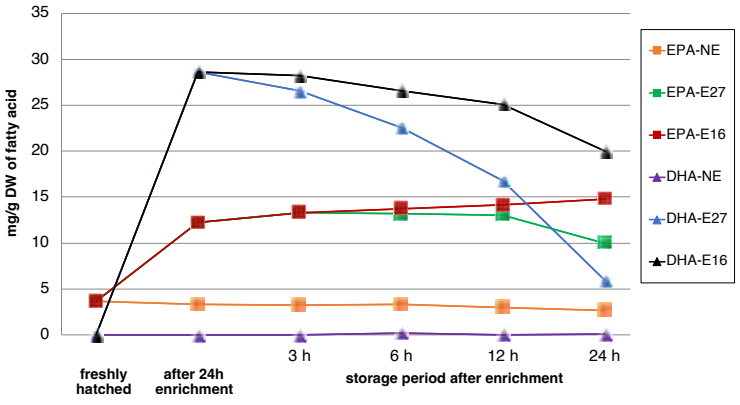


TABLE 22
Example of HUFA levels (in mg/g dry weight) obtained in metanauplii of Great Salt Lake *Artemia franciscana* enriched with different commercial emulsions over a 12 h and 24 h enrichment period versus non-enriched instar I nauplii

Fatty acid (mg/g dry weight)	GSL instar I non-enriched	Enriched with product A		Enriched with product B		Enriched with product C	
		12 h	24 h	12 h	24 h	12 h	24 h
LA: 18:2ω6	8.5	9.1	8.6	10.4	15.2	10.1	13.3
ALA: 18:3ω3	45.2	36.2	29.7	35.5	37.5	41.6	34.9
ARA: 20:4ω6	0.8	2.2	4.0	1.6	1.6	1.2	2.9
EPA: 20:5ω3	2.7	6.3	11.2	7.8	13.5	6.9	18.5
DHA: 22:6ω3	Traces	20.9	35.5	12.5	34.9	5.0	17.3
DHA/EPA	–	3.3	3.2	1.6	2.6	0.7	0.9
Sum ω3 > 20:3ω3	5.4	29.8	50.1	24.6	54.7	14.1	40.0
Sum ω6 > 18:2ω6-t	10.2	18.2	24.3	14.6	19.7	13.1	1.2
ω3/ω6	0.5	1.6	2.1	1.7	2.8	1.1	2.1

Note: ALA = alpha-linolenic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; GSL = Great Salt Lake; HUFA = highly unsaturated fatty acid; LA = linoleic acid.

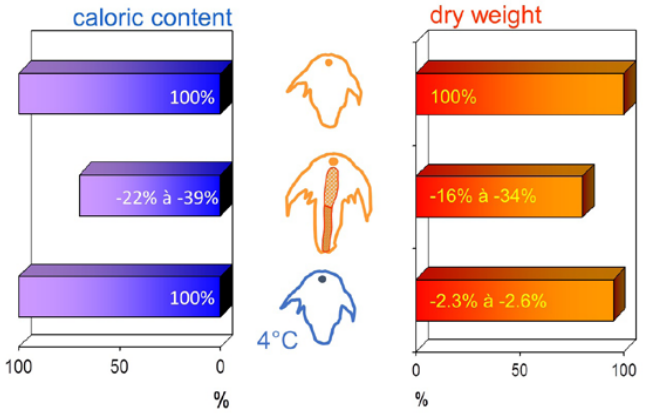
Source: Authors' own elaboration (LAARC).

3.1.8. Cold storage of nauplii

Refrigeration and quality protection of the *Artemia* nauplii are essential for the prevention of bacterial growth and possible negative changes in the nutritional quality of the *Artemia* nauplii during storage. Today, many hatcheries still overlook the fact that an *Artemia* nauplius is not feeding in its first stage of development (instar I) and thus consumes its own energy reserves to grow and further develop. At elevated temperatures, which are applied for cyst hatching incubation, the freshly hatched *Artemia* nauplii develop into the second larval stage within a matter of hours, consuming a substantial part (up to more than 30 percent) of their energy reserves within that time frame (Figure 44). Instar II are less visible, as they are more transparent, and they are larger and swim faster than instar I larvae, as a result of which they are less acceptable as prey.

Consequently, as hatcheries should feed the best *Artemia* for a prolonged time, cold storage facilities are needed as they will help to guarantee the high quality of the live food. Moulting of the *Artemia* nauplii to the second instar stage may be avoided and their energy metabolism may be greatly reduced by storage of the freshly hatched nauplii at a temperature below 10 °C (Léger, Vanhaecke and Sorgeloos, 1983). Densities can be as high as 5 000 individuals/ml when no pure oxygen is applied, and even up to 10 000 individuals/ml with pure oxygen. In this way, nauplii can be stored for periods up to 24 h without significant mortalities with the reduction of energy being less than 5 percent. In terms of bacterial growth, it is important to consider that, even though cool rooms and refrigeration greatly suppress the growth of bacteria, this suppression is not 100 percent and proper hygiene and biosecurity procedures need to be respected (see Section 3.1.1).

FIGURE 44
Decrease in energy (caloric content) and dry weight of different forms of *Artemia*
Newly hatched instar I nauplii (top) are considered to have 100 percent values. The percent decrease is shown for instar II–III metanauplii (middle) and instar I nauplii stored at 4 °C for 24 h (below)



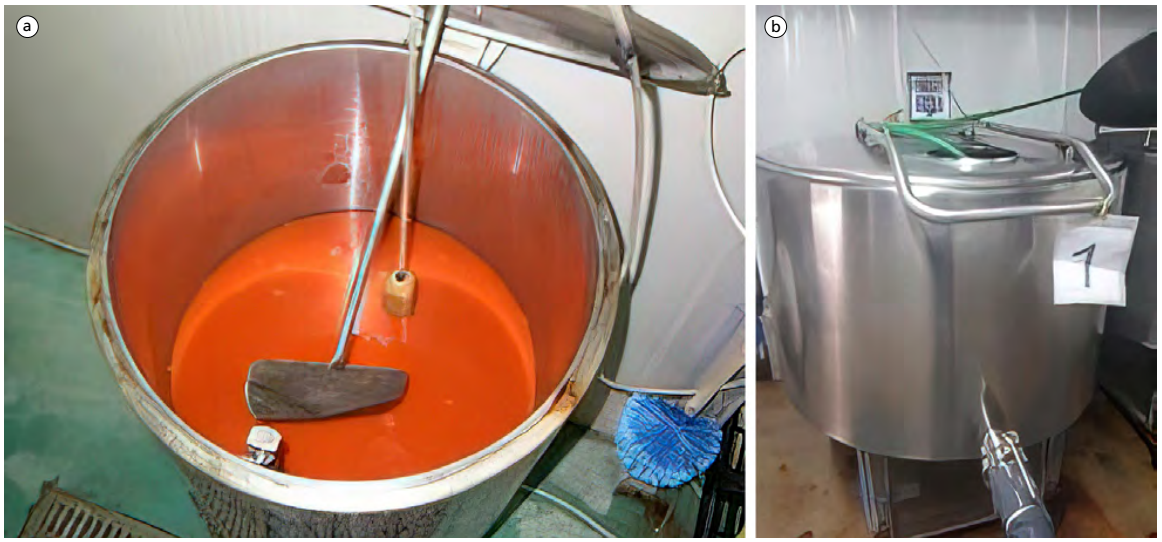
For the storage of live *Artemia* nauplii for a prolonged time at low temperatures, a variety of equipment can be used, such as immersion tanks (where the collected nauplii are stored in a tank, immersed in water and kept at a low temperature using conventional refrigeration tools such as Styrofoam isolation of the tank, ice packs or ice packed in closed plastic bags), refrigerators, cold rooms or "milk" cooling tanks (Plate 57). In these cooling containers, only a slight aeration is needed to prevent the nauplii from accumulating at the bottom of the tank where they would experience oxygen stress and ultimately mortality. The use of a rotating system together with the supply of air or oxygen to keep the nauplii in suspension is recommended. It is important that the suspension is cooled as quickly as possible after harvesting the nauplii. This can be done by submerging disinfected plastic-packed ice blocks into the *Artemia* nauplii suspension or by using disinfected precooled water at the end of harvesting.

Applying 24 h cold storage allows commercial hatcheries to economize on cyst hatching efforts (i.e. reduction of the daily number of hatchings and harvests, fewer tanks, bigger volumes). Cold storage allows the farmer to consider more frequent and even automated food distributions of an optimal live food. This is 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 larviculture tank can be minimized. Furthermore, when working with poor hunters, such as the larvae of turbot (*Scophthalmus maximus*) and tiger shrimp (*Penaeus monodon*) and some early stages of whiteleg shrimp (*Litopenaeus vannamei*), feeding cold stored *Artemia* that are less active for a while after leaving cold storage conditions results in much more efficient food uptake.

Nowadays, *Artemia* nauplii are also produced and delivered to the hatcheries by specialized companies as a concentrated paste or as a frozen product. The concentration of this fresh or frozen paste is, for example, up to 80 million nauplii/L, and the product is ready to be used in the tank. Depending on the company, if the product is delivered in a fresh paste it may be certified to be pathogen-free and of guaranteed quality of the nauplii (viability and recovery after introduction in the larviculture tank). If frozen, the nauplii are killed by the freezing process and thus immobilized (which may be advantageous for feeding, for example, the first postlarvae stages of shrimp), and can be kept for a number of days or weeks in the freezer. It is important to underline that the procedure of concentration to extremely high density can cause physical damage to the nauplii if not done properly. Under these conditions, the nauplii can lose their

PLATE 57

Milk cooling tank used for cold storage of *Artemia* nauplii; Top (a) and side (b) view



nutritional value because of the degradation following physical damage, or they may sink to the bottom of the larviculture tank without being predated, increasing the bacterial load.

Summarizing, the following parameters are critical to achieve optimal cold storage, guaranteeing maximal quality of the live food:

Nauplius stage (instar I/II)

The stage that will be stored depends on the moment when the nauplii are harvested (duration of hatching incubation), the hatching kinetics of the batch/strain in the given hatching conditions, and the time needed to harvest, rinse and transfer the nauplii to cold storage facilities.

Temperature is a key parameter

- Fast cooling of the nauplii suspension is mandatory.
- Optimal storage temperature: +4 ±1 °C.
- Minimal temperature: 0 °C (frozen animals will be damaged and leak nutrients).
- Maximal temperature: 10 °C (the moulting process is slowed down, yet moulting will happen, leading to energy consumption and increase of size).

Motion

The nauplii suspension should be kept in motion to prevent the nauplii to settle and die.

Oxygen/air

Even though metabolic processes are slowed down, the animals are still alive and need oxygen to survive during cold storage. The use of pure oxygen is preferred. If this is not available, air also can be used and supplied at high pressure through a normal air compressor and a porous stone suited for high pressure (such as ceramic stone). In this case, the air should be filtered to avoid introducing bacteria in the *Artemia* suspension. A minimal dissolved oxygen level of 6 mg/L should be maintained. The aeration should be strong enough to prevent nauplii to sink to the bottom where they would suffocate, but gentle enough to prevent the *Artemia* to be damaged.

Density

Up to 5 000 nauplii/ml when no pure oxygen is applied; up to 10 000 nauplii/ml when pure oxygen is applied.

Duration of storage

Maximum 24 h.

Bacterial control

Low temperature substantially decreases the growth of bacteria but does not stop it. Neither is it a disinfection process: If the bacterial load is already high after harvesting, it will also be high at the end of the storage phase. Therefore, it is important to follow disinfection procedures for the hatching and harvesting process and for the material used for cold storage, and to respect overall proper hygiene and biosecurity procedures (see Section 3.1.1).

Feeding

When *Artemia* nauplii are needed, the right amount of live food can be sampled from the cold storage containers and fed immediately. The *Artemia* nauplii will recover in a few minutes from the cold condition. The remaining suspension should immediately be returned to refrigerated conditions until the next use.

3.2. QUALITY EVALUATION OF *ARTEMIA* CYST PRODUCTS

Artemia cysts are offered on the market in a number of brands originating from different strains, corresponding with a variety of quality criteria. These criteria contribute to the value of each brand. To characterize the quality of *Artemia* products, the aquaculture industry therefore employs an array of evaluation tools, of which the following are very common: cyst and nauplii biometry, cyst hatching characteristics and the nutritional profile of the nauplii.

3.2.1. Biometric characteristics

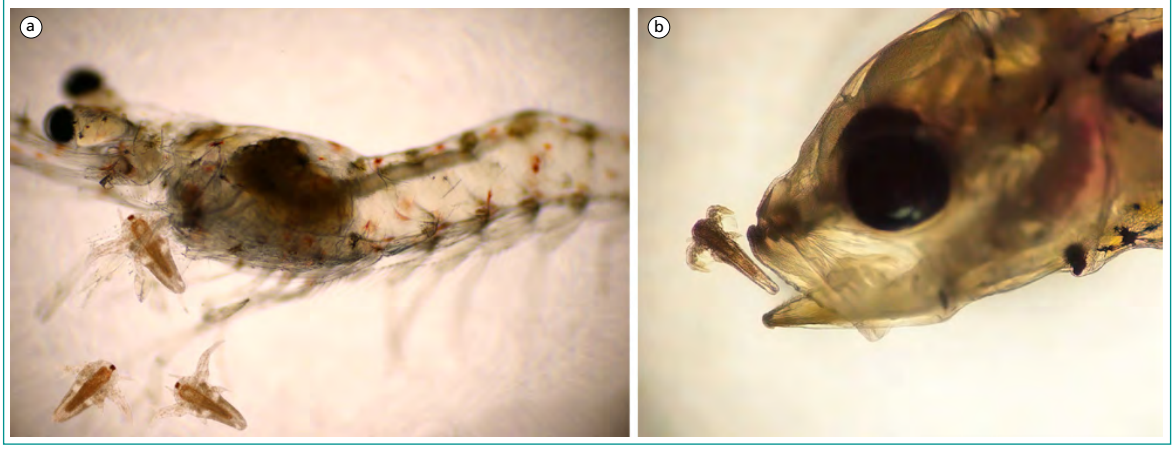
Biometry relates to the size and the shape of the food organism, which affect its ingestibility and eventually also its nutritional effectiveness. Naupliar size, varying greatly from one geographical source of *Artemia* to another (see Section 1.4.1), is often not critical for crustacean larvae, which can capture and tear apart food particles with their feeding appendages (Plate 58). On the other hand, for marine fish larvae that have a very small mouth and swallow their prey in one bite, the size of the nauplii is particularly critical. For example, fish larvae that are offered oversized *Artemia* nauplii may starve because they cannot ingest the prey. Fish that produce small eggs, such as gilthead seabream, turbot and grouper, must be fed rotifers as a first food because the nauplii from all *Artemia* strains are too large. In these cases, the size of nauplii (of a selected strain) will determine when the fish can be switched from a rotifer to an *Artemia* diet.

Quality evaluation from a biometric point of view thus uses the following parameters:

- Diameter of hydrated, non–decapsulated (regular) and decapsulated cysts. Full hydration is required as in this condition the fully spherical cysts reach their maximal size;
- chorion thickness; and
- length and width of freshly hatched nauplii.

Whereas in the past, cyst biometrics was done by manual measurements under the microscope (see Annex 8), nowadays, computer image analysis using specialized software and hardware is more common in cases of routine application.

PLATE 58
Shrimp (a) and fish (b) larva feeding on *Artemia* larvae



Cyst biometrics

For all biometric parameters, important differences may be observed between *Artemia* strains (see Section 1.4.1), and strains can to a certain extent be differentiated based on their biometric characteristics. For example, the mean diameter of most non–decapsulated hydrated cysts varies between 220 and 290 µm. According to size, three groups can be distinguished:

- Those with the smallest size: 220–230 µm, e.g. San Francisco Bay (United States of America) and strains originating from it, such as Vinh Chau (Viet Nam).
- Cysts of intermediate size: 240–260 µm, e.g. Great Salt Lake (United States of America).
- Cysts of bigger size: several parthenogenetic strains: 260–290 µm, such as Bolshoe Yarovoe (Russian Federation); strains from Tibet, China, such as Lagkor Co.

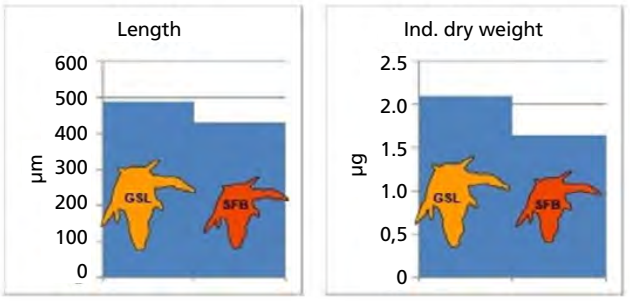
As small cysts also have a lower individual weight, cyst biometrics also has an effect on hatching efficiency (see Section 3.2.2). Within a same strain, the mean cyst size remains more or less constant between batches that are collected at different periods of the year or over different years. Nevertheless, environmental conditions, such as temperature, salinity and variations in food conditions, may cause minor differences in cyst size. Consequently, cyst diameter can be considered as being mainly strain–specific.

For the decapsulated cysts, a similar range of differences as in the untreated cysts is observed between the different strains, with the mean decapsulated cyst diameter varying between 200 and 270 µm. There is no relationship between non–decapsulated cyst size and chorion thickness.

Nauplii biometrics

As cyst and naupliar biometric parameters are mutually significantly correlated, naupliar biometric data follow the differences between the strains, as observed for cyst size (Figure 45); cyst diameter is one of the factors determining the price of the cyst product on the market, with small cysts fetching higher prices. Knowledge on naupliar biometric characteristics becomes of practical value when selecting the most appropriate strain as an adequate food source for predators such as larval fishes:

FIGURE 45
Strain-specific differences in instar I nauplius length (left) and dry weight (right) between Great Salt Lake (GSL) and San Francisco Bay (SFB) *Artemia*



- Large nauplii with higher individual weight are beneficial if naupliar size is not critical for the ingestion mechanism: The predator spends less energy when taking up a lower number of larger nauplii to fulfil its food demand. This is especially the case for fish larvae, which are not efficient in prey hunting.
- If the naupliar size is critical for the ingestion mechanism, better results might be obtained when using small nauplii.

3.2.2. Hatching quality

To have a good hatching quality, an acceptable cyst product should firstly contain minimal quantities of impurities, such as sand, cracked and empty shells, feathers and salt crystals. Together with a low water content, this will contribute to a high number of cysts per gram product. As most common parameters, hatching efficiency (the number of nauplii hatched per gram of [dry] cyst product) and hatching percentage (the total percentage of full cysts that actually hatch) are observed after a standard hatching incubation period, generally 24 h. These parameters often vary considerably between different commercial products and obviously account for much of the price differences. In this respect, hatching efficiency is regarded as a better overall commercial criterion than hatching percentage, as it reflects the amount of prey (nauplii) provided per gram of the cyst product as it is, taking into account its impurities, water content,

etc. Hatching efficiency values of commercial cyst product may be as low as 10 000 nauplii/g, while, for example, premium quality cysts from Great Salt Lake yield 250 000 nauplii/g (with an equivalent hatching percentage of >90 percent). Batches of small cysts (e.g. San Francisco Bay type Viet Nam cysts) may yield even higher numbers of nauplii, (i.e. >300 000 nauplii/g): Because a small cyst also has a lower weight (see Section 3.2.1), 1 gram of cyst product of a strain characterized by small cysts contains more cysts than 1 gram of a bigger cyst strain. The hatching efficiency potential of the smaller strain product is therefore correspondingly higher.

Another aspect of hatching quality does not relate to the hatching obtained after a standard hatching incubation period, but to the rate of a cyst sample to come to maximal hatching; this is the so-called hatching synchrony. Hatching speed and synchrony must be high enough; when incubated in 32 g/L seawater at 28 °C, the first nauplii should appear after 8–12 h of incubation (T0; see further), and the last nauplii should have hatched within more or less 8 h thereafter (T_{100}). Analogously, other T values can be formulated (e.g. T_{90} is the time needed for 90 percent of all hatchable cysts to hatch). When hatching is not sufficiently synchronous (T_{100}–T_0 >10 h), the first–hatched nauplii will have moulted into the second larval stage and have consumed much of their energy reserves by the time that the last nauplii have hatched and harvesting is done. Moreover, these later larval stages may present a higher biosecurity risk as they may have taken up the pathogenic *Vibrio*, present in the hatching environment (whereas first stage instar do not feed). When the total incubation period required to obtain maximal hatching exceeds 24 h, the cyst user will not be able to restock the same hatching containers for the next day's harvest, which in turn implies higher infrastructure costs.

To evaluate the hatching quality of a cyst product, the following criteria are therefore being used:

Water content (% H_2O) (see Annex 9):
To study the interrelationship between water content and metabolism in *Artemia* cysts, various approaches for quantification can be followed (for example, water content of the whole cyst versus of the embryo versus of the cyst shell; and water content per gram cyst product or per gram dry weight of cysts). For practical use, and to ensure a long shelf-life with high hatching, the water content of a cyst sample, dried and calculated according to a standard protocol, must have a water content below 10 percent (see Section 2.4.2.4). Moreover, a higher water content of a commercial cyst sample leads to a correspondingly lower hatching efficiency (as the water in the cysts also contributes to their weight, and hatching efficiency is expressed on weight basis. Usually, a water content of 8 percent is aimed for.

Cyst count (cysts/g) (see Annex 10):
= Number of cysts (full + empty) per gram product. This criterion gives a first indication of:

- Quality of previous processing of the sample (degree of impurities, cracked cyst shells, etc.).
- The amount of empty cysts (in combination with hatching percentage and efficiency data).
- The individual cyst weight and thus cyst size (i.e. more cysts/g for smaller strains). For example, typical values are the following (see Section 3.2.1 for example strains of the different size categories):
  - Small–size cysts: >300 000 cysts/g
  - Intermediate–size cysts: 250 000 to 300 000 cysts/g
  - Large–size cysts: <250 000 cysts/g

As an alternative calculation, embryo count, in which only the number of full cysts per gram product is considered, may be used.

Hatching percentage (H%) (see Annex 10):
= Number of nauplii that can be produced under standard hatching conditions from 100 full cysts within a standard hatching incubation period; this criterion does not take into account cyst impurities (cracked shells, sand, salt, etc.), and refers only to the hatching capacity of the full cysts, which in turn depends upon:

- Degree of diapause termination: Cysts that are still in diapause do not hatch, even under favourable hatching conditions (see Section 1.5.4).
- Energy content of cysts: Energy may be too low to build up sufficient levels of glycerol to enable breaking and hatching (see Section 1.5.2), as a consequence of, for example, improper processing and/or storage, and environmental or genotypical conditions affecting the parental generation.
- Amount of dead/non-viable/abortic embryos, either naturally or resulting from improper processing and/or storage.

For determination of H%, normally only free-swimming nauplii are taken into account and considered as "hatched cysts". However, within the standard hatching incubation period of 24 h, not all hatching embryos may have reached the nauplius stage, and some may still be in the umbrella stage. To have a more complete view on the sample's hatching potential, sometimes these umbrellae are also considered as "hatched cysts" and included in the calculation, resulting in the so–called "hatching percentage +" (H+%) value. The difference between H+% and H% gives an indication of the amount of cysts hatching relatively late.

Hatching efficiency (HEff) (see Annex 10):
= Number of nauplii that can be produced from 1 g dry cyst product under standard hatching conditions within a standard hatching incubation period. As this criterion generally refers 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. As described above, this criterion reflects:

- The hatching percentage (see above).
- The embryo (full cyst) count per gram. This parameter is in its turn affected by:
  - the presence of other components apart from full cysts in the cyst product (i.e. empty shells, salt, sand, water content of the cysts); and
  - the individual dry weight (i.e. higher number of cysts/g for smaller strains).

Consequently, the calculation "hatching % × embryo" count may be used to express the number of nauplii produced from 1 g of cyst product (or hatching efficiency).

Analogously to the calculation of H+% (see above), the HEff+ value can also be calculated.

Hatching rate (see Annex 10):
Hatching rate refers to the time period for full hatching from the start of hatching incubation (= start of hydration of cysts) until nauplius release (hatching). It is not expressed as a single value, but considers a number of time intervals, such as T_{10} (incubation period needed until 10 percent of hatchable cysts have hatched), T_{90} (90 percent hatching), T_{100} (maximal hatching). Data on the hatching rate allow the calculation of the optimal incubation period so as to harvest a maximum of freshly hatched instar I nauplii, i.e. containing the highest energy content.

Hatching synchrony is the time lapse during which most nauplii hatch, generally considering the period between T_{90} and T_{10}, so $Ts = T_{90}-T_{10}$. A high hatching synchrony ensures a maximal number of instar I nauplii available within a short time span; in case of poor synchrony, the same hatching tank needs to be harvested several times in order to avoid a mixed instar I–II–III population when harvesting just once, at T_{90} or T_{100}.

Hatching output:
= Dry weight biomass of nauplii that can be produced from 1 g dry cyst product incubated under standard hatching conditions within a standard incubation period; the best products yield about 600 mg nauplii dry weight per gram cysts.

Hatching output is calculated as follows: hatching efficiency × 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 (which is strain–dependent). The hatching output criterion is related to the total amount of food biomass available for the predator per gram of cyst product (cf. calculation of food conversion). Although in the field it is common to use live food biomass (thus based on wet [live] weight of nauplii), wet weight determination is difficult to standardize, and therefore the dry weight is a much more reproducible parameter to express the amount of food supplied to the predator.

Given the definition of the hatching criteria, as described above, and the correlation between biometric parameters, there are some direct relationships between some of the hatching quality criteria of a cyst product and the biometric characteristics of the strain it belongs to (Table 23). Other relationships are more complex: For example, big cysts with large nauplii (Bolshoe Yarovoe in Table 23) may reach a higher hatching output than small–size cysts (e.g. Vinh Chau) even if the hatching efficiency is much lower.

TABLE 23
Example of hatching characteristics of a sample of a small-strain cyst (VC: Vinh Chau, Viet Nam); intermediate-strain cyst (GSL: Great Salt Lake, United States of America); and large–cyst strain (By: Bolshoe Yarovoe, Russian Federation), with corresponding biometric characteristics

Strain	Cyst size (µm)	Instar I length (µm)	Cyst count (cysts/g)	Embryo count (full cysts/g)	% empty cysts	HEff (nauplii/g)	H%	Ind. DW of nauplii (µg)	Hatching output (mg)
VC (small)	239	472*	333 211**	322 566	3.19	285 342**	88.46	1.65*	471
GSL (intermediate)	248	483	309 444	273 780	11.53	253 000	92.41	2.40	607
BY (large)	262	498*	260 428**	240 442	7.68	204 665**	85.12	3.30*	675

Note: For the VC and BY strains, the asterisk (*) indicates parameters that are positively correlated with the cyst size; double asterisk (**) indicates parameters negatively correlated with cyst size. H% = hatching percentage; HEff = hatching efficiency; Ind. DW = individual dry weight.

Source: Authors' own elaboration (LAARC).

3.2.3. Nutritional quality of (meta) nauplii

3.2.3.1. Nutritional composition and digestibility of live food

Larvae of nearly all marine and of many freshwater fish species and larvae of crustaceans require live organisms as first food. The inclusion of live food in feeding protocols still gives better results in terms of growth and survival than when artificial diets alone are applied. Several explanations have been put forward for this, e.g. chemical and visual stimuli from the live food organisms, but also many factors related to food composition and nutritional quality such as the structure and digestibility of proteins. While live food is also a source of exogenous digestive enzymes, the importance of this for the digestive process in fish larvae is still under debate.

The overall nutritional quality of the live food organisms depends on the content and nutritional balance of carbohydrates, protein and lipids, its so-called proximate composition. The concentrations of protein, lipid, carbohydrate and also ash may change significantly during hatching and subsequent *Artemia* development, as described further in this section. Table 24 provides a general overview of the macronutrient, vitamin and mineral composition of freshly hatched Great Salt Lake *Artemia franciscana* nauplii.

TABLE 24
Macronutrient, vitamin and mineral composition of freshly hatched Great Salt Lake *Artemia franciscana*

Macronutrients (g/kg dry matter)	**Amount**
Total amino acids (TAA)	471-503
Nitrogen	85-102
Protein/nitrogen factor	4.7-5.6
Soluble amino acids (% of TAA)	54 ± 4
Free amino acids (% of TAA)	9-10
Total lipids (TL)	102
Polar lipids (% of TL)	31
Neutral lipids (% of TL)	69
Total fatty acids	119
Glycogen	74-96
Ash	90
Vitamins (mg/kg dry matter)	**Amount**
Vitamin A (retinol)	0
Provitamin A (carotenoids)	630-750
Vitamin B1 (thiamine)	4.2
Vitamin B2 (riboflavin)	37
Vitamin B3 (niacin)	159
Vitamin B6 (pyridoxine)	28
Vitamin B7 (biotin)	4.5
Vitamin B9 (folic acid)	14
Vitamin B12 (cobalamin)	0
Vitamin C (ascorbic acid)	798
Macrominerals (g/kg dry matter)	**Amount**
Phosphorus	12-19
Calcium	1.9-2.0
Magnesium	2.0-5.0
Microminerals (g/kg dry matter)	**Amount**
Iodine	0.5-4.6
Manganese	4-30
Copper	7-40
Zinc	120-310
Selenium	2.2
Iron	63-130

Sources:
Hamre, K., Yú Fera, M., Rønnestad, I., Boglione, C., Conceiçã, L.E.C. & Izquierdo, M. 2013. Fish larval nutrition and feed formulation: Knowledge gaps and bottlenecks for advances in larval rearing. *Reviews in Aquaculture*, 5(s1): S26–S58. https://doi.org/10.1111/j.1753-5131.2012.01086.x

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Hamre, K., Erstad, B., de Kok, J., Norberg, B. & Harboe, T. 2020. Change in nutrient composition of *Artemia* grown for 3–4 days and effects of feeding on-grown *Artemia* on performance of Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae. *Aquaculture Nutrition*, 26: 1542–1554. https://onlinelibrary.wiley.com/doi/abs/10.1111/anu.13101

Fish and shrimp larvae have high dietary amino acid requirements owing to their high growth potential and extensive mobilization of amino acids in their energy metabolism. Moreover, during the early stages, it has been shown that larvae absorb free amino acids (FAA) faster and retain them more efficiently than polypeptides.

FAA and small peptides are usually water soluble and are efficiently absorbed from the intestine without previous digestion and can thus be regarded as pre–digested dietary protein. *Artemia* nauplii, unlike formulated feeds, contain a high proportion of water-soluble protein (67 percent of total nitrogen; Table 25) and small peptides, which are highly digestible by larvae. Additionally, the insoluble fraction of *Artemia* seems to be highly digestible as well (digestibility of 71 percent), which allows to estimate the digestibility of a complete *Artemia* nauplius to be up to 84 percent, while the digestibility of inert diets is often substantially lower (Table 26).

TABLE 25
Distribution of crude protein into water-soluble and water-insoluble fraction in *Artemia* versus alternative live food and formulated feed ingredients

Sample	Water-soluble fraction (%)	Insoluble fraction (%)
Artemia (nauplii)	67	33
Calanus (copepods)	54	46
Squid meal	11	89
Fishmeal	17	83
Roe meal	11	89

Source: Tonheim, S.K., Nordgreen, A., Høgøy, I., Hamre, K. & Rønnestad, I. 2007. *In vitro* digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients. *Aquaculture*, 262(2–4): 426–435. https://doi.org/10.1016/J.AQUACULTURE.2006.10.030

TABLE 26
In vitro digestibility of live food and feed ingredients used in starter diets for fish larvae in aquaculture

Sample	Digestibility of water-soluble fraction (%)			Digestibility of insoluble fraction (%)			Total digestibility (%)		
	0 h	1 h	12 h	0 h	1 h	12 h	0 h	1 h	12 h
Artemia (nauplii)	37	66	91	17	46	71	30	59	84
Calanus (copepods)	93	88	96	9	45	72	54	68	87
Squid meal	79	85	90	7	42	75	15	47	77
Fishmeal	56	99	103	6	46	71	15	55	77
Roe meal	57	71	91	6	21	43	12	27	49
Protein-encapsulated diet							1	29	53
Mixed ingredients for protein-encapsulated diet							8	54	74

Note: In this study, digestibility was assessed as the percentage of nitrogen in the soluble fraction after treatment of the protein source with proteases and subsequent precipitation of undigested protein by trichloroacetic acid. For each component analysed, samples for digestibility were analysed at 0, 1 and 12 h after addition of the proteases.

Source: Tonheim, S.K., Nordgreen, A., Høgøy, I., Hamre, K. & Rønnestad, I. 2007. *In vitro* digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients. *Aquaculture*, 262(2–4): 426–435. https://doi.org/10.1016/J.AQUACULTURE.2006.10.030

3.2.3.2. Proteins and amino acids

The quality of the dietary protein has a primary relevance. Inclusions of low to medium levels of highly digestible proteins in diets fed to larval fish and shrimp have been shown to improve survival and growth.

The protein concentration of *Artemia* can be measured by two different methods, i.e. by measuring total hydrolysed amino acids (TAA) and by nitrogen analysis. The TAA method does not detect cysteine, cystine and tryptophane and therefore underestimates the protein content. When using nitrogen analysis, the amount of nitrogen is determined and multiplied with a standard nitrogen-to-protein conversion factor (the Jones factor) to obtain the total protein. In animal protein, protein concentration is generally assumed to be N × 6.25. In reality, however, every organism has its own ratio (or Jones factor), and it has been found that the nitrogen-to-protein factor is rather 4.7 in *Artemia* nauplii and 5.3 in ongrown *Artemia* (Hamre *et al.*, 2013, 2020).

The protein content of *Artemia* varies between 47 percent and 51 percent for the cysts and the umbrella stage and decreases gradually during further development. As cysts (and instar I nauplii) belonging to different *Artemia* species and strains may show strain-specific size differences (see Section 1.4.1), the total amount of protein contained within an individual nauplius may be different (Table 27), with for example bigger parthenogenetic strains containing more protein per individual than small- or medium-sized San Francisco Bay or Great Salt Lake *A. franciscana*, respectively.

TABLE 27
Protein content in instar I nauplii for different populations hatched at 35 g/L

***Artemia* population**	**Protein (µg/individual)**
Macau (MAC), Brazil	1.12
Vinh Chau (VC), Viet Nam	1.00
Great Salt Lake (GSL), United States of America	1.19
San Francisco Bay (SFB), United States of America	0.91
Megalon Embolon (MEG), Greece	1.56
Swakopmund (SWA), Namibia	1.38

Notes: MAC, VC, GSL and SFB are Artemia franciscana strains, whereas MEG and SWA are parthenogenetic strains.

Source: Helland, S., Triantaphyllidis, G.V., Fyhn, H.J., Evjen, M.S., Lavens, P. & Sorgeloos, P. 2000. Modulation of the free amino acid pool and protein content in populations of the brine shrimp *Artemia* spp. *Marine Biology*, 137: 1005–1016. https://link.springer.com/article/10.1007/s002270000409

The total amino acid composition of proteins in *Artemia* seems to be remarkably similar from strain to strain, suggesting that it is not environmentally determined in the manner that the fatty acids are. Yet, given the strain-specific size differences of cysts, the total content of free amino acids (FAA) of decapsulated cysts may differ among *Artemia* populations (Table 28). Although it is difficult to define a general pattern, these inter–strain differences in FAA contents are sometimes smaller in the newly hatched nauplii (Table 29).

TABLE 28
Composition of free amino acids pool (pmol/individual) in decapsulated cysts for various populations of *Artemia*

Amino acid	***Artemia* populations**					
	MAC	**VC**	**GSL**	**SFB**	**MEG**	**SWA**
Essential						
Leucine	1.0	1.5	1.1	1.7	5.4	2.4
Valine	1.1	1.7	1.4	1.6	6.0	2.7
Isoleucine	0.6	1.2	0.8	1.1	3.0	1.9
Lysine	5.8	3.8	2.7	4.2	9.9	7.0
Threonine	1.9	2.4	2.6	2.3	9.6	3.0
Arginine	Traces	2.1	1.6	1.1	7.3	3.4
Phenylalanine	Traces	0.5	0.9	1.5	3.8	1.8
Histidine	4.3	2.4	2.0	3.0	14.0	6.5
Tyrosine	1.7	1.0	1.5	3.4	4.3	1.9
Methionine	Traces	Traces	0.1	Traces	Traces	Traces
Non-essential						
Alanine	18.6	32.6	24.9	28.8	80.7	63.1
Serine	8.4	8.3	8.3	9.2	19.5	11.3
Glutamic acid	7.6	19.4	5.8	19.9	42.5	23.1
Glycine	7.6	8.4	7.8	14.6	49.7	28.5
Taurine	10.3	18.1	11.5	32.0	194.3	103.6
Proline	3.2	6.3	5.6	5.7	24.0	7.3

TABLE 28 (CONTINUED)

Amino acid	*Artemia* populations					
	MAC	VC	GSL	SFB	MEG	SWA
Essential						
Aspartic acid	11.0	19.3	5.4	20.8	61.7	20.1
α -Aminobutyric acid	Traces	Traces	0.1	Traces	Traces	0.4
Glutamine	Traces	1.2	0.8	0.5	0.8	Traces
Phosphoserine	Traces	Traces	Traces	Traces	Traces	Traces
Total FAA	**82**	**131**	**85**	**151**	**536**	**288**
% essential	**20.0**	**13.2**	**17.4**	**13.0**	**14.5**	**13.7**

Note: FAA = free amino acids; GSL = Great Salt Lake, United States of America; MAC = Macau, Brazil; MEG = Megalon Embolon, Greece; SFB = San Francisco Bay, United States of America; SWA = Swakopmund, Namibia; VC = Vinh Chau, Viet Nam.

Source: Helland, S., Triantaphyllidis, G.V., Fyhn, H.J., Evjen, M.S., Lavens, P. & Sorgeloos, P. 2000. Modulation of the free amino acid pool and protein content in populations of the brine shrimp *Artemia* spp. *Marine Biology*, 137: 1005–1016. https://link.springer.com/article/10.1007/s002270000409

TABLE 29
Composition of free amino acids pool (pmol/individual) in instar I nauplii for various populations of *Artemia*

Amino acid	*Artemia* populations					
	MAC	VC	GSL	SFB	MEG	SWA
Essential						
Leucine	3.6	3.7	4.0	3.1	6.9	4.7
Valine	5.2	4.2	5.3	4.4	11.2	5.4
Isoleucine	2.8	2.3	2.5	2.5	4.0	2.5
Lysine	11.9	14.2	15.3	9.5	15.4	12.5
Threonine	17.3	12.5	18.6	12.0	30.7	18.0
Arginine	20.5	12.6	20.2	15.5	33.6	21.4
Phenylalanine	2.7	2.2	2.7	2.4	3.7	3.2
Histidine	13.6	18.2	15.3	13.3	18.6	13.6
Tyrosine	10.6	5.3	12.8	6.8	11.3	11.0
Methionine	Traces	Traces	0.4	1.3	Traces	Traces
Non-essential						
Alanine	22.1	19.6	25.8	20.9	50.0	48.6
Serine	37.4	32.7	39.2	26.7	49.2	49.8
Glutamic acid	30.3	23.8	39.2	26.6	66.4	61.9
Glycine	36.5	34.7	48.8	32.6	116.2	123.7
Taurine	48.0	29.5	57.9	41.5	149.0	172.3
Proline	15.5	14.9	79.1	21.0	70.1	27.7
Aspartic acid	29.9	25.8	31.8	27.2	54.4	33.4
α -Aminobutyric acid	0.7	Traces	1.0	Traces	1.1	Traces
Glutamine	3.2	28.8	8.1	2.3	Traces	1.1
Phosphoserine	Traces	Traces	10.4	5.2	30.3	22.6
Total FAA	**312**	**312**	**431**	**306**	**722**	**634**
% essential	**28.2**	**26.0**	**22.5**	**26.5**	**18.7**	**14.7**

Note: FAA = free amino acids; GSL = Great Salt Lake, United States of America; MAC = Macau, Brazil; MEG = Megalon Embolon, Greece; SFB = San Francisco Bay, United States of America; SWA = Swakopmund, Namibia; VC = Vinh Chau, Viet Nam.

Source: Helland, S., Triantaphyllidis, G.V., Fyhn, H.J., Evjen, M.S., Lavens, P. & Sorgeloos, P. 2000. Modulation of the free amino acid pool and protein content in populations of the brine shrimp *Artemia* spp. *Marine Biology*, 137: 1005–1016. https://link.springer.com/article/10.1007/s002270000409

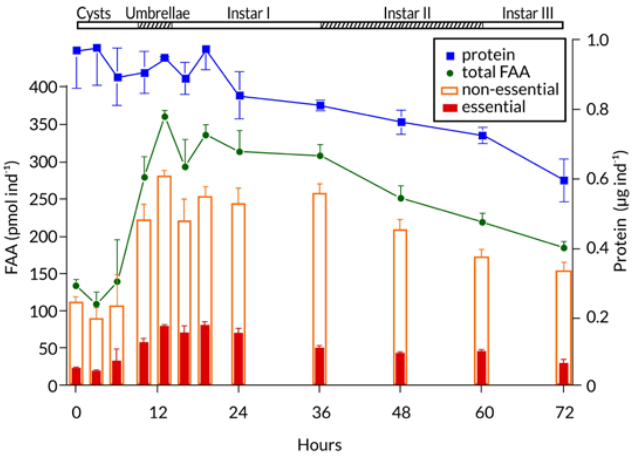
The relationship between individual protein and FAA content in developing *Artemia* nauplii has been analysed in Vinh Chau–type *A. franciscana* (see Figure 46). As *Artemia* cyst development proceeds towards the umbrella stage and hatching

occurs, the individual FAA content increases about threefold and then slowly decreases towards instar III. Relative to the protein content, the pool of FAA amounts to approximately 1.5 percent in cysts and about 4 percent in late umbrella and naupliar stages. The general trend for the FAA content in the individual *Artemia* nauplius is to decrease after hatching, although some amino acids (such as aspartate and glutamate) might peak. The amino acid analogue taurine seems to increase and accumulate in developing *Artemia* and accounts for approximately 50 percent of the total FAA at 72 h post–hatching. Overall, the levels of essential amino acids in *Artemia* are generally not seen as a major problem in view of its nutritional value.

3.2.3.3. Lipids and fatty acids

An important nutritional characteristic of *Artemia* nauplii was identified in the late 1970s and early 1980s, when many fish and shrimp hatcheries scaled up their production and reported unexpected problems when switching from one source of *Artemia* to another. American, European and Japanese researchers studied these problems and soon confirmed variations in nutritional value when using different geographical sources (strains) of *Artemia* for fish and shrimp species. The situation became even more critical when very significant differences in production yields were obtained with distinct batches of the same strain of *Artemia*.

FIGURE 46
Content of free amino acids (FAA) and protein during hatching and early development in *Artemia franciscana* from Vinh Chau, Viet Nam at 35 g/L



Source: Helland, S., Triantaphyllidis, G.V., Fyhn, H.J., Evjen, M.S., Lavens, P. & Sorgeloos, P. 2000. Modulation of the free amino acid pool and protein content in populations of the brine shrimp *Artemia* spp. *Marine Biology*, 137: 1005–1016. https://link.springer.com/article/10.1007/s002270000409

Studies in Japan and the multidisciplinary International Study on *Artemia* revealed that the concentration of the essential fatty acid 20:5ω3 eicosapentaenoic acid (EPA) in *Artemia* nauplii was what determined its nutritional value for larvae of various marine fish and crustaceans. Various results were obtained with different batches of the same geographical *Artemia* source, which contained different amounts of EPA and accordingly yielded variable results in growth and survival of the test organism *Mysidopsis bahia* shrimp, fed with these *Artemia*. The levels of the essential fatty acid EPA in *Artemia* vary substantially from strain to strain and even from batch to batch (Table 30; see also Sections 1.4.6 and 3.1.7), the causative factor being the fluctuations in biochemical composition of the primary producers (microalgae) available to the adult reproducing brine shrimp population. The levels of this and of other dietary ω3 highly unsaturated fatty acids (HUFAs), such as 22:6ω3 (docosahexaenoic acid, DHA), in *Artemia* affect

TABLE 30
Examples of intra–strain variability of 20:5ω3 (EPA) content in different populations of *Artemia*

***Artemia* population**	**20:5ω3 range (mg/g dry weight)**
Vinh Chau, Viet Nam	14.0–21.8
Great Salt Lake, United States of America	0.6–2.9
Bolshoe Yarovoe, Russian Federation	0.7–4.9
Lake Aibi, China	2.9–14.5

Note: Values represent a range (mg/g dry weight) and are based on a number of random samples, analysed at the Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University, Belgium. They thus do not necessarily represent the maximal range that can be found in the populations concerned.

Source: Authors' own élaboration (LAARC).

various aspects of (shell)fish larval performance, including the survival rate, growth, swim bladder inflation, survival after handling stress, (flatfish) pigmentation, and development in general, as has been found in numerous fish and crustacean species. Commercial provisions of *Artemia* cysts containing high EPA levels are limited and, consequently, these cysts are more expensive. Therefore, the use of the high–EPA cysts should be restricted to the feeding period when feeding of freshly hatched nauplii of a small size is required.

The levels of different categories of lipids, which together make the lipid profile (e.g. lysophospholipids, phospholipids, pigments, cholesterol and other neutral lipids), and how these evolve over different *Artemia* developmental stages (cysts, instar I, instar II) have been studied in detail in Great Salt Lake *A. franciscana* using thin layer chromatography (Table 31). The phospholipid phosphatidylcholine (PC), for example, is relatively abundant in cysts (about 22 percent of total lipids) but decreases in the two successive naupliar stages (to about 13 percent). Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) together make up about 9–10 percent throughout these three stages; also, phosphatidylserine (PS) content remains stable, albeit at a much lower level (≤ 1 percent). Some lysophospholipids, on the other hand, show an increase from cysts to instar II.

Generally, the abundance of neutral lipids in cysts (80 percent of total lipids, the remaining being polar lipids) and in nauplii (60 percent of total lipids) indicates a high availability of energy as storage lipids in these developmental stages of *Artemia*. In cysts, these neutral lipids constitute 67 percent of triacylglycerols, which decreases to 47–49 percent through development to instar I and instar II stages. Furthermore, while the free fatty acids (FFAs) and diacylglycerol (DAG) levels are very low in cysts (3.7 percent and 2.0 percent of total neutral lipids, respectively), their levels increase to 20 percent (FFA) and 6 percent (DAG) in both naupliar stages. Finally, cholesterol and cholesteryl–ester levels do not change much during *Artemia* development.

TABLE 31
Polar and neutral lipid classes in cysts, instar I nauplii and instar II metanauplii of *Artemia franciscana* from Great Salt Lake, United States of America

Polar and neutral lipid classes identified in cysts, instar I and instar II			
Polar lipid classes	**Cysts**	**Instar I % total lipids**	**Instar II**
LPC	0.9 ± 0.6	1.8 ± 0.4	2.6 ± 0.5
PI + SM + LPE	4.6 ± 1.5	4.0 ± 1.0	4.3 ± 1.1
PS	0.8 ± 0.1	0.6 ± 0.2	1.0 ± 0.3
PC	21.8 ± 5.6	12.9 ± 2.2	12.7 ± 1.3
PE + PG	9.7 ± 2.8	8.6 ± 0.9	9.3 ± 1.3
CL	1.8 ± 0.7	3.9 ± 0.8	4.1 ± 0.9
Neutral lipid classes	**% total neutral lipids**		
Chol–esters	21.3 ± 7.2	20.3 ± 8.1	20.7 ± 11.1
TAG	67.0 ± 3.5	49.0 ± 3.5	47.3 ± 6.6
FFA	3.7 ± 0.6	20.0 ± 1.0	20.3 ± 1.1
Chol	5.7 ± 2.9	4.0 ± 1.7	4.7 ± 2.1
DAG	2.0 ± 1.0	6.3 ± 1.5	6.3 ± 1.5

Note: Chol = cholesterol; Chol–esters = cholesterol esters; CL = cardiolipin; DAG = diacylglycerol; FFA = free fatty acids; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; PC = phosphatidylcholine; PE = phoshatidylethanolamine; PG = phosphatidylglycerol; PI = phosphatidylinositol; PS = phosphatidylserine; SM = sphingomyelin; TAG = triacylglycerol.

Source: Lopalco, P., Lobasso, S., Alfama Lopes Dos Santos, R. M., Van Stappen, G., & Corcelli, A. 2019. Lipid profile changes during the development of *Artemia franciscana*, from cysts to the first two naupliar stages. Frontiers in Physiology, 9. https://doi.org/10.3389/fphys.2018.01872

Following these observations, appropriate techniques have been developed for improving the lipid profile of HUFA–deficient *Artemia* strains (see Section 3.1.7). Successful enhancement of HUFAs together with polar lipids in enriched *Artemia* metanauplii has been obtained using either a combination of a commercial, neutral lipid–based HUFA–rich emulsion and soya lecithin, or HUFA–rich phospholipids. Phospholipids are structural constituents of biomembranes and are therefore highly demanded in the fast-growing larvae. Phospholipids are also involved in the digestion, absorption and transport of lipids from the intestine to the rest of the body. There are several indications that larvae are unable to synthesize phospholipids efficiently at a rate fast enough to cover their high demand; therefore, phospholipids need to be provided by the diet.

3.2.3.4. Glycerol and carbohydrate metabolism

Glycerol is present in dry cysts before hatching incubation to the extent of about 5 percent of the cyst dry weight (Clegg, 1962). This glycerol content in the embryo increases progressively during formation of the nauplius while also glycogen levels increase (see Table 32). The source of this glycogen is trehalose, a non-reducing disaccharide of glucose, which is present in the cysts at relatively high concentrations (up to 15–17 percent of dry weight) and which is the major storage substrate for the energy metabolism and carbohydrate synthesis in hydrated cysts. As explained more in detail in Section 1.5.2, the increased levels of glycerol result in increased osmotic pressure within the cyst, eventually leading to the breaking stage, as a function of salinity of the hatching medium: Earlier breaking will be attained in lower salinities.

TABLE 32
Glycerol and glycogen content of cysts (split up between embryo and shell) and of newly hatched *Artemia* nauplii (assuming that the embryo dry weight is 78 percent of the entire cyst dry weight)

Developmental stage	Glycerol (% of dry weight)	Glycogen (% of dry weight)
Entire cyst (shell + embryo)	4.91	1.13
Shell	0.19	0.04
Embryo	6.30	1.86
Newly emerged nauplius	4.85	15.1

Source: Clegg, J.S. 1962. Free glycerol in dormant cysts of the brine shrimp *Artemia salina*, and its disappearance during development. The Biological Bulletin, 123: 295–301. https://www.journals.uchicago.edu/doi/pdf/10.2307/1539275

3.2.3.5. Ash

The levels of certain minerals in *Artemia* are listed in Table 24. The mineral requirements of marine organisms are poorly understood, and it is generally accepted that they may be satisfied through the uptake of seawater. The main concern regarding the mineral composition of *Artemia* is whether they meet the requirements of fish or crustacean larvae reared in freshwater. The levels of trace elements, with the exception of iodine and zinc, are adequate in *Artemia*. The iodine concentration can easily be increased by dissolving potassium iodide in the water used for enrichment. Alternatively, iodine can be delivered by incorporating it in the enrichment emulsion itself. A similar strategy can be applied to increase the zinc content using zinc salts.

3.2.3.6. Vitamins

Vitamin levels have been analysed in freshly hatched nauplii of Great Salt Lake *Artemia* franciscana (Table 24). According to the requirements of juvenile and adult fish as references, most of the water soluble vitamins are present in adequate amounts in these unenriched *Artemia*. Exceptions are thiamine and cobalamine (Table 24). Of the lipid-soluble vitamins, vitamin A and vitamin E are well characterized, together with carotenoids, which are the provitamin A forms in live prey. Non-enriched live

prey normally do not contain vitamin A; however, *Artemia* contain large amounts of cantaxanthin. A stable form of the water-soluble vitamin C (ascorbic acid 2-sulphate) is present in *Artemia* cysts. This derivative is hydrolysed to free ascorbic acid during hatching; ascorbic acid levels in the range of 310–520 µg/g dry weight were found in freshly hatched *Artemia* of different strains (Merchie, Lavens and Sorgeloos, 1997). As far as is known, there are no data on typical levels of vitamin D and vitamin K in non-enriched *Artemia*, but these levels can be manipulated using vitamin-fortified enrichment emulsions.

3.2.3.7. Enzymes

The presence of several proteolytic enzymes in developing *Artemia* embryos and *Artemia* nauplii has led to the assumption that these exogenous enzymes play a significant role in the breakdown of the *Artemia* nauplii in the digestive tract of the predator larvae. This has become an important question in view of the relatively low levels of digestive enzymes in many first-feeding larvae coupled with the limited suitability of artificial feeds versus live prey. Encysted embryos and larvae of *A. franciscana* have been shown to contain a cysteine protease that represents over 90 percent of the protease activity in these organisms. It is thought that this cysteine protease in embryos and larvae of *Artemia* functions in yolk utilization as a hatching enzyme in apolysis during the moulting cycle and as a digestive enzyme when the swimming larvae begin to feed. Furthermore, large amounts of two serine proteases are found in the hatching incubation medium during the first two larval stages of *A. franciscana*, concomitant with N acetyl-β-glucosaminidase, a well-described chitin degrading enzyme. These proteases were found to be nearly identical to two serine proteases synthesized during early larval development (Warner *et al.*, 1995; Warner and Matheson, 1998) in *Artemia* and thought to be digestive enzymes. The appearance or secretion of these serine proteases into the hatching medium during larval development is required for apolysis and ecdysis and appears to be dependent on cysteine protease activity in the epidermal layer under the cuticle. A systematic study of the levels of various enzymes in decapsulated cysts and in successive instar stages has been done for a number of parthenogenetic *Artemia* strains from coastal saltworks in Greece (Table 33).

TABLE 33

Enzymatic activities (mmoles/100 µg dry weight within 5 minutes) determined in parthenogenetic Greek *Artemia* populations (Milos, Polychnitos and Messolongi saltworks) at different developmental *Artemia* stages

Enzyme (mmoles/100 µg dry weight within 5 min)	Decapsulated cysts	Instar I	Instar II	Instar III
Alkaline phosphatase	0.57	27.19	66.09	68.99
Trypsin	0.00	0.00	0.00	3.12
Leucine aminopeptidase	12.84	101.77	140.90	92.45
Valine aminopeptidase	2.06	11.74	24.88	16.56
Cystine aminopeptidase	0.38	9.07	21.55	13.83
Lipase (C14)	0.20	0.00	4.63	3.12
Esterase lipase (C8)	1.69	12.85	18.50	15.81
Esterase (C4)	2.07	24.39	45.47	32.43
Acid phosphatase	12.43	22.19	28.36	19.76
β-Galactosidase	10.95	77.27	133.62	79.59
β-Glucosidase	1.89	12.88	21.78	14.71
N-acetyl-β-glucosaminidase	11.63	19.54	35.17	25.62
α-Fucosidase	5.24	15.63	26.42	15.71
Naphtol-AS-BI-phosphohydrolase	0.95	5.16	14.99	8.25

Source: Moraiti-Ioannidou, M., Castritsi-Catharios, J., Miliou, H. & Kotzamanis, Y.P. 2007. Fatty acid composition and biometry of five Greek *Artemia* populations suitable for aquaculture purposes. *Aquaculture Research*, 38(15): 1664–1672. https://doi.org/10.1111/J.1365-2109.2007.01835.X

3.2.3.8. Analytical methods

The nutrient composition of *Artemia* can be measured by ISO (International Organization for Standardization) certified routine methods. Table 34 presents an overview of the most common biochemical methods with the analytical principles used along with the related literature references.

TABLE 34
Analytical methods for different nutrients

Analyte	Analytical method – principle	Reference
Dry matter	Gravimetric method (103 °C – 4 h)	ISO 6496:2001
Protein*	Kjeldahl analysis	García–Ortega *et al.* (1998)
	LECO N Analyzer	Hamre and Mangor–Jensen (2006)
Total amino acids	Hydrolysis, derivatization and HPLC analysis	Espe *et al.* (2006)
Free amino acids	HPLC and post-column derivatization	Srivastava *et al.* (2006)
Total lipids	Folch	Folch *et al.* (1957)
Fatty acids	Transmethylation extraction and GC/FID	Lepage and Roy (1984)
Lipid classes	HPTLC	Jordal *et al.* (2007)
Total carbohydrate	Phenol-sulphuric acid reaction	García–Ortega *et al.* (1998)
Glycogen	Hydrolysis and spectrometric detection	Hemre *et al.* (1989)
In vitro digestibility	Modified filtration method from Babinszky *et al*. (1990)	García–Ortega *et al.* (1998)
Thiamine = vitamin B1	HPLC	CEN (2003a)
Riboflavin = vitamin B2	HPLC	CEN (2003a)
Niacine = vitamin B3	Microbiological	Mæland *et al*. (2000)
Folic acid = vitamin B9	Microbiological	Mæland *et al*. (2000)
[Sum]Vitamin B6	HPLC	CEN (2005)
Vitamin C	HPLC	Maeland and Waagbø (1998)
[Sum]Vitamin A	HPLC	CEN (2000a)
[Sum]Vitamin D	HPLC	CEN (1999)
[Sum]Vitamin E	HPLC	CEN (2000b)
[Sum]Vitamin K	HPLC	CEN (2003b)
Microminerals	ICP–MS	Julshamn, Dahl and Eckhoff (2001)
Macrominerals	ICP–MS	Liaset, Julshamn and Espe (2003)
Iodine	ICP–MS	Julshamn, Dahl and Eckhoff (2001)
Total protease	Modified casein method from Walter (1984) on homogenized and sonicated sample	García–Ortega *et al.* (1998)
Trypsin activity	Modified method of Hofer and Köck and Bergmeyer	García–Ortega *et al*. (1998)

* Protein is calculated as N × 6.25 (general Jones factor); for *Artemia* instar I and II nauplii N × 4.7 should be used; for ongrown *Artemia* N × 5.3.

Note: GC/FID = gas chromatography flame ionization detector; HPLC = high-performance liquid chromatography; HPTLC = high-performance thin-layer chromatography; ICP–MS = inductively coupled plasma mass spectrometry.

Source: Hamre, K., Erstad, B., de Kok, J., Norberg, B. & Harboe, T. 2020. Change in nutrient composition of *Artemia* grown for 3–4 days and effects of feeding on-grown *Artemia* on performance of Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae. Aquaculture Nutrition, 26: 1542–1554. https://onlinelibrary.wiley.com/doi/abs/10.1111/anu.13101

3.2.4. Microbiological aspects of the hatching process (by *Peter De Schryver*)

3.2.4.1. Introduction

Bacteria are by far the most important (and generally the only) types of microorganisms under study when considering the hatching process. The bacterial quality of the fed *Artemia* nauplii is an important factor in the culture success of fish and shrimp larvae. The presence and growth of bacteria during the *Artemia* hatching process should therefore be a continuous point of attention. Adequate analysis methods to evaluate

the bacterial populations during and after hatching, both in the hatching water and in the nauplii, are required for this purpose.

A plethora of technologies exist that can be used for bacterial analysis of samples, each with its own characteristics, advantages and disadvantages. In the following section, the most relevant technologies that are currently available for studying the bacterial composition of the hatching water and *Artemia* nauplii are briefly introduced.

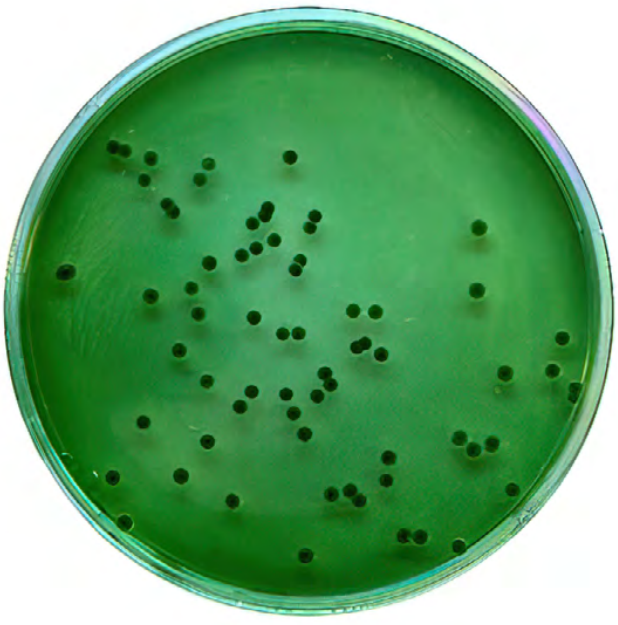
3.2.4.2. Agar plating

Agar plating is a cultivation-based method. A sample from the hatching water or from the *Artemia* nauplii is placed on a nutrient-rich agar plate, allowing the bacteria from the sample to grow on the plate. By subsequently counting the number of grown colonies, an estimation can be made of the number of bacteria in the analysed sample, which can then be recalculated to the number of "colony forming units (CFUs)/ml hatching water" or "colony forming units (CFUs)/nauplius".

Aside from non-selective agar media that allow a broad range of bacterial types to grow, selective compositions of the agar medium can also be used. The goal is then to allow specific bacterial groups from the sample to grow to get insight in the presence and number of bacterial groups of interest. In the context of *Artemia* hatching, the most widely used selective agar type is thiosulphate–citrate–bile salts–sucrose (TCBS) agar with the purpose to estimate the number of *Vibrio* in the sample (Plate 59).

Agar plating has been used for decades in microbiological research and environmental monitoring, including in the aquaculture industry. It is very widespread in use, as it is cheap, relatively fast and easy in execution. The main disadvantage of the method is that results are often misinterpreted. In the first place, on average only 1 percent of the bacterial populations in an environmental sample can grow on an agar plate. The remaining 99 percent is considered "unculturable". This is called the "great plate count anomaly". In practice, this means that the number of bacteria determined by plate counting is, on average, underestimated about 100 times. A second reason for misinterpretation is that the so-called selective media often are not completely selective, meaning that either bacterial populations from non targeted groups can grow on the selective medium, or that not all populations from a targeted group can grow on the selective medium. This is, for example, the case for TCBS agar. The results from the selective media should thus be used as an estimate for the bacterial group(s) of interest. When both disadvantages are taken into consideration when evaluating the results, the method can be of high value.

PLATE 59
TCBS plate



© LAARC

Agar plating is currently the most widespread method in use to evaluate bacterial quality of *Artemia* hatching water and *Artemia* nauplii, mainly because of its practicality and possibility to easily execute onsite. However, the easiness in execution of the method quickly leads to the feeling that "anyone can do it", which often results in an underestimated need to work accurately and with high care. Non-proper sampling and sample storage, inaccurate dilutions during sample preparation and contamination by bacteria not originating from the sample,

among others, easily lead to wrong results. A prerequisite is, therefore, that a good protocol is used and that this is followed carefully.

A protocol to determine the bacterial load in *Artemia* hatching water and in *Artemia* nauplii can be found in Annex 11.

3.2.4.3. 16S rRNA gene amplicon sequencing

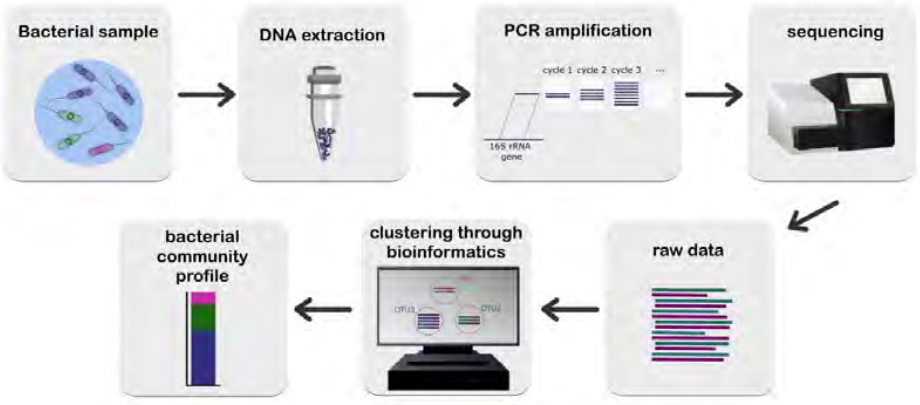
Amplicon sequencing is a bacterial community analysis method based on molecular detection and identification of all bacteria in a sample. Because it works by detecting the genes of the bacteria in the sample, the method works without the need for cultivation. The 16S ribosomal ribonucleic acid (rRNA) gene is the commonly used marker gene because it is found in all bacterial taxa and it contains both conserved regions and variable regions. The conserved regions ensure that all bacteria in the sample are included in the analysis, while the variable regions allow for a phylogenetic differentiation of the detected bacteria. Each detected bacterium in the sample is identified during the analysis, so that a list of taxonomic names – also called operational taxonomic units (OTUs) – is obtained.

In short, during 16S rRNA gene amplicon sequencing, the bacterial 16S rRNA gene (= DNA) from all bacteria in the sample is first extracted. A selected region (or fragment) of all extracted 16S rRNA genes in the sample is subsequently amplified (= copied) in a process called polymerase chain reaction (PCR). As a result, many fragment copies for each bacterium are obtained, and the higher the number of a specific bacterium in the original sample, the higher the number of fragment copies thus will be. Next, the genetic sequence of each copy is determined in a process called sequencing. The result is a huge set of known fragment sequences (= raw read data). During the step of clustering, the identical copies are then determined and counted, so that the number of different fragment sequences and the abundance of each become known. Finally, each different sequence is compared to a reference database to obtain the taxonomic identity (or the OTU) associated with those sequences. The outcome is a list of the different OTUs detected in the sample, each with its detected frequency, and thus this gives information on the composition of the microbial community in the sample (Figure 47).

FIGURE 47

Illustration of a typical workflow for investigating the composition and structure of a bacterial community

The DNA of a sample is extracted, after which a marker gene sequence – for example (part of) the 16S rRNA gene – is amplified using PCR and sequenced. The raw read data are processed through a bioinformatics pipeline to: (1) remove erroneous and low–quality data; and (2) construct discrete ecological units that comprise the biological diversity (e.g. operational taxonomic units). The resulting contingency table provides information on the bacterial community



Sources:

Props, R. 2018. Microbial population and community dynamics in natural and managed freshwater systems: From methodology development to mechanistic insights. Ghent University, Belgium. PhD dissertation. 282 pp. https://biblio.ugent.be/publication/8576485

Heyse, J. 2021. *Identifying drivers of microbial dynamics in aquaculture: from single–cell technology to metagenomics*. Ghent University, Ghent, Belgium PhD dissertation. 206 pp. https://lib.ugent.be/catalog/pug01:8734215

Although the method has become widely used for microbiological research and environmental monitoring, it is not straightforward in execution, not fast and not as cheap as agar plating. For aquaculture applications, it is therefore not often used for routine analysis but mainly in a research context. That being said, the possibilities to have such analysis executed are very broad, as many academic research institutes and commercial companies offer this service.

The main disadvantage of the method is that no absolute quantification of the OTUs detected in the sample is obtained. As a result of the amplification step, only relative abundances are obtained, or in other words, which percentage of the total bacterial community the OTU represents. The total number of bacteria in the sample and the number of each bacterial type are thus not known.

Ideally, taxa-specific genome abundances could be converted to more tangible cell numbers. However, this requires taxa-specific information on the ploidy, i.e. the number of genome copies per cell. Prokaryotes have historically been considered as monoploid (1 genome copy per cell). However, several recent studies have found oligoploid (<10 genome copies per cell) and polyploid (>10 genome copies per cell) archaea and bacteria, and it appears that monoploid prokaryotes are rather the exception than the rule. A correction for ploidy is difficult. Consequently, the accurate conversion between genome abundances and cell numbers as well as biomasses remains a challenge.

3.2.4.4. Flow cytometry

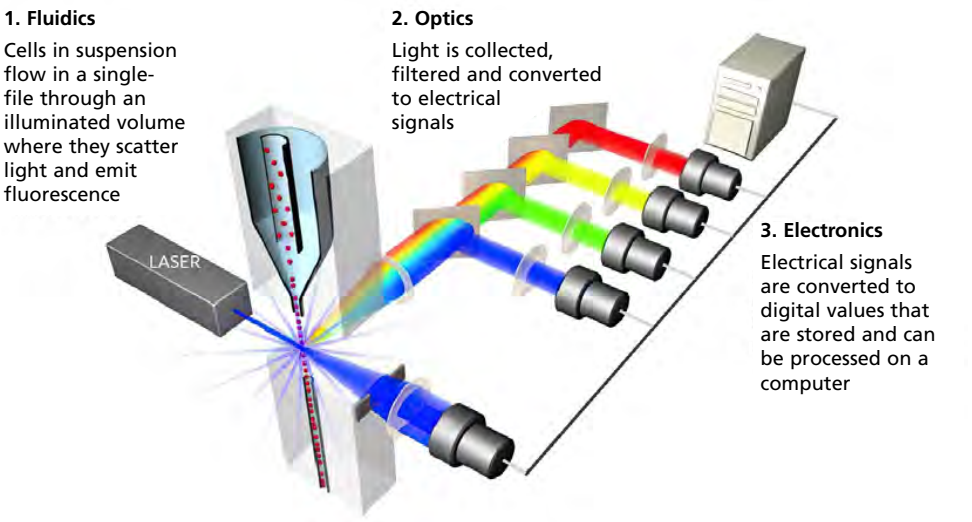
Flow cytometry is a bacterial community analysis technique that uses optical detection of individual bacteria in a sample, but that does not provide information on bacterial identities. The optical detection of each bacterial cell in the sample is achieved by fluorescently labelling them, and then passing them one by one in a directed fluid stream through a laser beam (Figure 48). When the laser beam hits a cell, this produces both visible light scattering and fluorescence, which are detected so that the cell is counted. As this is done for all bacteria in the sample, the total number of bacteria in the sample is counted. The technology allows to detect thousands of cells per second, and thus output for a sample can be generated within a matter of minutes.

In addition to simply counting, the signals from light scattering and fluorescence are also converted into electrical signals. As these signals differ in function of the phenotypic traits (physical and chemical structure) of the detected cells, flow cytometry allows to detect the different phenotypic populations of bacteria and their quantity and, as a result, the technique can also be used for microbial community profiling.

Although flow cytometry is a well-known technology in microbiological research and environmental monitoring, it is however only relatively recently that the technique is being used for microbial community profiling. Certainly, within an aquaculture context, the technology is still in a very initial phase of use, and only very few providers are able to offer flow cytometry as a service. Therefore, while undoubtedly the technology shows much potential, a main disadvantage is the fact that the bacteria within the community currently cannot be identified yet. For some needs, this clearly represents a limitation.

FIGURE 48

Schematic diagram of a flow cytometer. Source: Illustration by Thermo Fisher Scientific – Life Sciences Solutions



Source: Illustration by Thermo Fisher Scientific - Life Sciences Solutions.

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Annex 1

SALINITY CONVERSION TABLE

TABLE A1
Conversion table for various units of salinity for density readings of concentrated seawater[1]

Density (g/ml)	Degree Baumé (°Bé)	Salinity (g/L)	Density (g/ml)	Degree Baumé (°Bé)	Salinity (g/L)
1.020	2.8	28.6	1.061	8.4	
1.021	3.0		1.062	8.5	
1.022	3.1		1.063	8.7	
1.023	3.3		1.064	8.8	
1.024	3.4		1.065	8.9	
1.025	3.6		1.066	9.0	
1.026	3.7		1.067	9.2	
1.027	3.8		1.068	9.3	
1.028	4.0		1.069	9.4	
1.029	4.1		1.070	9.5	99.4
1.030	4.2	42.4	1.071	9.6	
1.031	4.4		1.072	9.7	
1.032	4.5		1.073	9.9	
1.033	4.7		1.074	10.0	
1.034	4.8		1.075	10.1	
1.035	4.9		1.076	10.2	
1.036	5.0		1.077	10.3	
1.037	5.1		1.078	10.5	
1.038	5.3		1.079	10.6	
1.039	5.4		1.080	10.7	114.1
1.040	5.5	56.4	1.081	10.8	
1.041	5.7		1.082	11.0	
1.042	5.8		1.083	11.1	
1.043	6.0		1.084	11.2	
1.044	6.1		1.085	11.3	
1.045	6.2		1.086	11.5	
1.046	6.4		1.087	11.6	
1.047	6.5		1.088	11.7	
1.048	6.7		1.089	11.8	
1.049	6.8		1.090	11.9	128.6
1.050	6.9	70.6	1.091	12.0	
1.051	7.0		1.092	12.1	
1.052	7.2		1.093	12.3	
1.053	7.3		1.094	12.4	
1.054	7.5		1.095	12.5	
1.055	7.6		1.096	12.6	
1.056	7.7		1.097	12.7	
1.057	7.9		1.098	12.8	
1.058	8.0		1.099	13.0	
1.059	8.1		1.100	13.1	144
1.060	8.2	84.9	1.101	13.2	

[1] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

TABLE A1 (CONTINUED)

Density (g/ml)	Degree Baumé (°Bé)	Salinity (g/L)	Density (g/ml)	Degree Baumé (°Bé)	Salinity (g/L)
1.102	13.4		1.141	17.8	
1.103	13.5		1.142	17.9	
1.104	13.6		1.143	18.0	
1.105	13.7		1.144	18.1	
1.106	13.8		1.145	18.2	
1.107	14.0		1.146	18.3	
1.108	14.2		1.147	18.5	
1.109	14.3		1.148	18.6	
1.110	14.4	159.5	1.149	18.7	
1.111	14.5		1.150	18.8	222.1
1.112	14.6		1.151	19.0	
1.113	14.7		1.152	19.1	
1.114	14.9		1.153	19.2	
1.115	15.0		1.154	19.3	
1.116	15.1		1.155	19.4	
1.117	15.2		1.156	19.5	
1.118	15.3		1.157	19.6	
1.119	15.4		1.158	19.7	
1.120	15.5	175.1	1.159	19.8	
1.121	15.6		1.160	19.9	237.8
1.122	15.7		1.161	20.0	
1.123	15.8		1.162	20.2	
1.124	15.9		1.163	20.3	
1.125	16.0		1.164	20.4	
1.126	16.2		1.165	20.5	
1.127	16.3		1.166	20.6	
1.128	16.4		1.167	20.7	
1.129	16.5		1.168	20.8	
1.130	16.6	190.6	1.169	20.9	
1.131	16.7		1.170	21.0	253.7
1.132	16.8		1.171	21.1	
1.133	16.9		1.172	21.2	
1.134	17.0		1.173	21.3	
1.135	17.1		1.174	21.4	
1.136	17.3		1.175	21.5	
1.137	17.4		1.176	21.6	
1.138	17.5		1.177	21.7	
1.139	17.6		1.178	21.8	
1.140	17.7	206.3			

Annex 2

FEEDING STRATEGY FOR INTENSIVE *ARTEMIA* CULTURE[2]

In the first week

- T* <15 cm; stop feeding and/or increase water renewal.
- <15 T <20 cm; maintain actual feeding ratio.
- T >20 cm; increase feeding ratio and/or add food manually.

In the second week

- T <20 cm; stop feeding and/or increase water renewal.
- <20 T <25 cm: maintain actual feeding ratio.
- T >25 cm; increase feeding ratio and/or add food manually.

From pre-adult stage: daily food ratio = 10% of WW** biomass/L culture water. The WW biomass/L is measured as follows

- Collect some liters of culture water over a sieve that withholds the animals.
- Rinse with tap water.
- Let water drip out and dip the sieve with paper cloth.
- Weigh the filter; WW biomass/L= (total weight - weight of empty filter)/ (volume of sampled culture water).

* T = transparency, as read with a Secchi disk

** WW = wet (live) weight

[2] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

Annex 3

DISINFECTION OF NON–DECAPSULATED *ARTEMIA* CYSTS[3]

Calculation of the volumes

The disinfection solution can be made up (in advance) of liquid bleach, i.e. sodium hypochlorite (NaOCl) in aqueous solution. For example, technical grade product of VWR Chemicals, labelled 14% Cl_2 (active chlorine), if fresh, has an activity generally in the range 14–15% w/w. Use the following proportions:

- 50–100 g cysts per litre seawater.
- Prepare a hypochlorite solution having 200 µL active chlorine/L or 10 000 µl/L (by diluting for example a commercial aqueous NaOCl solution with active chlorine concentration of minimally 14%). Different concentrations of the solution require a different incubation time (see below).
- Calculation: see Excel sheet 1 for practical example and detailed calculations.

Web link: EXCEL 1: Disinfection of *Artemia* cysts

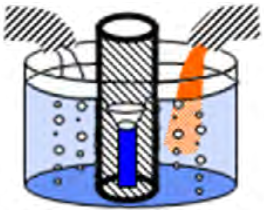
Hydration step

- Tank is filled with the calculated amount of seawater
- Add cysts
- Strong aeration is provided from an open tube
- Hydration time: 45 min.

Disinfection step

- Add calculated amount of sodium hypochlorite solution (NaOCl)
- Disinfect for:
  - 20 min in 200 µl/L active chlorine solution or
  - 1 min in 10 000 µl/L active chlorine solution

Washing step



- The cysts are harvested into a concentrator–rinser.
- Rinse cysts thoroughly with seawater
- Cysts are ready for hatching incubation

[3] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

Annex 4

PROCEDURE FOR THE DECAPSULATION OF *ARTEMIA* CYSTS[4]

Calculation of the volumes

The decapsulation solution can be made up (in advance) of liquid bleach, i.e. sodium hypochlorite (NaOCl) in aqueous solution. For example, technical grade product of VWR Chemicals labeled 14% Cl_2 (active chlorine), if fresh, has an activity generally in the range14–15% w/w. Use the following proportions:

- 0.5 kg active chlorine per kg of cysts
- 0.33 L sodium hydroxide (NaOH–technical grade) 30% in aqueous solution, per kg of cysts
- seawater to make up for the final solution with a volume of 14 L per kg of cysts.
- Calculation: see Excel sheet 2 for practical example and detailed calculations.

Web link: EXCEL 2: Decapsulation of *Artemia* cysts

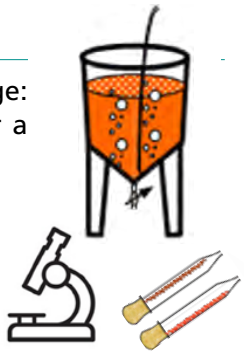
Hydration step

- Tank is filled with the calculated amount of seawater at 20 °C.
- Add cysts
- Strong aeration is provided from an open tube
- Hydration time: 50–60 min.

Decapsulation step

- After 50–60 min, add sodium hydroxide (NaOH)
- Then add the calculated amount of sodium hypochlorite (NaOCl)
- Finally add 250–500 µl antifoam/L (it's good practice to dissolve the antifoam beforehand in a small quantity of water, before adding it to the tank)

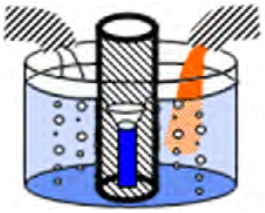
Monitoring the decapsulation process



- Follow the colour change of the cysts from brown to orange: this can be done by taking samples with a pipet, or under a binocular microscope.
- When the cysts are changing colour from brown–grey to orange and 90% of the cysts are sinking, the process is at its end.

[4] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

Washing and deactivation step



- The cysts are harvested into a concentrator-rinser filled beforehand with water and sodium thiosulfate ($Na_2S_2O_3$) at 50 g per kg cysts
- Rinsing is done at a flow range of ± 100% per minute
- Addition of 250–500 µl/L antifoam is recommended.
- Rinsing is continued until all chlorine is neutralized and the outflowing water is crystal clear.
- 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.

Annex 5

TITRIMETRIC METHOD FOR THE DETERMINATION OF ACTIVE CHLORINE IN HYPOCHLORITE SOLUTIONS[5]

Principle

Active chlorine will liberate free iodine from a potassium iodide (KI) solution at pH 8 or less. The liberated iodine is titrated with a standard solution using sodium thiosulfate ($Na_2S_2O_3$), with starch $(C_6H_6O_6)n$ as the indicator. Percent active chlorine is a unit of concentration used for hypochlorite-based bleaches (aqueous chlorine solutions). One gram of a 100% active chlorine bleach has the same bleaching power as one gram of chlorine. The term "active chlorine" is used because most commercial bleaches contain substantial amounts of chlorine in the form of chloride ions, which have no bleaching properties.

Reagents

Sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$) 0.1 mol/L:

In a volumetric flask (1 000 ml):

- Add the sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$) → 24.8 g
- Add distilled water to fill up the quantity to the correct volume of 1 L
- Shake the volumetric flask slightly until complete dissolution.

Starch solution of 10 g/L

In a 250 ml glass beaker, add:

- Distilled water → 100 ml
- Starch $(C_6H_6O_6)n$ → 0.5 g
- Insert a magnetic bar and place the beaker on the magnetic stirrer.
- Heat until boiling and until starch has completely dissolved.
- Allow to cool down to room temperature.

Indicator solution

In a 250 ml Erlenmeyer flask add:

- Distilled water → 50 ml
- Acetic acid, glacial (100 %) (CH_3COOH) → 5 ml
- Potassium iodide (KI) → a teaspoon
- 1 ml sample of hypochlorite solution

[5] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

Titration of the active chlorine

- Rinse and fill the burette with 0.1 mol/L sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$).
- Do the titration protected from direct sunlight and add carefully the sodium thiosulfate 0.1 M from the burette into the Erlenmeyer with the indicator solution. The yellow–brown colour of the liberated iodine must disappear.
- Add 1 ml of the starch solution $(C_6H_6O_6)n$; the solution should darken (dark blue).
- Using the burette, add sodium thiosulfate solution drop by drop until the liquid completely loses its colour and becomes clear.
- Wait 2 minutes and make sure that the solution has not turned blue again; if the solution is coloured once again, add more sodium thiosulfate, one drop at a time, until the liquid becomes colourless.

Calculation

- 1 ml of 0.1 mol/L sodium thiosulfate pentahydrate equals 3.54 mg active chlorine.

Annex 6

TANK PREPARATION AND *ARTEMIA* CYST HATCHING[6]

Some conditions need to be respected to ensure optimal cyst hatching at the hatchery:

Storage conditions

Store cysts in a dry place at temperature below +4 °C. Temperature above 4 °C can reduce the quality of the product. During storage the packaging should be kept carefully closed. Once opened, the product should be used immediately.

Cyst density

Cyst density of 2 g/L is advised.

Water salinity

For practical reasons, a salinity between 25–30 g/L is used.

External light

Optimal light conditions should be available during the complete hatching process and should be at least 2 000 lux at the water surface.

Water temperature

Optimal hatching can be achieved when the water is kept constant between 25–30 °C.

pH

Should be 8.0–8.5 during the entire hatching process; if necessary add dissolved sodium bicarbonate ($NaHCO_3$) or sodium carbonate (Na_2CO_3). Preferably add bicarbonate half an hour before incubation, and immediately before adding the cysts add also 120 mg/L of sodium hydroxide (NaOH). In general, a second dose of 120 mg/L of sodium hydroxide will be necessary after 12 h of hatching incubation.

Tank preparation

- Take out all removable parts (e.g. pipes) and clean them separately with soap; rinse and disinfect by immersion in chlorine (bleach) solution (150 mg/L active chlorine).
- Brush the tank thoroughly with soap after rinsing.
- Repeat exercise with chlorine solution.
- Rinse again extensively with water and fill the tank with filtered seawater. Make sure that all cysts and cyst shells are removed (e.g. remaining in outlet and in valves of the tank from previous hatching).

[6] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

- Disinfect the hatching water with e.g. 10 mg/L active chlorine and aerate gently for ±1 h.
- Deactivate any remaining chlorine by adding 8 mg/L sodium thiosulphate ($Na_2S_2O_3$).

Hatching protocol

- Check temperature.
- Open aeration (must be vigorous).
- Confirm water quality at start: take sample for microbiological analysis (see Annex 11).
- Weigh the required quantity of cysts.
- Switch on lights above hatching tank.
- Add cysts to the hatching tank.
- Check pH.

Annex 7

ARTEMIA INSTAR I NAUPLII HARVESTING[7]

- Cover the top of the hatching tank.
- Stop aeration.
- Allow the separation of nauplii and shells during about 10 min.

During this period, unhatched cysts will sink, the nauplii will migrate to the bottom of the tank and the empty shells will rise in the water and will collect on the surface of the tank. Since nauplii are positively phototactic, their concentration into the lower part of the tank 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.

- After about 5–10 min of separation, start draining the contents of the hatching tank. Nauplii should not be kept for too long (i.e. maximum 5 to 10 min) in the point of the conical container, to prevent dying off due to oxygen depletion.
  - ✔ Firstly, unhatched cysts and other debris that have accumulated underneath the nauplii are flushed and removed.
  - ✔ The nauplii are then collected on a net using a fine mesh screen (<150 µm), which should be submerged all the time so as to prevent physical damage of the nauplii, or better in a concentrator–rinser device (see Plate).
  - ✔ Stop draining the hatching tank and keep the floating cysts in the hatching tank.

The *Artemia* nauplii are then rinsed thoroughly with clean and disinfected seawater in order to remove possible contaminants and hatching metabolites like glycerol and *Vibrio* that has developed in the hatching tank once the glycerol became available as a suitable substrate for its proliferation. Installation of automated systems simplify production techniques in commercial operations (i.e. by the use of a concentrator–rinser (Plate A7.1), enable fast harvesting of large volumes of *Artemia* nauplii and allow complete removal of debris from the hatching medium. This results in a significant reduction of labour and production costs.

After thorough rinsing with clean, disinfected water, the nauplii can be fed directly to the larvae or stored in cold conditions (see Section 3.1.8.) or transferred to a prepared tank for enrichment (see Section 3.1.7.).

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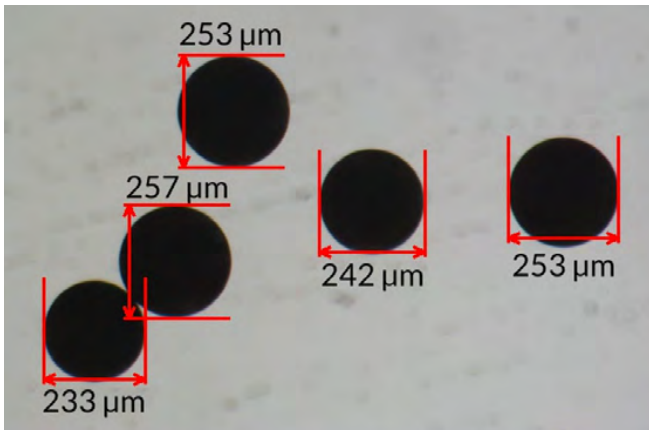
PLATE A7.1
Examples of operational concentrator–rinsers

[7] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

Annex 8

DETERMINATION OF *ARTEMIA* CYST DIAMETER, CHORION THICKNESS, LENGTH AND DRY WEIGHT (DW) OF THE INDIVIDUAL INSTAR I NAUPLIUS[8]

PLATE A8.1
Cyst diameter of non–decapsulated Great Salt Lake cysts



Cyst diameter of a non–decapsulated cyst

- Incubate a small sample of cysts in 35 g/L seawater; add 1% lugol solution (see below); incubate for 2 h.
- Add again 1% lugol solution.
- When measuring manually, measure cyst diameter of at least 100 fully hydrated cysts with a microscope, equipped with an eyepiece with a calibrated ruler (Plate A8.1).
- Calculate mean value and standard deviation.

Cyst diameter of a decapsulated cyst

- Incubate a small sample of cysts in 35 g/L seawater for 1 h.
- Decapsulate cysts with sodium hydroxide (NaOH) and sodium hypochlorite (NaOCl); check under microscope if decapsulation is complete.
- Rinse cysts well and incubate in seawater, add 1% lugol and incubate for 1 h more.
- Add again 1% lugol solution.
- When measuring manually, measure cyst diameter of at least 100 fully hydrated decapsulated cysts with a microscope, equipped with an eyepiece with a calibrated ruler.
- Calculate mean value and standard deviation.

Chorion thickness

- Calculate the chorion thickness:

$$\frac{\text{average cyst diameter of a non decapsulated cyst} - \text{average cyst diameter of a decapsulated cyst}}{2}$$

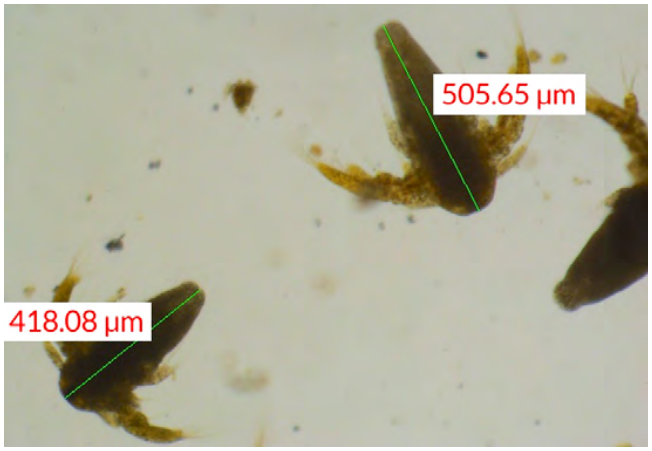
Instar I length

- Incubate cysts under standard hatching conditions (See Annex 6). Harvest nauplii after 22 h (to ensure a maximum of nauplii still in the instar I stage); fixate the nauplii with lugol solution.
- When measuring manually, measure the nauplius length of at least 100 instar I nauplii with a microscope, equipped with an eyepiece with a calibrated ruler (Plate A8.2).

[8] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

- Calculate mean value and standard deviation.

PLATE A8.2
Instar I nauplii length measurement



Determination of the individual instar I nauplius dry weight (DW)

- Incubate a small amount of cysts for hatching in standard hatching conditions (see Annex 6).
- Harvest the *Artemia* after 22 h (to ensure a maximum of nauplii still in the instar I stage) and concentrate in a cylindroconical tube; provide aeration from the bottom.
- Determine accurately (triplicate counts) the concentration of the nauplii in the cylindroconical tube (= Y nauplii/ml) by taking 3 subsamples of 250 µl with a micropipette and counting the nauplii under a binocular.
- Filter an exact volume (= Z ml) of nauplii suspension on pre–weighed glass microfibre filters (= A g) using a Büchner setup (in triplicate).
- Calculate the total amount of filtered nauplii: $Y \frac{nauplii}{ml} \times Z\ ml = N\ nauplii$
- Wash the filter with a solution (0.5 mol/L) of ammonium formate (NH_4HCO_2) to remove salts.
- Dry the filters at 103 °C for 4 h to volatilize the ammonium formate.
- Weigh the filters (= B g).
- Calculate the individual instar I nauplius DW (in µg):

See Excel sheet 3 for practical example and detailed calculations.

Web link: EXCEL 3: Dry Weight of 1 *Artemia*

NOTE: Composition and preparation of lugol solution

SOLUTION 1: weigh 300 g potassium iodide (KI) and 150 g iodine (I_2) in a glass beaker and add 600 ml distilled water. Stir (with magnetic stirrer) until dissolved.

SOLUTION 2: weigh 150 g sodium acetate ($NaCH_3COO$) in a glass beaker and add 1 500 ml distilled water. Stir (with magnetic stirrer) until dissolved.

Mix solution 1 and 2; the lugol solution can be stored at room temperature, but in darkness.

Annex 9

DETERMINATION OF WATER CONTENT (PERCENT H_2O) OF *ARTEMIA* CYSTS[9]

- Tare 3 small aluminium foil cups = T_1, T_2, T_3.
- Fill each cup with cyst sample, determine the gross weight = tare weight + wet weight of cyst sample = W_1, W_2, W_3.
- Calculate mass of the sample = M_1, M_2, M_3.
- Place the filled aluminium foil cups in a drying oven: 4 h at 103 °C.
- Determine gross water-free weight = tare weight + dry weight of cyst sample = D_1, D_2, D_3.
- Calculate the water content for each of three replicates:

$$\text{Water content (in \%)} = \frac{(T + M - D)}{M} \times 100$$

- Calculate water content per g dry weight:

$$\text{g } H_2O \text{ / g dry weight} = \frac{\frac{(T + M - D)}{M}}{1 - \left(\frac{T + M - D}{M}\right)} \times 100$$

- Calculate average and standard deviation of the three replicates.

See Excel sheet 4.1 and 4.2 for practical example and detailed calculations.

Web link: EXCEL 4.1: Determination of water content of *Artemia* (%H_2O)
EXCEL 4.2: Determination of water content of *Artemia* (g H_2O/g DW)

[9] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

Annex 10

DETERMINATION OF CYST COUNT, HATCHING PERCENTAGE, HATCHING EFFICIENCY AND HATCHING RATE OF *ARTEMIA* CYSTS[10]

For quality control of *Artemia* cysts, always work under standard conditions (see Plate A10.1)

- Use preferentially a cylindroconical or conical tube (e.g. Imhoff cone) or a graduated cylinder. Run test in triplicate.
- Incubate exactly 2 g cysts in exactly 1 000 ml of 32 g/L seawater.
- Apply continuous illumination (>2 000 lux).
- Keep temperature constant at 28 °C.
- Provide aeration from the bottom as to keep all cysts in suspension (aeration not too strong as to prevent foaming; oxygen should be >2 mg/L).
- pH should be between 8.0 and 8.5.

PLATE A10.1
Set–up for hatching quality control

Cyst count

- After 2 h incubation (= fully hydrated cysts) take 6 subsamples of 250 µl each out of each hatching cone with a micropipette.
- Pipet each subsample into a small vial and fixate cysts by adding a few drops of lugol solution (for preparation lugol: see Annex 8).
- Per hatching cone (= 6 subsamples), count cysts under a microscope and calculate the mean value per cone.
- Calculate the average and standard deviation per 3 cones:

$$Cyst\ count = \frac{(cysts \times 4 \times 1000)}{2} \quad or\ Cyst\ count = cysts \times 2000$$

(= multiplying the number of counted cysts with the conversion factor 2 000)

See Excel sheet 5 for practical example and detailed calculations.

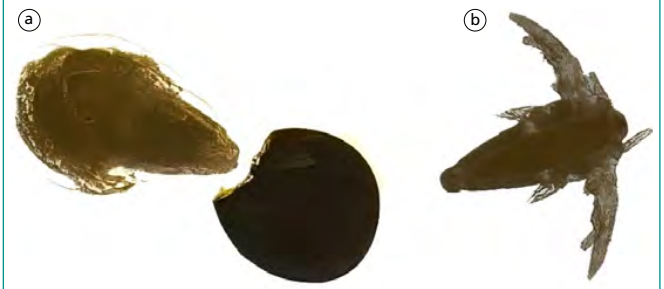
Web link: EXCEL 5: Cyst count

[10] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

Hatching percentage (H%) and hatching efficiency (HEff)

- After 24 h incubation take 6 subsamples of 250 µl each out of each hatching cone.
- Pipet each subsample into a small vial and fixate nauplii by adding a few drops of lugol solution.
- Per cone (= 6 subsamples), count nauplii under a microscope and calculate the mean value of the nauplii; count umbrellae and calculate mean value of the umbrellae (Plate A10.2).

PLATE A10.2
Umbrella (a) and instar I (b) nauplius



- Decapsulate unhatched cysts and dissolve empty cyst shells by adding a few drops of sodium hypochlorite (NaOCl) to each vial.
- Per cone, count unhatched (orange coloured) embryos and calculate mean value.
- Calculation of hatching percentage:

$$H\% = \left(\frac{nauplii}{nauplii + umbrellae + embryos}\right) \times 100$$

$$H+\% = \left(\frac{nauplii + umbrellae}{nauplii + umbrellae + embryos}\right) \times 100$$

- Palculation of hatching efficiency:

$$HEff = \frac{nauplii \times 4 \times 1000\ \text{ml}}{2\ \text{g}} \quad \text{or EEff} = nauplii \times 2000$$

$$HEff+ = \frac{(nauplii + umbrellae) \times 4 \times 1000}{2}$$

See Excel sheet 6 for practical example and detailed calculations.

Web link: EXCEL 6: Cysts hatching percentage and hatching efficiency

Ratio full and empty cysts

- Calculate the number of full cysts per gram cyst sample, using hatching percentage and hatching efficiency:

$$Full\ cysts = \frac{Hatching\ efficiency}{Hatching\ percentage} \times 100$$

- Calculate the percentage of full cysts and percentage of empty cysts using the number of full cysts per gram, and the cyst count value (see above):

$$\%\ Full\ cysts = \frac{Fulll\ cysts}{Cyst\ count} \times 100$$

$$\%\ Empty\ cysts = 100 - \%\ full\ cysts$$

Hatching rate

- Start taking subsamples and calculating the hatching efficiency from 8 h incubation onwards (follow procedure above).
- Continue sampling/counting procedures until mean value for hatching efficiency remains constant for 3 consecutive hours.
- The mean values per hour are then expressed as percentage of this maximal hatching efficiency.
- A hatching curve can be plotted; T_{10}, T_{90} and other T values can be extrapolated from the graph.
- A simplified procedure consists in sample taking e.g. every 3 or more hours.

See Excel sheet 7 for practical example and detailed calculations.

Web link: EXCEL 7: Cysts hatching rate and hatching synchrony

Annex 11

DETERMINATION OF THE BACTERIAL LOAD IN *ARTEMIA* HATCHING WATER OR IN HATCHED *ARTEMIA* NAUPLII[11]

Sample preparation for *Artemia* hatching water

- Take a 20 ml subsample from 1 L *Artemia* hatching water with a sterile pipette and immediately bring it over a sterile 40 µm cell strainer to remove particles.
- Collect the filtrate in a sterile vial. This represents dilution 10^0.
- Proceed with the rest of the protocol as soon as possible, and do not leave the filtrate standing at room temperature as the bacterial numbers can change quickly. If it is not possible to further process the sample immediately, store the filtrate at 4 °C but analyze within a matter of hours.
- The rest of the steps need to be executed in a sterile way. So, either work in a laminar flow, or very close to a burning Bunsen burner.
- Make a 10-fold dilution series of the filtrate:
  - Using a sterile pipette, transfer 1 ml of the filtrate into a sterile test tube filled with 9 ml sterile seawater. Close the test tube and homogenize. This represents dilution 10^{-1}.
  - Then using another sterile pipette, transfer 1 ml from the test tube with dilution 10^{-1} into a second test tube filled with 9 ml sterile seawater. Close the second test tube and homogenize. This represents dilution 10^{-2}.
  - Repeat this process to make dilutions 10^{-3}, 10^{-4}, 10^{-5}, … and so on until the target dilution is reached. The target dilution 10^{-X} is the dilution that results in the number of colonies being between 30 and 300 following growth on the agar plate. Less than 30 colonies is considered as not acceptable for statistical reasons, while more than 300 colonies is considered as too difficult to clearly distinguish the separate CFUs (colony forming units).
  - Finally, make 1 more dilution $10^{-(X+1)}$.
  - When doing plating for the first time, the target dilution is not known and so enough dilutions need to be prepared. Based on experience, the target dilution becomes known after some times of doing agar plating.
- The dilutions are now ready for agar plating (see below: Spreading of dilution samples on the agar plates).

Sample preparation for *Artemia* hatching water

- Take approximately 1 L *Artemia* hatching water, and separate the hatched nauplii from unhatched cysts and empty cyst shells (see Section 3.1.6). All tools used during the separation step should be sterile when brought in contact with the hatching medium or nauplii to prevent contamination
- Gently rinse the collected *Artemia* nauplii with 1 L sterile seawater on a clean disinfected sieve, and then transfer them into a clean 1 L container filled with clean disinfected seawater.

[11] Produced by the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium.

- Bring up the water volume in the container with the *Artemia* to 1 L with sterile seawater, and apply gentle aeration to the 1 L container
- Take a 100 ml volume out of the 1 L container with a sterile volumetric pipette and transfer onto a 40 µm sterile cell strainer. Make sure that all nauplii from the 100 ml are put on the cell strainer, which is very important.
- The rest of the steps need to be executed in a laminar flow, or very close to a burning Bunsen burner.
- Rinse the nauplii that are on the cell strainer 3 times with 10 ml sterile seawater, and then dry the bottom of the cell strainer with a clean tissue paper.
- Transfer all the nauplii from the cell strainer into a 50 ml sterile vial (when a mixer is used in the next step) or into a sterile stomacher bag (when a stomacher lab blender is used in the next step). Do this by turning the cell strainer upside down above the vial or bag and rinsing the bottom with exact 10 ml sterile seawater using a sterile 10 ml pipette.
- Homogenize the *Artemia* nauplii in the vial or stomacher bag using a laboratory mixer (for example Ultra Turrax mixer) or stomacher lab blender, respectively, as follows:
- When using a laboratory mixer, it first needs to be cleaned. Fill 3 clean plastic vials with a volume that allows to submerge the mixing part of the mixer. The liquids to be added in each are:
  - ✔ Vial 1: sterile seawater
  - ✔ Vial 2: ethanol (EtOH)
  - ✔ Vial 3: antiseptic cleaning liquid
    - – Mix the antiseptic cleaning liquid with the mixer for a few seconds, followed by drying the mixer with a clean paper tissue. Then mix the ethanol for a few seconds with the mixer, followed by drying the mixer with a clean paper tissue. Finally, mix the seawater for a few seconds, followed by drying the mixer with a clean paper tissue.
    - – When multiple *Artemia* samples need to be mixed with the mixer, repeat this cleaning process in between each sample.
- When using a stomacher laboratory blender, no specific cleaning steps need to be taken.
- Mix the nauplii in the sterile vial using the mixer at high speed, or homogenize the nauplii in the sterile stomacher bag at high speed, until only debris is observed and no more intact *Artemia* nauplii are present in the suspension. This *Artemia* nauplii debris suspension represents dilution 10^0.
- Proceed with the rest of the protocol as soon as possible, and do not leave the *Artemia* nauplii debris suspension standing at room temperature as the bacterial numbers can change quickly. If it is not possible to further process the sample immediately, store it at 4 °C but analyze as soon as possible.
- Make a 10-fold dilution series of the *Artemia* nauplii debris suspension:
  - ✔ Using a sterile pipette, transfer 1 ml of *Artemia* nauplii debris suspension into a sterile test tube filled with 9 ml sterile seawater. Close the test tube and homogenize. This represents dilution 10^{-1}.

- Then using another sterile pipette, transfer 1 ml from the test tube with dilution 10^{-1} into a second test tube filled with 9 ml sterile seawater. Close the second test tube and homogenize. This represents dilution 10^{-2}.
- Repeat this process to make dilutions 10^{-3}, 10^{-4}, 10^{-5} and so on until the target dilution is reached. The target dilution 10^{-X} is the dilution that results in the number of colonies being between 30 and 300 following growth on the agar plate.
- Finally, make 1 more dilution $10^{-(X+1)}$.
- When doing plating for the first time, the target dilution is not known and so enough dilutions need to be prepared. Based on experience, the target dilution becomes known after some times of doing agar plating.

Determine the nauplii density in the 1 L container from which the nauplii were sampled for plating:

- Take 1 mL sample out the 1 L container using a 1 000 µl pipette. Make sure the opening of the pipette tip is large enough to allow the *Artemia* nauplii to be sampled.
- Transfer the sample onto a petri dish foreseen of a counting grid, and that contains a thin layer of seawater.
- Repeat the 2 previous steps 10 times to make 10 petri dishes containing *Artemia* nauplii.
- Add to each petri dish a few drops of Lugol solution to fix and stain the *Artemia* nauplii.
- Count the amount of *Artemia* nauplii on each petri dish.
- Calculate the average density of the *Artemia* nauplii (***Artemia*/ml**) in the 1 L container by taking the average of the 10 petri dishes.
- Multiply this volume with 100 to obtain the number of *Artemia* nauplii in the *Artemia* nauplii debris suspension (**#*Artemia* / *Artemia* nauplii debris suspension**). This value is needed to calculate the bacterial load in the *Artemia* nauplii (see below: Calculate the bacterial load).
- **#*Artemia* / *Artemia* nauplii debris suspension = *Artemia* / ml × 100 ml**

Spreading of dilution samples on the agar plates

- The spreading of dilution samples needs to be executed in a sterile way. So, either work in a laminar flow, or very close to a burning Bunsen burner.
- Plate each of the dilutions from the *Artemia* hatching water or from the *Artemia* nauplii debris suspension on the agar medium of choice (typically Marine agar or TCBS agar):
  - Take 2 sterile plastic Drigalski spatulas, or alternatively take 2 metal Drigalski spatulas and put them in ethanol.
  - In case of metal spatulas, hold them for 2 seconds in the flame of the Bunsen burner until the ethanol has evaporated. Let the metal Drigalski spatulas cool down. Now they are sterile: do not touch the spatulas until needed to spread the samples on the plate.

- ✔ Vortex the highest dilution (= most diluted sample), take 100 µl using a micropipette with a sterile pipette tip, and put it on a non-used agar plate labeled with "$10^{-(x+1)}$, A". Repeat exactly the same using a new sterile pipette tip, but put this second sample on another agar plate labeled "$10^{-(x+1)}$, B". These two plates are duplicates.
- ✔ Repeat the previous steps for all dilutions to be plated. This results in the following set of agar plates, each containing 100 µl of diluted sample:
  - $10^{-(X+1)}$, A and $10^{-(X+1)}$, B
  - 10^{-x}, A and 10^{-x}, B
  - $10^{-(X-1)}$, A and $10^{-(X-1)}$, B
  - $10^{-(X-2)}$, A and $10^{-(X-2)}$, B
  - …
  - 10^{0}, A and 10^{0}, B

  When – based on experience – the target dilution is known, only the following plates can be inoculated with 100 µl: $10^{-(X+1)}$, 10-x, $10^{-(X-1)}$
- ✔ Take the "A" plate with the highest dilution $10^{-(X+1)}$, and take a sterile Drigalski spatula.
- ✔ Spread the 100 µl sample homogeneously over the plate by moving the Drigalski spatula from left to right and from bottom to top of the plate. Next, keep the plate open shortly until it is dry and close the plate again.
- ✔ Proceed to "A" plate of the second highest dilution 10^{-X} and repeat the spreading procedure. There is no need to disinfect the spatula in between.
- ✔ Repeat this for all "A" plates going from highest dilution to lowest dilution.
- ✔ Next, repeat the previous 3 steps for the duplicate "B" plates but use the other Drigalski spatula.
- ✔ When all plates have been spread, seal them with parafilm, and incubate for 48 h at 28–30 °C upside down.

Colony forming units (CFU) counting

- ▶ After 24 h of incubation, the number of colonies on each plate is counted a first time and written down. The colonies counted are indicated with a marker on the lid or bottom of the plates. Following this first counting, the plates are incubated for another 24 h at 28–30 °C upside down. After this additional 24 h of incubation (= 48 h of incubation in total), the colonies that have grown between 24 h and 48 h are counted as well, and added to the number of colonies at 24 h to obtain the total number of CFU.
- ▶ Plates with <30 colonies and >300 colonies per plate, are not taken into account to calculate the bacterial load. Less than 30 colonies is considered as not acceptable for statistical reasons, while more than 300 colonies is considered as too difficult to clearly distinguish the separate CFUs.
- ▶ Colonies that overlap and that do not have a clearly separated nucleus are counted as 1 colony.
- ▶ When using TCBS agar, green and yellow colonies must be counted.

Calculate the bacterial load

- Before calculating the bacterial load, it should be checked if the "A" plates and "B" plates of the same dilution show a similar number of colonies (within the same 10-fold). If not, the protocol has not been executed correctly and the results will not be reliable.
- To calculate the bacterial load in the *Artemia* hatching water "CFU/ml hatching water", the following formula is used:

$$\text{CFU/ml} = [Z / V_{tot}]$$

Where:
Z = sum of all colonies counted on all plates across dilutions and duplicates after 48 h of incubation;

$V_{tot.}$ = sum of all volumes (in ml) of sample inoculated on all the plates.

V_{tot} is calculated as:

$$V_{tot} = n \times V \times (d_1 + d_2 + ... + d_{x+1})$$

Where:
n = number of replicate plates per dilution = 2 (plate A and plate B);

V = volume of sample inoculated on each plate in ml = 0.1 ml;

$d_1 + d_2 + ... + d_{x+1}$ = dilutions of samples inoculated on the plates (d = 1 for dilution 10^0, d= 0.1 for dilution 10^{-1}, d = 0.01 for dilution 10^{-2}, etc, until d = 0.00…01 for dilution $10^{-(x+1)}$).

- If less than 30 colonies are present on plates for dilution 10^0, the result is shown as < 300 CFU/ml.
- If more than 300 colonies grow on the plates for the highest dilution $10^{-(X+1)}$, the result is shown as $>3.0 \times 10^{(X+3)}$ CFU/ml).
- To calculate the bacterial load in the *Artemia* nauplii, first the bacterial load in *Artemia* nauplii debris suspension "CFU/ml *Artemia* nauplii debris suspension" needs to be calculated. This is done exactly the same as for the bacterial load in the *Artemia* hatching water, but now using the colony counts from the plates inoculated with the (dilutions of the) *Artemia* nauplii debris suspension. This results in the number of "CFU/ml *Artemia* nauplii debris suspension".
- Next, the bacterial load per *Artemia* nauplius (CFU/*Artemia* nauplius) can be calculated in steps by considering the folowing formulas:
  - ✔ To calculate the bacterial load in the *Artemia* nauplii debris suspension, the following formula is used:

$$\mathbf{CFU}_{\text{ArtSus}} = \text{CFU/ml } \textit{Artemia} \text{ nauplii debris suspension} = [Z / V_{tot}]$$

Where:
Z = sum of all colonies counted on all plates across dilutions and duplicates after 48 h of incubation;

$V_{tot.}$ = sum of all volumes (in ml) of sample inoculated on all the plates.

V_{tot} is calculated as:

$$V_{tot} = n \times V \times (d_1 + d_2 + ... + d_{x+1})$$

Annex 12

THE INTERNATIONAL ARTEMIA AQUACULTURE CONSORTIUM

More than 40 years ago the International Study on *Artemia*, an interdisciplinary study on *Artemia* species and strains, generated foundational knowledge that led to important developments in fish and crustacean aquaculture. Through this study, the publication of the first *Artemia* manual (Sorgeloos *et al*., 1986) and organisation of local training courses, many new *Artemia* resources were identified, and the use of cysts in hatcheries expanded.

Today, *Artemia* underpin the production of around 10 million tonnes of high value aquaculture product. Around 3 500–4 000 tonnes of cysts are consumed annually, but about 90 percent of these cysts are still harvested from natural salt lakes, which are at constant risk from human activities and climatic variation. A sustainable supply of cysts from alternative sources must be developed to assure supply to hatcheries for aquaculture seed production.

The International *Artemia* Aquaculture Consortium (IAAC) is a new initiative that brings together 46 universities and R&D institutions from over 30 countries, hosted by the Network of Aquaculture Centres in Asia-Pacific. The consortium aims to guarantee the sustainable provision of *Artemia* to the aquaculture industry and to push the frontier of knowledge forwards, exploring new opportunities in:

- Conservation of *Artemia* biodiversity and salt lake habitats.
- Use of science-based protocols/guidelines for sustainable harvesting of wild sources.
- Socio-economic opportunities for integration of *Artemia* production as extra income in the many seasonal artisanal salt farms in Asia and Africa.
- Study of the impact of climate change on *Artemia* production.
- Further knowledge on basic biology issues in *Artemia* (parthenogenesis, diapause, lipid metabolism, etc).
- Development of new applications through strain selection and selective breeding.
- Integration of extractive *Artemia* farming with intensive fish/crustacean aquaculture.
- Use of *Artemia* biomass as a high-value protein ingredient in local human diets.
- Propagation of improved utilisation guidelines and training and extension services.
- Development of guidelines on certification of *Artemia* cyst products with regards to issues such as species, strain, geographical source, conditions to obtain ideal hatch rates, and sustainable harvesting protocols.

The role of IAAC in development of technology and the sustainable management of natural *Artemia* resources was formally recognized by the Eleventh FAO Committee on Fisheries (COFI), Sub-Committee on Aquaculture (SCA) held in Rome in 2022. The Twelfth COFI-SCA held in Mexico (Hermosillo, 2023) further recommended the preparation of protocols on sustainable harvesting practices of wild resources and certification of cyst products, along with initiatives to conserve biodiversity.

Further information on the IAAC including its activities, membership, steering and advisory committees, is available from the Consortium website, www.artemia.info.

The IAAC was conceptualised in November 2019 at an aquaculture meeting in Kuala Lumpur. From left to right: Meezanur Rahman, Yeong Yik Sung, Sui Liying, Patrick Sorgeloos, Gilbert Van Stappen and Nguyen Van Hoa.

Reference:

Sorgeloos, P., Lavens, P., Léger, Ph., Tackaert, W. & Versichele, P. 1986. *Manual for the culture and use of brine shrimp Artemia in aquaculture*. State University of Ghent, Belgium, 319 pp.

This FAO publication on brine shrimp *Artemia* is a manual for all those who are using *Artemia* or have an interest in this organism, whether as a source of live food in the hatchery, as a model organism in research, or for whatever purpose. It is intended for those who wish to update their knowledge on its biology, production or its use, but also for those who want to learn about *Artemia* for the first time. A team of leading *Artemia* experts from around the globe with diverse backgrounds, expertise and working in research, education and/or the industry have jointly contributed to its writing.

This manual presents in a concise form essential information on *Artemia* biology and the most important natural cyst resources that find their way to the aquaculture market. It also provides detailed information on general principles and practical procedures to produce *Artemia* in ponds and in tank systems. Finally, the manual offers a compilation of state-of-the-art guidelines and methodologies related to the adequate use of this crucial live food organism in aquaculture. The illustrations, tables and practical worksheets will help the reader to implement the correct procedures in the production and use of *Artemia*. This publication is a must for anyone working with this unique organism.

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